SA1

Oxygen binding heme proteins

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Myoglobin (Mb) and Hemoglobin (Hb), the respiratory pigments of mammals and some molluscs, annelids and arthropods, belong to an ancient super-family of heme associated globin proteins. Members of this family share common structural and spectral features. They also share some general functional characteristics such as the ability to bind ligands, e.g. O_2, CO and NO, at the iron atom and to undergo redox changes. These properties are used *in vivo* to perform a wide range of biochemical and physiological roles.

While it is acknowledged that the major role of Hb is to bind oxygen reversibly and deliver this to the tissues, this is not the sole function of the protein. In addition, the often-stated role of Mb as an oxygen storage protein is possibly a misconception. Both Hb and Mb may, for example, express enzymic activities that are important to their function e.g. NO oxidase activity or peroxidatic activities that may be partially responsible for pathophysiology following haemorrhage.

The biochemical and biophysical evidence for these functions will be described and the discussion extended to include proteins that have been discovered comparatively recently and that are expressed at low levels within the cell, e.g. the neuroglobins and cytoglobins. These proteins are wide spread throughout the animal and plant kingdoms and may have specialist roles in oxygen delivery to particular sites within the cell but may also perform roles associated with O_2 sensing and signalling and in the organism's response to stress e.g. by providing protection from reactive oxygen species. Similarly, hemoglobins are widespread in plants and bacteria and may serve similar protective functions. The talk will present the essential features shared by these proteins and discuss how these are tuned in different organisms to accomplish an extensive range of physiological tasks.

Support from the BBSRC and Wellcome Trust is gratefully acknowledged.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA2

Oxygen sensing by the mitochondrial electron transport chain: Role of reactive oxygen species in signal transduction in hypoxia

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Oxygen sensing is a fundamental biological process that is required for development, for successful transition from placental to lung respiration at birth, for normal oxygen homeostasis throughout life, and for tumor angiogenesis and progression at the end of life. Despite the importance of this process in health and disease, the molecular mechanisms by which cells trigger transcriptional and post-translational responses to hypoxia are not fully understood. Our model proposes that mitochondria function as cellular oxygen sensors through a process that involves interaction of molecular oxygen with Complex III of the electron transport chain. Hypoxia results in a paradoxical increase in the release of reactive oxygen species from the outer surface of the inner mitochondrial membrane. These signaling levels of oxidant stress lead to the activation of transcription factors including Hypoxia-Inducible Factor (HIF-1 and HIF-2) and NF-kB, and they trigger cell-specific post-translational responses to hypoxia. Stabilization of HIF-α protein in hypoxia is abrogated when genetic modifications to the electron transport chain lead to loss-of-function in terms of the ability to generate ROS signals during hypoxia. Conversely, genetic modifications to Complex II that induce a chronic increase in mitochondrial ROS production lead to the stabilization of HIF-α under normoxic conditions. In tumor cells, this gain-of-function leads to an increase in tumor cell growth in tissue culture and in vivo, which is mediated by the increase in HIF activation. These findings suggest that Complex III plays a dual role in the cell, through its involvement in energy transduction and in the detection of cellular hypoxia.

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SA3

Potassium channel protein partners: gas sensing in the cardiovascular system

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The ability to react rapidly to dynamic changes in arterial blood gas composition is crucial for optimal delivery of molecular oxygen (O_2) to respiring tissues. The principal sensory components of this homeostatic mechanism are the carotid bodies. Ideally situated in the bifurcation of the common carotid artery, they respond muliplicatively to hypoxia, hypercapnia, pH and hypoglycaemia. When activated, secretion of a variety of transmitters by the carotid body glomus cells results in augmented input to the respiratory centres of the brain stem. Thus, during reduced O_2 availability, activation of the carotid bodies promotes increased rate and depth of ventilation as a compensatory response to systemic hypoxia. At the cellular level, hypoxia promotes inhibition of plasma membrane potassium channels of carotid body glomus cells which leads to calcium influx and transmitter release. In rat glomus cells, two potassium channels have been implicated in the hypoxia-dependent depolarization - a specific member of the tandem P-domain potassium channel family (almost certainly the TASK sub-type (Buckler et al., 2000) and the calcium-activated, large conductance potassium channel (BK_{Ca} - (Peers, 1990)). It appears likely that both BK_{Ca} and the TASK-like potassium channel contribute to carotid body O_2 sensing but, until recently, the identity of the O_2 sensor had remained elusive.

In the search for a potential O_2 sensing mechanism which could account for rapid and reversible inhibition of BK_{Ca} channels, we carried out a proteomic screen for potential protein partners of the BK_{Ca} α-subunit using immunoprecipitation, two-dimensional electrophoresis and mass spectrometry. Of particular note was the protein partnership (verified by double-label immunocytochemistry) of BKα with an O_2-dependent enzyme, hemeoxygenase-2 (HO-2). In the presence of O_2 and NADPH, this enzyme oxidizes cellular heme to generate carbon monoxide (CO), iron and biliverdin. Downstream products of HO-dependent heme catalysis have been reported to play important roles in a wide variety of biological tissues including the immune, the central nervous and the cardiovascular systems. Of particular interest is the observation that HO-2 is expressed in the carotid body and that CO has a major impact on carotid body chemotransduction (Prabhakar et al., 1995).

To define the molecular mechanism linking HO-2 activity to channel inhibition during hypoxia, we have employed single channel studies to show that BK_{Ca} channel (expressed in both HEK293 cells and natively in carotid body glomus cells) activity is robustly and reversibly activated by the downstream products of HO-2, biliverdin and CO (the latter via addition of the chemical CO-donor, $[Ru(CO)_3Cl_2]$). In the presence of O_2, addition the HO-2 co-substrates, heme and NADPH, evoke an increase in channel activity. Importantly, in their continued presence, hypoxia evokes a depression in channel activity which is much larger than that observed in the absence of HO-2 co-substrates. These observations suggest that HO-2 enzymatic activity confers a significant enhancement to the O2 sensing ability of the HO-2/BK_{Ca} protein complex. In support of this notion, selective knock-down of HO-2 protein by RNA interference dramatically depresses tonic channel activity and the NADPH/heme-dependent hypoxic channel suppression is absent. Crucially, CO rescues this loss-of-function (Williams et al., 2004). The mechanism of such gas/channel interactions is complex, and may involve interactions with heme (Jaggar et al., 2005). Using chemical and molecular modifications of the BKα subunit in combination with channel chimera studies, we are beginning to appreciate the kinetic and structural basis of the dynamic regulation of this potassium channel by endogenous production of CO, a gas which is emerging as an important second messenger. Together, such data have led to our proposal that significant O_2-sensing is conferred upon the BK_{Ca} channel by co-localization with HO-2. In normoxia, tonic HO-2 activity generates CO which maintains the open state of the channel. During hypoxic challenge, cellular CO levels are reduced and rapidly fall below the critical threshold for the maintenance of BK_{Ca} channel activity. In other words, HO-2 functions as a sensor of acute reduction in environmental O_2 by changing the balance between intracellular heme concentration and the production of CO.

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This work was supported by a British Heart Foundation Programme Grant (RG/03/001) to PJK and CP.

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SA4

Oxygen sensing by protein hydroxylases

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Recent work has defined novel oxygen sensitive pathways that signal hypoxia by modulating post-translational amino acid hydroxylation at specific sites. Hypoxia inducible factor (HIF) is an alpha/beta heterodimeric transcriptional complex that plays a key role in directing cellular responses to hypoxia. The activity of HIF is itself controlled by post-translational hydroxylation at prolyl and asparaginyl residues within the alpha-sub-units. HIF prolyl hydroxylation governs proteolytic regulation of HIF whereas HIF asparaginyl hydroxylation (FIH) modulates interaction with transcriptional co-activators. These hydroxylations are catalysed by a set of non-haem Fe(II) 2-oxoglutarate (2OG) dependent dioxygenases. During catalysis, the splitting of molecular oxygen is coupled to the hydroxylation of HIF and the oxidative decarboxylation of 2-oxoglutarate to give succinate and CO2. Hydroxylation at two prolyl residues within the central 'degradation domain' of HIF-alpha increases the affinity for the von Hippel-Lindau (pVHL) E3 ligase complex by at least three orders of magnitude, thus directing HIF-alpha polypeptides for proteolytic destruction by the ubiquitin/proteasome pathway. Since the HIF hydroxylases have an absolute requirement for molecular oxygen this process is suppressed in hypoxia allowing the HIF-alpha to escape destruction and activate transcription. Co-substrate and co-factor requirements for Fe(II), ascorbate, and the Kreb's cycle intermediate (2OG) and inducible changes in the cellular abundance of three closely related HIF prolyl hydroxylases (PHD1-3) provide additional interfaces with cellular oxygen status that may be important in regulating the oxygen sensitive signal. Further work has defined other sites of FIH dependent asparaginyl hydroxylation, notably at specific residues within ankyrin repeat domains, indicating that intracellular protein hydroxylation is more common has been so far appreciated. The implications for understanding physiological oxygen homeostasis will be discussed.

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C1

Carbon monoxide exerts two different temporal effects on heterologously expressed rat $P2X_2$ receptors *in vitro*

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Carbon monoxide (CO) is produced constitutively in an oxygen-dependent manner by heme oxygenase enzymes and is increasingly being recognised as an important gas transmitter.

It is of particular interest in the carotid body, where it has been shown both to decrease (Prabhakar *et al.*, 1995) and to increase (Barbé *et al.*, 2002) carotid sinus nerve (CSN) activity, in a manner that is sensitive to purinergic receptor antagonists. ATP is an important co-transmitter in carotid body chemotransduction, where it is released from glomus cells following hypercapnic or hypoxic stimulation. ATP then acts via $P2X_2$ and $P2X_3$ subunits found on petrosal neurons (Zhang *et al.*, 2000). Here we use whole-cell voltage-clamp to show that CO exerts two different temporal responses on $P2X_2$ receptors expressed in HEK 293 cells. Data shown are mean ± s.e.m., p values are from Student's t-test. Acute pre-application (5-10 s) of 30 μM CO donor (tricarbonyldichlororuthenium (II) dimer) caused a reversible sensitization of P2X receptors. The breakdown product of this CO donor was utilized as a control, and had no effect. Currents elicited by sub-maximal ATP (10 μM) were potentiated by 34.6 ± 7.2 % ($n = 8$). 30 μM CO did not increase the maximum ATP-evoked response, and did not significantly alter the EC_{50} for ATP (control = 17.5 ± 2.2 μM; 30 μM CO = 15.6 ± 2.2 μM, $n = 4$, $p > 0.1$). However, 30 μM CO did significantly alter the Hill coefficient of the fitted concentration-response curves for each cell (control = 2.14 ± 0.4; 30 μM CO = 1.56 ± 0.1, $n = 4$, $p < 0.05$). This may suggest that CO alters the gating kinetics of the receptor, a hypothesis strengthened by the observation that CO also decreased the deactivation kinetics of the channel following removal of ATP ($\tau = 167 \pm 13$ ms for control; $\tau = 345 \pm 16$ ms for 30 μM CO, $n = 4$, $p < 0.005$). Both current potentiation and changes to deactivation kinetics were fully reversed following a 90 s wash. Chronic application (3-15 min) of 30 μM CO donor caused an inhibition of ATP-induced currents (9.6 ± 2.7 % current remaining after 15 min, $n = 3$). This inhibition appeared to be irreversible since there was no recovery following 6 min wash, though this may be due to accumulation of CO donor within the cell. The alteration in channel deactivation kinetics was maintained during chronic application of CO donor. Given the likely accumulation of the CO donor within the cell, these temporally distinct effects may represent two concentration dependent CO mechanisms of $P2X_2$ regulation. These observations may go some way to explain how CO may be both an activator and inhibitor of CNS activity, depending upon concentration.

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This work was funded by the BBSRC.

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SA5

Neurotransmitter release at a tonic synapse

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At the first synapse of the vertebrate visual pathway, photoreceptors perform the extraordinary task of releasing neurotransmitter via exocytosis in a graded manner for extended periods of time. Although the release of neurotransmitter via exocytosis is a highly conserved, fundamental feature of nervous system function, the extent and pattern of neurotransmitter release can be shaped by a variety of intrinsic and extrinsic factors. To gain a better understanding of the intrinsic properties that shape synaptic output and contribute to the ability of photoreceptors to release neurotransmitter in a tonic and graded manner, we performed time-resolved measurements of synaptic vesicle dynamics in isolated vertebrate photoreceptors.

Photoreceptors were acutely dissociated from the retina of aquatic tiger salamanders. Changes in membrane surface area indicative of synaptic vesicle fusion and the exocytotic release of neurotransmitter were detected using membrane capacitance measurements. Calcium currents were measured using standard whole-cell patch clamp techniques, and intracellular calcium was measured using ratiometric fluorescent calcium indicator dyes. Exocytosis was evoked either by membrane depolarization and the activation of calcium entry through voltage-gated channels or via the rapid and global release of intraterminal calcium by the flash-photolysis of caged calcium.

When exocytosis was stimulated via membrane depolarization in cone photoreceptors, two kinetic components of release were revealed. The first component was discrete in size, reaching a steady-state value of ≈ 45 fF, corresponding to approximately 1,000 vesicles. Fusion of vesicles in this pool was blocked by low millimolar EGTA. This pool could be depleted with a time constant of a few hundred milliseconds. Thus, the first pool has features similar to the releasable pool of synaptic vesicles described in other neurons. However, unlike other releasable pools, the cone releasable pool recovered from depletion quite rapidly ($t \leq 1$ s). Endocytosis was sufficiently slow that it is unlikely that refilling of the releasable pool occurred via newly-retrieved vesicles. The second component of release was approximately twice the size of the releasable pool and had a time constant for depletion that was substantially slower ($t \approx 3$ s). Simulations using a computational model of release demonstrated that the rapid replenishment of the releasable pool from the secondary pool was sufficient to maintain release evoked by near-maximal stimulations of up to 5 seconds in duration. The inclusion of a tertiary pool of vesicles, corresponding to the remaining cytoplasmic pool of vesicles, allowed for maintained release in the face of even longer periods of near-maximal stimulation.

The present data, combined with our earlier findings that the relationship between exocytosis and calcium is unusually shallow in photoreceptors, can account for several of the unique features of exocytosis in these neurons. First, in the range of intraterminal calcium believed to be physiologically-relevant, the rate of exocytosis almost linearly tracks the local calcium concentration, contributing to the graded nature of transmitter release. Secondly, a relatively large releasable pool with a fast rate of refilling from preformed vesicles contributes to the ability to maintain release for extended periods.

We gratefully acknowledge the support of the National Eye Institute.

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C2

Modulation of synaptic exocytosis by Akt phosphorylation

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Regulated exocytosis is the process by which specialised secretory cells including neurons and endocrine cells specifically release their contents. This process occurs in response to a specific cellular signal, usually an increase in the intracellular calcium concentration. While an increase in the calcium concentration is widely considered as the activator of the process, protein phosphorylation can be considered as a mechanism to regulate or fine tune this process. Recent evidence has indicated that the serine/threonine kinase, Akt/PKB, may play an important role in this modulation of the exocytic response. Evidence for the role of Akt has come predominantly from studies in glucose sensing and insulin secreting cells, such as adipocytes and pancreatic cells. We have used of range or cellular and biochemistry approaches to determine whether the role of Akt in the modulation of exocytosis extends to neurotransmitter release at the synapse.

Acute stimulation of neuroendocrine chromaffin cells with physiological secretogogues or depolarisation of cerebellar granule neurons (CGNs) resulted in increased expression of activated Akt (P-Akt), demonstrating a relationship between an exocytotic signal and Akt activation. The functional effect of Akt in the modulation of exocytosis was then considered in both chromaffin cells and CGNs, with expression of wild-type Akt in chromaffin cells resulting in a slowing of the catecholamine release kinetics. This effect was shown to be phosphorylation specific, as expression of kinase-dead Akt had no effect on kinetics.

Having identified a functional role for Akt in the modulation of exocytosis, we endeavoured to identify the substrates that may mediate these effects. In vitro phosphorylation assays demonstrated that Akt strongly phosphorylates Cystine String Protein (CSP) on Ser10 and Rab3a Interacting Molecule (Rim1) on Ser413. Furthermore, co-expression of CSP with wild-type Akt in chromaffin cells results in increased Ser10 phosphorylation, which was not observed when CSP was co-expressed with a kinase-dead Akt. These findings indicate that CSP is a cellular Akt substrate that may in part be responsible for the modulation of release kinetics. Taken together, these findings indicate that Akt may be an important kinase in the modulation of exocytosis at the synapse and that these effects may be mediated through the phosphorylation of exocytic proteins including CSP and Rim1.

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C3

The dual role of caveolin-1 in para- and transcellular permeability in endothelial cells

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Endothelial cells control water and solute exchange between the intravascular- and the interstitial space by a transcellular and paracellular pathway. In the regulation of both permeability pathways caveolin-1, the main scaffold protein of caveolae seems to be critically involved. We show that caveolin-1 plays a dual role regarding the paracellular and transcellular barrier function of endothelial cells. Using caveolin-1 negative endothelioma cells generated from caveolin-1 deficient mice or siRNA of caveolin-1 we demonstrate a critical role of caveolin-1 in the down regulation of the paracellular endothelial barrier function after thrombin stimulation by dynamin II-dependent endocytosis of junctional proteins. In contrast, in caveolin-1 negative endothelioma cells the constitutive endocytosis of albumin was two fold enhanced compared to caveolin-1 wt cells or after re-expression of caveolin-1 in caveolin-1 negative cells by lentiviral gene transfer. These data indicate that caveolin-1 has a dual role on the endothelial barrier function. It is required for the opening of intercellular junctions but it negatively regulates albumin uptake and in turn the transcellular albumin transport in endothelial cells.

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SA6

Mapping and integrating virus entry and endocytic pathways in human cells

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Our work has focused on basic molecular mechanisms of endocytosis, in particular caveolae/raft-mediated endocytosis, and on infectious virus entry. We have developed single virus particle-tracking and applied it to study how Simian Virus 40 particles hijack endocytosis, and studied basic principles of caveolar vesicle formation, dynamics, activation of traffic, and ligand sorting. In addition, we have developed a systems approach to study endocytosis quantitatively and comprehensively (e.g. the collective behaviour of vesicles in a particular system), to identify the genes regulating endocytic routes (using high-throughput systematic RNA interference), to reveal in an unbiased and quantitative manner perturbed states of endocytic systems, and to link specific endocytic routes with specific physiological functions in the cell. We are also providing a proof-of-principle for a new type of virus classification according to the HOST machineries hijacked, which allows the design of a new type of anti-viral compounds that target host components and not the virus itself. This has the potential to result in broad-antiviral drugs that inhibit several unrelated viruses in their infection that are not hampered by the development of anti-viral resistance. The combination of basic cell biology and virology with systems approaches is the current focus of our laboratory at the Institute of Molecular Systems Biology of the Swiss Federal Institute of Technology (ETH) in Zurich.

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SA7

Role of Munc18 and Rab proteins in the dynamics of dense-core granule exocytosis

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The SNARE proteins including syntaxin, SNAP-25 and VAMP, play an essential role in membrane fusion events such as those that occur during exocytosis to allow fusion of secretory vesicles with the plasma membrane and the release of vesicle contents to the exterior of the cell. Many other proteins are involved in the regulation of the events that trigger and mediate vesicle fusion and amongst these are members of the Sec/Munc and Rab protein families. In regulated exocytosis of synaptic vesicle and dense-core granules Munc18-1 and Rab3A are known to be important but their exact functions remain to be elucidated. Both Munc18-1 and Rab3A have been implicated in vesicle docking at the plasma membrane. We have used PC12 and adrenal chromaffin cells expressing various forms of these proteins to investigate the timing and sites of their action. Expression of mutated forms of Munc18-1 coupled with biochemical analysis of their protein-protein interactions and analysis of the kinetics of exocytosis has allowed us to establish syntaxin-dependent and syntaxin-independent roles for Munc18-1 that affect both early (recruitment and docking) and late stages (exocytosis dynamics) of exocytosis. In addition, we have been examining dynamic aspects of Rab3A on secretory granules using confocal microscopic imaging of GFP-tagged Rab3A in live cells. One of our major goals is now to understand how Munc18-1 and Rab3A interact to control exocytosis.

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Work in the author's laboratory is supported by the Wellcome Trust.

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SA8

Elementary properties of resting fusion

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Neurotransmitter and hormone release requires the fusion of secretory vesicles with the plasma membrane of neurons and neuroendocrine cells (i.e. exocytosis). Exocytosis begins with the formation of a fusion pore, an aqueous channel between the vesicle and the plasma membrane through which cargo molecules diffuse out of the vesicle lumen to the cell exterior. After the fusion pore formation it either closes and allows the vesicle to be reused in the next round of exocytosis ("kiss-and-run" exocytosis) or it fully opens leading to the complete merge of vesicle membrane with the plasma membrane (full fusion exocytosis).

Not only in stimulated but also in resting synapses and neuroendocrine cells, vesicle cargo appears to be released. For decades, stimulated and spontaneous exocytosis was thought to exhibit similar properties at elementary level, differing only in the probability of occurrence (1). However, recent studies indicate that spontaneous exocytosis differs from the stimulated one in many respects, including in distinct protein requirements for vesicle trafficking, fusion, recycling, and the kinetics of vesicle content discharge.

Stimulated hormone discharge from a single lactotroph vesicle of the anterior pituitary, is some 10 to 20 times faster than spontaneous hormone discharge (2), indicating differences in the fusion pore properties in resting and stimulated conditions, respectively. In particular, the fluorescent peptide hormone discharge was monitored with confocal microscopy and compared with the simultaneous loading of vesicle by FM styryl dye. In stimulated vesicles FM 4-64 loading and hormone release occurred within seconds. In contrast, in 50% of spontaneously releasing vesicles, the vesicle content release and the FM 4-64 loading were slow (~3 min). Membrane capacitance measurements revealed regular repetitive transient fusion pore openings ("the pulsing pore"), suggesting that flickering activity of the fusion pore may be the constraint that causes slow vesicle content discharge in resting neuroendocrine cells (2).

To see whether the slow release at rest observed by Stenovec et al. (2004) reflects also a relatively narrow fusion pore, we performed additional optical and electrophysiological studies on resting and stimulated pituitary lactotrophs. Kiss-and-run exocytosis, consisting of reversible fusion between the vesicle membrane and the plasma membrane, is considered to lead to full fusion upon stimulation of vesicles containing classical transmitters (3,4). Whether this is also the case in the fusion of peptidergic vesicles is unknown.

We analyzed the permeation of FM 4-64 dye and HEPES molecules through spontaneously forming fusion pores in lactotroph vesicles expressing synaptopHluorin, a pH-dependent fluorescent fusion marker (5). Confocal imaging showed that half of the spontaneous exocytotic events exhibited fusion pore openings associated with a change in synaptopHluorin fluorescence, but were impermeable to FM 4-64 (diameter = ~1 nm) and

HEPES (diameter = ~0.5 nm). Together with the results obtained by membrane capacitance measurements these findings indicate an open fusion pore diameter <0.5 nm, much smaller than the neuropeptides stored in these vesicles. In 100 mM KCl-stimulated cells, >70% of exocytotic events exhibited a larger, FM 4-64–permeable pore (>1 nm). Interestingly, membrane capacitance measurements showed that the majority of exocytotic events in spontaneous and stimulated conditions were transient. However, stimulation increased the frequency of transient events and their fusion pore dwell-time, but decreased the fraction of events with lowest measurable fusion pore.
Our results obtained by confocal imaging and membrane capacitance measurements show that kiss-and-run is the predominant mode of exocytosis in resting and in stimulated peptidergic vesicles. Furthermore, our study reveals at the single-vesicle level that stimuli prolong the effective fusion pore opening, which expands from its resting subnanometer diameter (<0.5 nm) to dimensions permitting hormone secretion (>1 nm). Although these findings support the view that spontaneous fusion of peptidergic vesicles is "release-unproductive", the nature of an energetically stable transient fusion pore and how this relates to membrane trafficking in eukaryotic cells remains to be investigated.

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We thank Ms. Sonja Grilc for continuous support. This work was supported by grants P3 310 381 from the Ministry of Education, Sciences and Sports of the Republic of Slovenia, NS36665 from the National Institutes of Health, EC grant #QLG3 2001-2004, EC support DECG, CLG3-CT-2001-02004, and EC GROWBETA QLG1-CT-2001-02233.

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SA9

The genetics of schizophrenia

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It has been clear for many years from genetic epidemiology that there is a substantial genetic component to schizophrenia. However, inheritance is complex and non-mendelian with liability to the disorder probably depending upon the combined effects of several, perhaps many, genes together with environmental factors. This genetic complexity together with the fact that the diagnosis of schizophrenia has no validating biology criteria has hampered attempts to identify specific risk genes. In recent years, replicated findings have begun to emerge from positional genetic and convergent genomic work implicating several specific genes; although in no case have nucleotide changes associated with direct risk or protective effects been unequivocally identified. Recent genetic findings are consistent with a primary deficit in synaptic function, but other explanations, such as defects in myelination, are equally plausible. Finally it is perhaps not surprising that some "schizophrenia genes" appear also to confer risk to schizoaffective disorder and bipolar disorder and show associations with clinical syndromes rather than specific categorically defined diagnoses.

MRC, Wellcome Trust, NIMH.

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SA10

Disease biomarkers in first-onset schizophrenia

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At present, little is known about the basic mechanisms that underlie the schizophrenia disease process. This lack of knowledge is most likely due to the fact that until recently large-scale expression profiling studies were technologically impossible. Thus, most researchers employed a "candidate gene/protein" approach. With recent technological advances in genomics, proteomics and metabolomics techniques, it is now possible to globally investigate the molecular underpinnings of psychiatric conditions which should result in improved knowledge and hopefully new (pre-symptomatic) diagnostic, therapeutic and preventative regimes.
My laboratory combines advanced computing and bioscience technologies with functional genomics studies. Using this powerful approach we explore the molecular "fingerprints" of psychotic disorders from early onset through their progressive stages, exploring alterations at the gene, protein, lipid and metabolite level. This in turn should reflect and reveal dynamic changes of interlinked pathways in the normal and disease brain.
I will present results from our biomarker discovery studies. To date we have identified a number of highly significant peptides and metabolites that distinguish first-onset paranoid schizophrenia patients from healthy controls. Our findings suggest brain-specific alterations in glucoregulatory processes in the CSF of drug-naïve patients with first-onset schizophrenia, implying that these abnormalities are intrinsic to the disease, rather than a side effect of antipsychotic medication. Short-term treatment with atypical antipsychotic medication resulted in a normalization of the CSF disease signature in half the patients well before a clinical improvement would be expected. Furthermore, our results suggest that the initiation of antipsychotic treatment during a first psychotic episode may influence treatment response and/or outcome.

Thank you to the Stanley Medical Research Institute and the Henry Smith Trust for Centre support.

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SA11

Modelling prefrontal cortex deficits in schizophrenia: Implications for treatment

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Cognitive deficits are a core feature of schizophrenia and are associated with dysfunction of prefrontal cortex activity (hypofrontality). Although cognitive deficits are highly debilitating to schizophrenic patients, antipsychotic drugs have limited efficacy in the treatment of these symptoms. Development of effective models that mirror these aspects of the disease should aid in the understanding and treatment of cognitive dysfunction in schizophrenia.

We have recently shown that following chronic intermittent low dose PCP treatment, rats exhibit reductions in prefrontal cortex glucose utilisation (hypofrontality) (Cochran et al., 2003) paralleling deoxyglucose imaging results in patients with schizophrenia. In order to understand more about the neurobiological processes that underlie these deficits we have employed genomic (see Winchester et al this meeting) neurochemical and behavioural techniques. Of particular relevance to human post mortem findings are the deficits in markers of GABAergic interneurone activity. Repeated PCP treatment produced deficits in parvalbumin mRNA, Kv3.1, but not calbindin mRNA expression in the prefrontal cortex indicating that the properties of the chandelier subset of fast spiking interneurones are affected. Chandelier cells occupy a key position in prefrontal cortex function in that they are primary targets of thalamocortical neurones and dopaminergic neurones and themselves strongly influence the output of pyramidal cells. Thus disruption of chandelier cell function is predicted to result in a disruption of the corticolimbothalamic network.

In schizophrenia there are deficits across several cognitive domains, including the capacity to effectively transfer attentional set between abstract properties of complex stimuli as shown in the Wisconsin Card Sort Test. In order to assess, whether similar deficits exists following chronic PCP treatment to rats, the attention set-shifting task was employed (Birrel and Brown 2000)which is formally equivalent to the human Wisconsin Card Sort Test. In a single session rats perform a series of seven two-choice discriminations, at the core of which is the extra-dimensional shift (EDS) test that necessitates a shift of attention between perceptual dimensions. Seventy two hours after the final PCP treatment, rats exhibited a significant deficit in their ability to shift attention between, but not within, perceptual dimensions. These findings suggest that repeated intermittent administration of PCP produced a persistent selective deficit in ability to transfer attentional set, but not in ability to reverse stimulus-reward associations akin to that observed in schizophrenia.

Existing antipsychotic medications have limited ability to improve cognitive deficits or hypofrontality in patients. Investigations of the ability of clozapine and haloperidol to reverse chronic PCP-induced hypofrontality and GABAergic interneurone deficits in rats showed that neither drug reversed hypofrontality. Unlike haloperidol, clozapine reversed parvalbumin deficits indicating a partial ability to restore prefrontal cortex activity. The ability of these drugs to reverse cognitive deficits is inconclusive partly due to their other behavioural confounding effects. The effects of novel cognitive enhancers in these models will be discussed.

In summary, repeated PCP treatment to rats, results in neuroadaptive processes in GABAergic interneurones within a corticolimbothalamic network that may contribute to hypofrontality and cognitive deficits similar to that observed in schizophrenia. This PCP treatment regime represents a valuable translational model for further understanding the neurobiological processes contributing to schizophrenia pathology and the validation of new therapeutic agents for the treatment of the cognitive deficits of the disease.

Cochran, SM et al., 2003. Neuropsychopharmacology 28: 265-275.

Birrel J and Brown V., 2000. J. Neurosci 20: 4320-4324.

YRING is a collaborative venture between the Universities of Strathclyde and Glasgow and Mitsubishi Pharma Co.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA12

Imaging genetics in subjects at high risk of schizophrenia

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There is impressive and growing evidence for the involvement of several specific genetic risk factors in the aetiopathogenesis of schizophrenia and/or bipolar disorder. Examining the relationship of well established genes to brain imaging abnormalities is arguably the best currently available method of examining these effects in vivo. Ideally, this would be done in large well characterised cohorts of patients and also in those at risk but without illness or illness related potential confounders such as antipsychotic medication. We have recently completed a ten year longitudinal study of brain structure, function, and risk of developing schizophrenia in a group of individuals at high risk of schizophrenia for familial reasons.

In a sample of young and initially healthy people at high genetic risk of schizophrenia, blood was taken and assays conducted for a small number of these best replicated risk factors for schizophrenia. Here we will discuss the effects of the Val(158)Met polymorphism in the Catechol-O-MethylTransferase (COMT) and the effects of a variant in the human Neuregulin 1 (NRG1) promoter region in subjects at high risk of schizophrenia on: (i) brain structure as measured with structural magnetic resonance imaging (sMRI), (ii) brain function as indexed with key neuropsychological tests and functional MRI, and (iii) the development of psychotic symptoms and/or schizophrenia itself.

The COMT Val allele increased the risk of schizophrenia in this cohort in a dose-dependent manner. Subjects with the COMT Val allele had reduced gray matter density in anterior cingulate cortex. In addition, there was evidence of increased fMRI activation in lateral prefrontal cortex and anterior and posterior cingulate, with increasing task difficulty in those with the COMT Val allele despite a similar level of performance. The risk allele

in the NRG1 promoter region, on the other hand, was associated with the development of psychotic symptoms, decreased premorbid IQ and decreased activation of pre-frontal and temporal lobe regions.
These patterns of altered brain structure and function have previously been associated with schizophrenia in this and other samples. In the Scottish population, the NRG1 gene appears to be a risk factor for an extended or intermediate phenotype while the COMT Val allele is associated with an increased risk of schizophrenia in subjects at increased familial risk. Determining how these and other risk factors interact will require studies which are an order of magnitude larger and establishing these will require collaborative clinical, genetic and multi-centre imaging research networks.

MRC and Sackler Foundation.

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C4

Identification of schizophrenia-related genes by global transcriptome analysis of the prefrontal cortex of PCP treated rats

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Schizophrenia is a complex psychiatric disorder, affecting 1% of the population. It is a multifactorial disease characterised by positive symptoms, negative symptoms and cognitive deficits. The cognitive deficits (impaired working memory, attentional and executive function deficits) are considered a core feature resulting primarily from dysfunction in the prefrontal cortex. Despite concerted efforts into the cause of schizophrenia, the genetic components of this complex disease remain elusive.
We performed a global transcriptome screen to identify schizophrenia-associated genes differentially expressed in the prefrontal cortex, utilising a phencyclidine (PCP) rat model of schizophrenia (Cochran et al 2003) and rat oligonucleotide GeneChips from Affymetrix. This PCP treatment regime developed in our laboratories produces a pattern of metabolic hypofunction, neurochemical changes and cognitive deficits in the rodent brain that closely mirror those observed in the brains of schizophrenic patients (Cochran et al 2003 and Morris et al 2005)
327 differentially expressed transcripts were identified. Many of the associated genes map to key schizophrenia loci, including the neurodevelopmental gene DPYSL2 that maps to 8p21-22 and has been previously implicated as a susceptibility gene for schizophrenia and the AMPA receptor subunit gene GRIA1 (GluR1) that maps to SCZD1 (schizophrenia susceptibility locus 1) on 5q33. We present the microarray identification of schizophrenia-associated genes and confirmatory real-time PCR data on differentially expressed genes mapping to key schizophrenia loci. We conclude that microarray analysis of a validated animal model of prefrontal cortex deficits in schizophrenia can lead to the identification of genes involved in the cognitive aspects of the disease. This approach has identified genes previously linked to schizophrenia together with novel genes that may represent a completely novel convergent pathway underlying the cognitive deficits associated with schizophrenia. Together these findings have implications for novel drug target development.

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Morris, BJ, Cochran, SM and Pratt JA, 2005, Current Opinions in Pharmacology 5: 101-106.

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C5

The Role of PDE11A1 in Schizophrenia

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Phosphodiesterases (PDEs) are enzymes that play a role in the regulation of the second messenger molecules, cAMP and cGMP. PDE enzymes are classified into 11 families, PDE1 to PDE11. Due to their physiological effects, PDE inhibitors have been identified as new potential therapeutics in areas such as pulmonary arterial hypertension, coronary heart disease, respiratory disease, metabolic disorders, dementia and more recently in some forms of depression. Here, we implicate PDE11 in the aetiology of schizophrenia. PDE11 expression is detected in the brain in small amounts and has been linked to the treatment of diseases or conditions that affect the prostate, reproduction and more recently major depressive disorder. We have identified a number of compounds with low to sub micromolar IC50 activity against PDE11A1. We have identified Compound A, which indicates an IC50 value of 300nM and shows selectivity of greater than 300 against all other PDE families. This compound has also been tested in a murine model of Pre Pulse Inhibition (PPI) an in vivo model of schizophrenia. Compound A indicates efficacy in this model at a dose of 50mg/kg and at a dose of 15mg/kg, when PCP is incorporated into the PPI model. The compound has no effect in open field activity models. This study indicates a potential role for the PDE11 family in the treatment of Schizophrenia.

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SA13

Trafficking of ATP-sensitive potassium channels in health and disease

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ATP-sensitive potassium (K_{ATP}) channels are octomeric complexes comprising four subunits each of Kir6.2 and the

sulphonylurea receptor (SUR1)[1;2]. Kir6.2 is encoded by *KCNJ11* and SUR1 by *ABCC8*. They control insulin secretion and glucose homeostasis by coupling the metabolic status (ATP/ADP ratio) of the cell to its membrane potential. This ability is conferred by the unique property of K_{ATP} channels: they are inhibited by ATP and activated by Mg-nucleotides. A rise in blood glucose increases the uptake and metabolism of glucose in pancreatic β-cells, resulting in an increase in the ATP/ADP ratio, which leads to the closure of K_{ATP} channels, and consequent membrane depolarisation and activation of voltage-gated calcium channels. Entry of Ca^{2+} through the activated calcium channels leads to a rise in intracellular Ca^{2+}, which triggers insulin secretion. As glucose levels return to normal, the channels begin to open and reduce insulin secretion. As such, mutations that lead to a loss of function, or impair trafficking to the cell surface, of K_{ATP} channels lead to congenital hyperinsulinism (CHI), a genetic disorder characterised by the unregulated insulin secretion and severe hypoglycaemia [3]. By contrast, mutations that lead to a gain of function [2], or an increase in the cell surface density [4], of the channels cause permanent neonatal diabetes mellitus (PNDM) where insulin secretion is subnormal and resting blood glucose levels are extremely high.

Here we investigated two types of genetic mutation found in Kir6.2: one (E282K) that causes CHI by abolishing surface expression of the channel by preventing the ER exit and the other (Y330C and F333I), found in PNDM patients, increases the cell surface density by inhibiting endocytosis. The E282K mutation prevented the surface expression of K_{ATP} channels, but did not affect the assembly of Kir6.2 with SUR1. The mutant subunits are retained in the ER, but this retention does not appear to be caused by the mis-folding of the protein. Instead, the mutation abrogates the di-acidic ER exit signal (^{280}DXE^{282}) in Kir6.2. Using the inhibitory forms of Sar1-GTPases, we demonstrate that wild-type K_{ATP} channels are recruited into COPII coat vesicles and that this recruitment requires a functional 'DXE' ER exit signal [5]. Co-assembly of the mutant subunits with the wild-type Kir6.2 and SUR1 formed functional channels indicating that, in the heterozygous state, the mutant subunits can support the function of the channel, although with diminished ability. These data explain the mild disease phenotype expressed by some members of the family carrying the E282K mutation. The PNDM causing Y330C and F333I mutations abolished the ability of the channel to undergo endocytosis. This defect could be rescued when the wild-type subunits were co-expressed with the mutant subunits. Thus in the heterozygous state, the mutant subunits are able to undergo endocytosis. However, the surface levels of the heterozygous channels were over 2-fold greater than those of wild-type channels, indicating that increased surface channel numbers may contribute to the disease phenotype [4]. In conclusion, our studies with the genetic mutations causing contrasting phenotypic effects illustrate the importance of the regulation of the surface density of K_{ATP} channels in glucose stimulated insulin secretion.

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The work was funded by the Medical Research Council, UK

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA14

HERG channel trafficking: Novel targets in drug-induced long QT syndrome

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The cardiac potassium channel hERG encodes the α-subunit of the rapid delayed rectifier current I_{Kr} in the heart which contributes to terminal repolarization in human cardiomyocytes. The crucial role played by hERG/I_{Kr} in cardiac repolarization was uncovered first in patients with inherited long QT syndrome (LQTS2) with the identification of mutations in the hERG gene that reduced hERG/I_{Kr} currents. In symptomatic patients, LQTS2 is characterized by a prolongation of the QT interval on the electrocardiogram and associated with an increased propensity to develop arrhythmias. More recently, trafficking deficient LQTS2 mutations have been recognized as an important disease mechanism. Although hERG currents have been studied extensively, only few proteins have been identified so far that are involved in hERG trafficking to the cell surface. We have isolated two cytosolic chaperones, Hsp70 and Hsp90 that interacted with newly synthesized hERG. HERG-chaperone interactions were transient with wildtype protein, but prolonged with trafficking deficient LQTS2 mutants. Inhibition of Hsp90 function with geldanamycin prevented the association of Hsp90 with hERG, inhibited hERG maturation and reduced expression of hERG/IKr currents in cardiomyocytes. These data suggested that Hsp90 is a crucial player in the maturation of the cardiac potassium channel hERG/I_{Kr} and that disruption of Hsp90 function will lead to a reduction in the number of functional channels at the cell surface with acquired long QT syndrome as a possible consequence. Based on our observations with geldanamycin, a specific inhibitor of Hsp90, we speculated that proteins in the processing pathway are not only crucial for the maturation of wildtype channels but at the same time provide targets for therapeutic compounds that have been linked in the past to acquired long QT syndrome with no indication of direct channel block as the underlying mechanism. In subsequent studies we have identified for the first time acquired trafficking inhibition of hERG as a novel mechanism causing acquired long QT syndrome.

For example, arsenic trioxide (As_2O_3) produces dramatic remissions in patients with acute promyelocytic leukemia. However, its therapeutic use is burdened by toxicity including QT prolongation and torsade de pointes arrhythmias. Using electrophysiological recordings, surface expression assays and Western blotting we have shown that As_2O_3 inhibits maturation and surface expression of the cardiac potassium channel hERG at clinically relevant concentrations. As_2O_3 interfered with the processing and maturation of hERG by inhibition of hERG-Hsp90 complexes. At the same time, direct hERG block was not detected. While arsenic trioxide as a metalloid is an unusual therapeutic

compound, pentamidine represents a more conventional small molecule compound that is clinically used for treatment of leishmaniasis, trypanosomiasis and Pneumocystis carinii pneumonia. Once again, pentamidine use was accompanied by electrographic abnormalities with no clear indication of direct hERG block. We were able to confirm that pentamidine -just like arsenic trioxide- inhibited maturation and surface expression of hERG. HERG currents and the fully-glycosylated cell surface form of hERG were suppressed after overnight exposure to clinically relevant concentrations while other membrane currents were not affected. Thus, pentamidine represents another example of a therapeutic compound that produces QT prolongation by suppression of hERG trafficking.

To survey therapeutic compounds in a more systematic manner for effects on hERG trafficking, we have developed a novel chemiluminesence-based surface expression assay performed in 96-well format. We have used this assay to identify novel compounds that interfere with hERG trafficking and found that cardiac glycosides represent a large class of potent inhibitors of hERG cell surface expression. Cardiac glycosides reduced expression of the fully-glycosylated cell surface form of hERG on Western blots indicating that channel exit from the endoplasmic reticulum was blocked. Similarly, hERG currents were reduced with nanomolar affinity on long-term exposure. Importantly, hERG trafficking inhibition was initiated by cardiac glycosides through direct block of Na/K pumps and not via off-target interactions with hERG or another closely associated protein in the processing or export pathway.

Taken together, we have identified several of a growing number of hERG trafficking inhibitors which poses a novel problem for the identification of compounds that impair hERG channel function in pre-clinical safety studies that are geared towards detection of acute channel block.

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Supported by NIH grant HL71789

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C6

Characterising the role of abnormal trafficking of KCNE1 in long QT syndrome 5 (LQT5)

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Mutations in the minK gene encoding the protein KCNE1 have been associated with the long QT syndrome 5 (LQT5). KCNE1 is an auxiliary subunit that associates with the potassium channel KCNQ1 to form a complex in cardiac myocytes that produces the slow delayed rectifier current IKs. Recent experiments have shown that abnormal trafficking of KCNQ1 mutants can influence disease pathogenesis in LQT1 (Wilson et al., 2005), but the role of abnormal trafficking of KCNE1 in LQT5 remains to be firmly established. To investigate whether abnormal trafficking of KCNE1 contributes to LQT5 three KCNE1 mutants were generated, G52R, T58P/L59P and R98W, and their effects on trafficking and channel function examined. To examine defects in trafficking the level of colocalisation of green fluorescent protein tagged KCNQ1 (KCNQ1-GFP) with an endoplasmic reticulum marker when overexpressed in conjunction with the KCNE1 mutants, in CHO-K1 cells, was investigated. For G52R, T58P/L59P and R98W a modest, but significant ($P<0.05$), trafficking defect was observed that was similar to the retention level seen when KCNQ1-GFP was expressed without KCNE1. To assess the effects of the KCNE1 mutants on channel function the mutants were coexpressed with KCNQ1-GFP, in CHO-K1 cells, and the currents recorded by whole-cell patch clamping. G52R and T58P/L59P produced currents that lacked the slow activating IKs current and were similar to currents produced by KCNQ1-GFP expression alone. R98W produced currents that had the slow activating IKs profile but with reduced amplitude and steady state activation kinetics that were shifted to the right. To determine whether the lack of IKs current produced by G52R and T58P/L59P was due to a lack of expression, these mutants were cotransfected with KCNQ1, in CHO-K1 cells, and subjected to western blotting. Both G52R and T58P/L59P were found to be expressed at a similar level to wild type KCNE1, which suggests that the inability of G52R and T58P/L59P to form a pore complex that produces a slow activating IKs current is not due to a lack of expression. These results highlight two possible mechanisms for a role of abnormal trafficking in LQT5. Firstly, that the modest trafficking defect and a functional defect combine to increase dysfunction or secondly that certain KCNE1 mutants fail to enter the pore complex which results in a severe loss of function. We are currently developing techniques with the aim to distinguish between these two potential mechanisms.

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C7

Are $GABA_A$ receptors dynamically trafficked during *in vitro* models of pathological conditions?

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$GABA_A$ receptors ($GABA_A$Rs) form the major inhibitory system in the CNS. Many pathological disorders result from an imbalance in excitation and inhibition, for example, the heterozygous $\gamma 2$ knockout displays increased anxiety-like behavior (Crestani *et al.*, 1999). Furthermore, several mutations in $GABA_A$R subunit genes ($\alpha 1$, δ and $\gamma 2$) are associated with the presentation of seizures in families with inherited epilepsies (reviewed by Mizielinska *et al.*, 2006). $GABA_A$Rs that are assembled from a combination of the different subunits (19 subunit genes plus splice variants) exhibit distinct pharmacological profiles and

contribute to different aspects of the behavioural responses to benzodiazepines (reviewed by Wafford *et al.*, 2004). The dynamic trafficking of excitatory receptors is pivotal in the formation of learning and memory. This study utilizes individually tagged wild type and mutant subunits in combination with *in vitro* neuronal models of epilepsy and ischaemia to analyze the dynamic movement of $GABA_AR$ subunits. The use of pH-sensitive fluorophores permits visualization of endocytosis by the quenching of fluorescence on internalization into acidic vesicles. Previous findings have shown a down-regulation of the γ2-subunit after one hour of epileptiform activity using immunohistological techniques (Blair *et al.*, 2004). Our approach is to monitor the actual time course of subunit trafficking following the onset of seizure-like activity and ischaemia.

Funding support by the BBSRC, Merck Sharpe & Dohme and Anonymous Trust.

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SA15

Activity-dependent regulation of voltage-gated potassium channels by bidirectional changes in phosphorylation state

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Electrical excitability is conferred by the expression of a diverse repertoire of voltage-gated ion channels, each with distinct functional properties, localization and modulation but that together determine the electrical characteristics of a given cell and how it receives, processes and responds to external stimuli. Modulation of ion channel abundance, distribution and function is crucial to dynamic regulation of cellular excitability, and the integrated function of the numerous tissues in response to developmental and environmental cues.

Among the most numerous and variable determinants of a cell's electrical signature are voltage-gated potassium (Kv) channels, which exert diverse effects on membrane excitability. In particular, the voltage-gated K+ channel Kv2.1 constitutes a major component of the total delayed rectifier Kv current in many mammalian central neurons, smooth and skeletal muscle cells, and pancreatic islet beta cells. In neurons, the subcellular localization and voltage-dependent gating properties of Kv2.1 are dramatically modulated by rapid calcineurin-dependent dephosphorylation of the constitutively phosphorylated channel protein in response to increased excitatory synaptic activity, epileptic seizures, and ischemia, which homeostatically suppresses neuronal firing. Similar modulation can also occur in response to neuromodulatory stimuli. The large (≈450 amino acid) cytoplasmic carboxyl terminus of the Kv2.1 polypeptide can act as an autonomous domain that is both necessary and sufficient to confer Kv2.1-like localization, function and modulation to diverse Kv channels. Attempts to identify specific phosphorylation sites critical to Kv2.1 modulation were confounded by the fact that over 100 cytoplasmic amino acids in the Kv2.1 polypeptide are residues (Ser, Thr or Tyr) susceptible to covalent phosphorylation, and up to 63 of these score as strong consensus phosphorylation sites. As such, we undertook an unbiased analysis of in vivo calcineurin-regulated phosphorylation sites on immunopurified Kv2.1 using tandem mass spectrometric (MS/MS) and SILAC approaches. Subsequent mutation of individual sites was used to identify sites critical for Kv2.1 modulation. Phosphospecific anti-Kv2.1 antibodies reveal complex bidirectional activity-dependent regulation of these sites in mammalian neurons. Other neuronal Kv channels also display complex patterns of in vivo phosphorylation and regulation. Such an unbiased proteomic strategy to identify complex sets of dynamically regulated phosphorylation sites is a powerful approach to pinpoint specific sites controlling physiologically important protein function.

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Supported by NIH grant NS042225

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SA16

Kv channel surface expression is modulated by endocytosis of channels and retrograde trafficking in endosomal compartments by the dynein motor

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Overexpression of p50/dynamitin, known to disrupt the dynein-dynactin complex responsible for carrying vesicle cargo, substantially increased outward K+ currents in HEK293 cells stably expressing Kv1.5 (0.57+0.07 nA/pF, n=12; to 1.18+0.2 nA/pF, n=12, $p < 0.01$), Kv4.2, and Kv2.1 channels, as did treatment of the cells with a dynamin inhibitory peptide, which blocks endocytosis. Nocodazole pretreatment, which depolymerizes the microtubule cytoskeleton along which dynein tracks, also doubled Kv1.5 currents in HEK cells and sustained K+ currents in isolated rat atrial myocytes. These increased currents were blocked by 1 mM 4-aminopyridine, and the specific Kv1.5 antagonist, DMM (100 nM). Confocal imaging of both HEK cells and myocytes, as well as experiments testing the sensitivity of the channel in living cells to external Proteinase K, showed that this increase in K+ current density was caused by a redistribution of channels toward the plasma membrane. Co-immunoprecipitation experiments demonstrated a direct interaction between Kv1.5 and the dynein motor complex in both heterologous cells and rat cardiac myocytes, supporting the role of this complex in Kv1.5 trafficking, which required an intact SH3-binding domain in the Kv1.5 N-terminus to occur. Throughout this post-internalization trafficking, the channels very probably reside in endosomes. As assayed by immunocytochemistry/confocal microscopy and by live-cell imaging, internalized Kv1.5 co-localized with both EEA1 and Rab5, markers of the early endosomal compartment, as well as with recycling endosome-specific Rab11.

Furthermore, co-expression of Kv1.5 with a Rab5 dominant negative, which prevents early endosome maturation, increased Kv1.5 currents to an extent similar to that seen with p50 or the dynamin inhibitor. These experiments highlight a pathway for Kv1.5 internalization from the cell surface involving early endosomes, followed by later trafficking in endosomal vesicles by the dynein motor along microtubules. This work has significant implications for our understanding of the way Kv channel surface expression is regulated.

Supported by the Heart and Stroke Foundation of BC and Yukon, and the Canadian Institutes of Health Research.

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SA17

The pH gradient and electrical potential of phagosomes: measurement and functional role

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Phagosomes employ lytic enzymes, cationic peptides and reactive oxygen intermediates to eliminate invading microorganisms. The effectiveness of these and other microbicidal mechanisms is potentiated by the acidic pH created by proton-pumping V-ATPases on the phagosomal membrane. The degree of phagosomal acidification varies greatly between cell types, and can be affected by diseases like cystic fibrosis. The development of acidification is affected by the electrical potential that develops across the phagosomal membrane. We describe a non-invasive procedure to estimate phagosomal potential in intact cells, based on fluorescence resonance energy transfer. At steady state the phagosomal voltage averaged 27 mV (lumen positive) and was only partially dissipated by inhibition of the V-ATPase with concanamycin A. The comparatively small contribution of the potential to the protonmotive force suggests that in macrophages phagosomal proton pumping is not limited by the counter-ion permeability. Maintenance of luminal acidification was found to be essential for proper phagosomal maturation to phago-lysosomes. Dissipation of the proton gradient arrested maturation prior to acquisition of Rab7, despite the association of active Rab5 with the phagosomes and with endosomes. In fact, when the luminal acidification was abrogated, endosomes became enlarged and Rab5 was stimulated, leading to the accumulation of inordinately large amounts of phosphatidylinositol 3-phosphate. The phosphoinositide was ultimately delivered to the endosomal lumen, where it was sequestered and became unavailable to promote membrane fusion. We suggest that luminal acidification controls endosome and phagosome maturation, at least in part by signaling the timely termination of Rab5 activity.

Original work in the authors' laboratory is supported by the Canadian Cystic Fibrosis Foundation and the Canadian Institutes for Health Research

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SA18

The role of calcium and other ions in sorting and delivery in the late endocytic pathway

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The passage of endocytosed receptor-bound ligands and membrane proteins through the endocytic pathway of mammalian cells to lysosomes occurs via early and late endosomes. The latter contain luminal vesicles and are also referred to as multivesicular bodies (MVBs). The overall morphology of endosomal compartments is, in major part, a consequence of the many fusion events occurring in the endocytic pathway. Kissing events and direct fusion between late endosomes and lysosomes provide a means of delivery to lysosomes. Using time lapse confocal microscopy we have studied content exchange between lysosomes and endosomes in living cells and found evidence for both these modes of delivery of endosomal content to lysosomal hydrolases [1]. Following fusion in living cells, tubulation and budding events occur that may be part of the process of lysosome re-formation. Fusion of lysosomes with late endosomes results in the formation of a hybrid compartment which acts as a 'cell stomach' in which hydrolysis of endocytosed macromolecules occurs and from which lysosomes are re-formed. The formation of hybrid organelles and some aspects of lysosome reformation have been reconstituted in cell-free systems [2]. Such systems have allowed the identification of some of the biochemical requirements for fusion and lysosome re-formation including the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex required for fusion [3]. The ionic composition of the lumen of endocytic organelles is known to be important for sorting and traffic through the endocytic pathway. Some endocytosed ligands such as low density lipoprotein (LDL) are delivered to lysosomal hydrolases for degradation after dissociation from their receptors in the acidic lumen of the early endosome, a process which also allows subsequent recycling of the empty receptors to the cell surface. Other ligands, such as epidermal growth factor (EGF), remain bound to their receptors which are ubiquitinated and, after endocytosis, are sorted into the luminal vesicles of MVBs. Formation of MVBs and sorting into the luminal vesicles requires a group of cytosolic proteins organised into endosomal sorting complexes required for transport (ESCRT) and probably also a sodium/proton exchanger which is the mammalian homologue of yeast protein Vps44p. This may be involved in maintaining the ionic balance of the endosome lumen.

The fusion of late endosomes with lysosomes and the re-formation of lysosomes from the resultant hybrid organelles both have a requirement for luminal Ca2+ [2]. Thus, fusion was inhibited by BAPTA, but not by EGTA, a chelator with a similar dissociation constant for Ca2+ but an on-rate much slower than BAPTA. Inhibition was reversed by adding additional Ca2+. Fusion was also inhibited by EGTA-AM, a membrane permeable, hydrolysable ester of EGTA. Taken together, the data suggested that it is release of luminal Ca2+ that is required for membrane fusion. . The recovery of electron dense lysosomes from hybrid organelles was shown to require ATP and was inhibited

by bafilomycin and EGTA-AM. The effect of EGTA-AM suggested that luminal Ca2+ was required for the condensation process necessary for re-formation of electron dense lysosomes. Ca2+ also appears to play an important role in the traffic of some lipids through the late endocytic pathway. Mutations in the gene MCOLN1 result in the lysosomal storage disorder mucolipidosis type IV (MLIV). MCOLN1 encodes mucolipin-1, a lysosomal membrane protein thought to be a cation channel. Aberrant lactosylceramide trafficking through the late endocytic pathway in MLIV cells may be rescued by wild type mucolipin-1 expression but not by lysosome-localised mucolipin-1 mutated in its predicted ion pore-selectivity region [4]. Thus, the Ca2+ content and other ionic composition of endocytic organelles together with the correct localisation of membrane proteins contributing to this composition are essential for sorting and delivery in the endocytic pathway.

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Experimental work was funded by a Research Grants from the Medical Research Council.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA19

The V-ATPase a2-subunit as a putative endosomal pH-sensor

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V-ATPase-driven intra-vesicular acidification is crucial for vesicular trafficking. Defects in vesicular acidification and trafficking have recently been recognized as essential determinants of various human diseases. An important role of endosomal acidification in receptor-ligand dissociation and in activation of lysosomal hydrolytic enzymes is well established. However, the molecular mechanisms by which lumenal pH information is transmitted to the cytosolic small GTPases that control trafficking events such as budding and coat formation are unknown. Here we discuss our recent discovery that endosomal V-ATPase is a pH-sensor regulating the degradative pathway (1). We also propose the hypothetical molecular mechanism involved.

Previously, work from Schulz and colleagues reported a pH-dependent interaction of Arf small GTPases with purified microsomal vesicles (2). However, the specific members of the Arf-family that are involved in this recruitment were not established. Also, the cellular compartment in which this interaction took place and the mechanism of pH-dependent Arf recruitment remained unknown. Subsequent studies from Gruenberg and colleagues demonstrated acidification-dependent recruitment of COP and Arf1 proteins onto early endosomes (3). These authors proposed that a hypothetical endosomal trans-membrane pH sensor is involved in a direct interaction with these proteins and that this biochemical event is necessary for the formation of transport carrier vesicles. In addition, the presence of a pH sensor in yeast vacuoles has been recently proposed (4). While the existence of a vesicular pH sensor was suggested in all these studies, its nature and pH-sensing molecular mechanism remained obscure.

Kidney proximal tubule (PT) cells have an extensive endocytic apparatus that is critical for the reabsorption and degradation of filtered proteins via the endosomal/lysosomal pathway. The acidification of PT endosomes and lysosomes is driven by V-ATPase. The importance of endosomal acidification is underlined by our finding that V-ATPase inhibitors and acidification uncouplers strongly abolish function of this pathway (1). Recent data from our laboratory provide new insights into the regulation of this process by trans-membrane V-ATPase and cytosolic small GTPases. In particular, we have demonstrated that Arf6 and ARNO are targeted to early endosomes and colocalize with V-ATPase (5). Moreover, specific recruitment of ARNO and Arf6 (but not Arf1) from the cytosol to endosomes depends upon V-ATPase-driven intra-endosomal acidification, so implicating this biochemical event in regulating the protein degradative pathway. Importantly, our study also postulated the existence of a trans-membrane pH-sensing protein (PSP) in early endosomes and its direct interaction with ARNO and Arf6 (5). However, the nature of PSP and the precise mechanism of its pH-dependent interaction with small GTPases remained illusive. In search of the pH-sensing protein, we recently examined the role of V-ATPase and small GTPases in the trafficking of albumin-Alexa594 via the PT protein degradative pathway (1). We found that inhibition of endosomal acidification by bafilomycin selectively affects the degradative pathway by preventing the delivery of albumin-Alexa594 from early to late endosomes. We also showed that the trans-membrane a2-isoform of V-ATPase is specifically targeted to early endosomes. Its cytosolic N-terminal tail directly interacts with ARNO and the trans-membrane c-subunit of V-ATPase interacts with Arf6. Importantly, the interaction between the V-ATPase a2-isoform and ARNO is modulated by acidification of the endosomal lumen and regulates the protein degradative pathway. These results led us to propose that V-ATPase itself might be the long sought-after PSP. According to our model V-ATPase is responsible for: i) the generation of a pH gradient between vesicular membranes; ii) sensing of intra-vesicular pH; and iii) transmitting this information to the cytosolic side of the membrane.

Hypothesis: Based on extensive experimental evidence on the crucial role of histidine residues in the function of pH-sensing proteins in eukaryotic cells, we hypothesize that pH-sensitive histidines within the intra-endosomal loops and C-terminal luminal tail of the a2-subunit of V-ATPase could also be involved in the pH-sensing function of V-ATPase. The crucial role of these histidine residues in pH-dependent conformational changes of the V-ATPase a2-isoform, its interaction with ARNO and ultimately in its acidification-dependent regulation of the endosomal/lysosomal protein degradative pathway remain to be demonstrated.

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This work was supported by a National Institute of Health Grant DK038452.

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SA20

The role of the NADPH oxidase in the killing of bacteria and fungi by neutrophils – replacing the paradigm of free radical damage with ion fluxes and pH optimisation

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The NADPH oxidase is the molecular machinery responsible for making O2- in the phagocytic vacuole. It is the prototype of a number of NADPH Oxidase or NOX enzymes that have been identified in a wide variety of tissues.

It consists of a flavocytochrome that spans the membrane and passes electrons from the substrate, NADPH, in the cytoplasm, across a short electron transport chain consisting of a FAD and two haems, to O2 in the vacuole.

Activation of this oxidase requires the participation of a number of cytosolic proteins including p47phox, p67phox, and the small GTP binding protein, p21rac. These proteins seem to activate electron transport, possibly by modifying the conformation of the flavocytochrome, and providing NADPH access to the active site of the enzyme.

Function of oxidase

Microbial killing - Neutrophils consume large amounts of oxygen when they phagocytose bacteria and fungi. This oxygen consumption, known as the "respiratory burst" is required for the efficient killing and digestion of the engulfed microbe which are killed poorly under anaerobic conditions or in Chronic Granulomatous Disease (CGD), in which the oxidase is dysfunctional. The oxidase produces superoxide, O2-, in the vacuole, and this dismutates to form hydrogen peroxide. The neutrophil granules contain myeloperoxidase which can use H2O2 as substrate to oxidise halides to hypohalous acids. The discovery that neutrophils produce oxygen free radicals and H2O2, coupled with the fact that the absence of this production results in impaired microbial killing, led to the belief that the oxygen radicals themselves killed the microbes.

This concept that the products of the oxidase were themselves toxic and directly responsible for killing the bacteria was attractive and gained almost universal support. However it was then shown that mice in whom the major neutrophil neutral proteases cathepsin G and elastase had been knocked out were susceptible to bacterial infection, and that their neutrophils demonstrated a profound defect of microbial killing, despite the production of normal amounts of ROS and normal activity of myeloperoxidase. The oxidase had to be doing something else!

Charge compensation - The oxidase is electrogenic, which means that the passage of electrons across the vacuolar membrane produces a charge across this membrane that will curtail further electron transport unless compensated. Electrons carry one negative charge so this charge compensation requires the passage of negatively charged ions in the opposite direction or positively charge ions in the same direction. We have found that combinations of the two occur.

The neutrophil granules contain a number of digestive enzymes and other proteins and high concentrations of ions, particularly Cl- (320mM), K+ (240mM) and Na+ (80mM). The main compensating ion is Cl- which passes from the vacuole to the cytoplasm. In addition K+ passes through BKCa channels into the vacuole.

The efflux of Cl- from, and influx of K+ into, the vacuole activate the granule enzymes to kill and digest the microbes.

pH regulation - Electrons are accepted in the vacuole by O2 to produce O2- which dismutates to form O22-(peroxide) which becomes protonated to form H2O2:

$4NADPH + 4O2 \rightarrow 4NADP + 4H+ + 4O2-$

$4O2- \rightarrow 2O22- + 2O2$

$2O22- + 4H+ \rightarrow 2H2O2$

$2H2O2 \rightarrow 2H2O + O2$

The H+s released from NADPH are left in the cytoplasm, which is acidified, whereas the consumption of H+s in the vacuole causes the pH to rise in this compartment. It is important that the pH in the vacuole is maintained at that optimal for the activity of the neutral proteases that kill and digest the microbes. This is accomplished by the buffering capacity of the granule contents, exchange of H+ for Na+ by NHE1 and compensation of some of the charge by the passage of H+s and K+ from the cytoplasm to the vacuole.

Implications for the role of free radicals in the pathogenesis of disease

ROS production by neutrophils provided the paradigm for the toxic effect of these molecules in a biological system, and by extrapolation, to their potentially toxic role in the pathogenesis of human diseases, including oncogenesis. Now that the true mechanism by which the NADPH oxidase promotes microbial killing has been established, the involvement of ROS as causal agents in disease processes must be reassessed.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA21

Insulin action on the liver in vivo

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Over the past decade knowledge relating to insulin's action regulating glucose metabolism in vivo has expanded. It is now clear that physiologic increases in plasma insulin can potently inhibit glucose production by both direct and indirect means. Selective changes in insulin within the liver sinusoids (no change elsewhere in the body) are capable of setting glucose production

between 0 (3 fold increase in insulin) and 7 (70% decrease in insulin) mg/kg-min. In addition insulin's effects on muscle, fat, the alpha cell and the brain can also indirectly modify hepatic glucose output. A tripling of arterial insulin in the absence of a change in hepatic sinusoidal insulin inhibits glucose output from the liver by 50%. This effect appears to relate primarily to the action of insulin on adipose tissue. The role of physiological changes in insulin within the brain modifying glucose production remains controversial. Data in rodents support a role for neural insulin action while data in the dog and human do not. Regardless of the mechanisms involved in the indirect control of hepatic glucose production by insulin it is clear that in the normal animal the direct affect of insulin on the liver is dominant.

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SA22

Gut peptides and energy balance

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The ability to maintain adequate nutrient intake is critical for survival. Complex interrelated neuronal circuits have therefore developed in the mammalian brain to regulate many aspects of feeding behaviour, from food-seeking to meal termination. The brainstem and hypothalamus serve as key homeostatic sites receiving and integrating the neural, nutrient, and hormonal signals that convey nutritional status and orchestrating appropriate efferent responses. Energy homeostasis is controlled by long-term adiposity hormones, such as leptin and insulin, which reflect overall energy stores and short-term gastrointestinal signals that convey information pertaining to individual meals.

Gut hormones released postprandially in a nutrient-dependent manner include cholecystokinin, glucagon-like peptide 1, peptide YY, oxyntomodulin and pancreatic polypeptide. All of these hormones have been shown to reduce food intake when administered peripherally to rodents or humans. In contrast, ghrelin produced primarily in the stomach, has been shown to stimulate food intake. These hormones have been shown to act via both brainstem and more recently hypothalamic arcuate nucleus pathways to modulate behavioural, autonomic and neuroendocrine responses to food intake. Studies in obese rodent models and in human subjects have started to reveal abnormalities in these gut hormone mechanisms in obesity.

The gut hormone peptide3-36 (PYY) is released in response to ingestion of nutrients and in proportion to the calories ingested. In lean, normal-weight and obese human volunteers venous infusion of PYY reduces hunger and food intake. The precise mechanism by which PYY exerts its anorectic effects remains to be determined and evidence exists for hypothalamic, brainstem and vagal sites of action. However, mechanistic data from rodents has confirmed that PYY mediates its anorectic actions via the NPY Y2 receptor.

Obese adults and children have been reported to have low circulating plasma concentrations of PYY, whilst increased levels have been reported in patients with anorexia nervosa. These findings suggest that PYY plays a role in the regulation of body weight and that PYY defiency might be involved in the pathogeneis of obesity. To test this hypothesis we genereated transgenic mice that lacked PYY. We found that from weaning PYY-null mice were hyperphagic and obese and that their obese phenotype was reversible with exogenous PYY treatment.

Chronic peripheral administration of PYY to rodents with diet-induced obesity also reduces food intake and adiposity. Increased protein diets have been shown to reduce hunger, increase satiety and cause greater weight loss than normal-protein diets. Recently, we have shown that high-protein diets cause the greatest satiety and release of PYY compared with high-fat or high-carbohydrate diets. Moreover, we found that PYY was critical for the beneificial effects of high-protein diets. Post-bariatric surgery PYY levels are markedly increased and part of the weight loss effects of this procedure have been attributed to increased PYY. Taken together these findings suggest that increasing PYY plasma concentration either by exogenous administration or by stimulating endogenous PYY release may offer an effective treatment for obesity.

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SA23

Nutrient and hormone sensing by hypothalamic neurons

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The hypothalamus integrates nutrient and hormonal signals of body energy status, which result in altered neuronal responses that act to maintain energy homeostasis. As this brain region plays an important role in the control of body fat content and peripheral glucose levels, defects in this system could, at least in part, account for loss of homeostatic mechanisms resulting in increased adiposity and excessive plasma glucose levels.

Type 2 diabetes is characterized by multiple defects in insulin action and glucose sensing, resulting in fasting hyperglycaemia. These high levels of glucose are mainly determined by increased hepatic glucose production, through increased gluconeogenesis. Consequently, an understanding of the mechanisms by which hepatic glucose production is controlled has important implications for the potential treatment of diabetes. Although leptin exhibits glucose lowering actions in rodents, insulin appears to play a dominant role in the central control of glucose metabolism. Numerous studies have concluded that insulin inhibits hepatic glucose production by both direct and indirect actions. A recently promulgated indirect site of action for insulin modulation of hepatic glucose production in rodents is the hypothalamus. It has been suggested that insulin reduces hepatic glucose output by altering hypothalamic neuron activity resulting in modified vagal control of liver gluconeogenic enzyme levels. Blockade of insulin signalling in the hypothalamus by delivery of insulin antibody, knockdown of insulin receptor by antisense oligonucleotides or inhibition of phosphatidylinositol 3-kinase all decrease the ability of insulin to suppress hepatic glucose production. Furthermore, this central effect of insulin is blocked by administration of glibenclamide, an inhibitor of ATP-sensitive potassium (KATP) channels. Hypothalamic administration of

diazoxide or oleic acid also inhibits hepatic glucose output and gluconeogenesis through increased KATP activity, and mice lacking the KATP channel subunit, SUR1 exhibit an impaired ability for hypothalamic insulin to suppress hepatic gluconeogenesis. It is also proposed that the activation of hypothalamic KATP channels is involved in the counter-regulatory response to hypoglycaemia. Here the fall in plasma glucose levels is detected by the hypothalamus and results in activation of counter-measures such as enhanced secretion of adrenaline and glucagon, which increase hepatic glycogenolysis and reduce insulin secretion, ultimately increasing glucose availability to the brain.
Thus, KATP channels are critical components of the sensing-transduction mechanisms by which hypothalamic neurons respond to changes in nutrient (glucose and fatty acids) and hormone (leptin and insulin) levels that initiate corrective neural-mediated peripheral responses to maintain glucose homeostasis. However, the molecular mechanisms and identities of the individual hypothalamic neurons and circuits responsible for these compensatory feedback systems are presently unclear. Using a combination of biochemical and electrophysiological methods, we are examining the actions of leptin, insulin and fatty acids and of altered glucose concentration on arcuate neuron signalling mechanisms and electrical activity. Two main signalling pathways have been targeted, PI3K and AMPK, as these have been reported to play key roles in the transduction of both hormone and nutrient signals in the hypothalamus. However, biochemical analysis of signalling and electrophysiological examination of unidentified neurons, although informative about mechanisms generally, do not reveal the hypothalamic neurons and pathways responsible for specific outputs. Evidence has implicated the NPY/AgRP and POMC neurons in the ARC as mediators of the central actions of insulin (and leptin) in the control of food intake and body weight. Central administration of NPY increases hepatic gluconeogenesis and weakens insulin suppression of hepatic glucose output. Conversely, activation of hypothalamic melanocortin receptors increases the rate of hepatic gluconeogenesis, whereas administration of melanocortin-4 receptor antagonist decreases hepatic glucose output. As leptin and insulin inhibit NPY and increase POMC hypothalamic gene expression, these neuron types also appear attractive candidates as central mediators of peripheral glucose. However, changes in neuronal peptide expression may not directly equate with neuronal functional outcomes, which are more likely to be dependent upon changes in neuron electrical activity. Our recent findings on the electrophysiological sensitivity of these neurons to leptin, insulin and glucose will be presented and discussed in relation to their potential physiological significance.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA24

Transgenic analysis of hypothalamic function

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The central nervous system plays a key role in regulating energy homeostasis and complex inter-related neuronal circuits have therefore developed to regulate feeding behaviour, energy expenditure and peripheral nutrient handling. The hypothalamus has long been recognised as one of the key brain regions involved in such processes receiving and integrating neural, nutrient, and hormonal signals that convey nutritional status and orchestrating appropriate efferent responses. Until recently both the small size of the hypothalamus and the complexity of its neuronal circuitry have made investigations into the precise anatomical and physiological properties of this brain region difficult. However, the advent in recent years of neuron-specific gene manipulation techniques in mice has permitted an increased understanding of the signals, both extra-cellular and intracellular, and the hypothalamic cell types that regulate energy homeostasis. Work from a number of groups has implicated hypothalamic arcuate nucleus pro-opiomelanocortin (POMC) and agouti-related protein/neuropeptide Y (AgrP/NPY) neurons as playing key roles in regulating energy homeostasis.
Our work has focussed on the role of two signalling pathways in POMC and AgrP/NPY neurons. The insulin receptor substrate (IRS)/ phosphoinositide- 3-OH kinase pathway mediates the effects of the long-term adiposity hormones, insulin and leptin in peripheral tissues. To establish the role of this pathway in hypothalamic function, we have deleted *Irs2* and other signalling components in POMC, AgrP and other neuronal populations. These studies have identified a novel population of neurons in the arcuate nucleus that respond to insulin and play a key role in body weight regulation. The adenosine monophosphate (AMP)-activated protein kinase (AMPK) is an evolutionarily conserved sensor of cellular energy status. Accumulating evidence indicates that AMPK regulates whole body energy homeostasis acting in metabolic tissues in response to nutrient and hormonal signals. We have deleted AMPK in POMC and AgrP neurons and these studies have revealed key roles for this pathway in nutrient sensing. We will discuss our findings in detail and review the phenotypes of other transgenic models to provide a current view of the hypothalamic regulation of energy and glucose homeostasis.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C8

Early life programming of energy balance

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There is growing evidence that early nutrition has a major impact on susceptibility to obesity. To determine the basis for energy balance programming we have established a rat model of altered early life nutrition with control animals (offspring of control dams fed a 20% protein diet), recuperated animals who experienced poor fetal growth then catch-up growth (offspring of dams fed an isocaloric low-protein (8%) diet during pregnancy, but nursed by control dams fed a 20% protein diet) and postnatal low-protein animals who grew slowly during lactation (offspring

of control dams (20% protein) diet nursed by low-protein (8%) diet fed dams)[1]. The impact of this early life nutritional manipulation on the energy balance system was evaluated by measuring hormone concentrations along with anatomically specific hypothalamic expression of genes measured by in situ hybridisation [2,3]. Two male pups were collected from each litter (giving n=8) at weaning. Data is shown as mean ± SEM unless otherwise stated and was analysed using one-way ANOVA. Recuperated pups were smaller at birth (6.6 ± 0.2g vs. 7.5 ± 0.2g, $p<0.001$), but caught up with controls by day 14 (34.9 ± 0.7g vs. 33.4 ± 1.0g). At weaning, despite similar body and fat pat weights, recuperated offspring were hypoleptinemic in the fed state compared to controls (1.9 ± 0.3 ng/ml vs. 4.4 ± 0.4 ng/ml, $p<0.001$). Postnatal low-protein offspring had lower body weights than control offspring (25.0 ± 1.2g vs. 54.6 ± 1.0g, $p<0.001$), and were hypoglycemic (6.2 ± 0.8 mM vs. 9.1 ± 0.6 mM, $p<0.05$) hypoinsulinemic (4 [3-5] pM vs. 96 [74-132] pM, $p<0.001$, data shown as geometric mean and 95% confidence intervals) and hypoleptinemic (1.5 ± 0.3 ng/ml vs. 4.4 ± 0.4 ng/ml, $p<0.001$) at weaning. Leptin receptor gene expression in the arcuate nucleus (ARC) was increased in postnatal low-protein offspring compared to controls (310 ± 54% vs. 100 ± 8%, $p<0.05$) and expression of the suppressor of cytokine signaling-3 (42 ± 14% vs. 100 ± 11%, $p<0.001$) was decreased. Consistent with reduced serum leptin in postnatal low-protein offspring, ARC gene expression for orexigenic neuropeptides, neuropeptide Y (151 ± 8% vs. 100 ± 6%, $p<0.001$) and, agouti-related peptide (201 ± 14% vs. 100 ± 5%, $p<0.001$) was increased, and that for anorexigenic neuropeptides, proopiomelanocortin (41 ± 8% vs. 100 ± 4%, $p<0.001$) and cocaine-and amphetamine-regulated transcript (26 ± 3% vs. 100 ± 4%, $p<0.001$) was decreased. This increase in anabolic drive could indicate that central pathways recognise the energy deficit, but fail to redress the imbalance. These results suggest that the early nutritional environment can affect the development of energy balance circuits and consequently alter future obesity risk.

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Funded by The British Heart Foundation

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA25

Protein quality control in the endoplasmic reticulum

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During biogenesis secretory proteins are translocated into the first compartment of the secretory pathway, the endoplasmic reticulum (ER), through a channel formed by the Sec61 protein. Proteins which fail to fold in the ER lumen are toxic and need to be degraded. Degradation of these proteins is mediated by cytosolic proteasomes and requires protein transport across the ER membrane back into the cytosol. This retrograde transport is also dependent on the Sec61 protein, but the mechanism of retrograde protein unknown. – We asked whether proteasomes were actively involved in extracting misfolded proteins from the ER. We used a cell-free assay (based on yeast ER membranes, in vitro translated proteins and cytosol) that reconstitutes export of a non-glycosylated misfolded protein from the ER and proteasomal degradation. We found that in this assay, proteasomes could fully substitute for cytosol, and were sufficient to promote ER export and degradation of the substrate. We next investigated whether proteasomes were able to directly bind to protein translocation channels in the ER, similar to ribosomes during cotranslational protein import into the ER. We found that proteasomes bind to both yeast and mammalian ER membranes. Ribosomes and proteasomes competed with each other for ER binding, and proteoliposomes containing only Sec61 channels were sufficient for proteasome binding. ER membranes from two *sec61* mutants, *sec61-32* which is defective in protein export from the ER, and a new mutant, *sec61-302*, displayed reduced affinity for proteasomes. Binding of proteasomes to the channel was mediated by the 6 AAA-ATPases (Rpt proteins) in the base of the 19S regulatory particle of the proteasome. These have non-equivalent functions in proteasome-mediated protein turnover and form a hetero-hexameric ring. We purified 19S particles with equivalent point mutations in the ATP-binding sites of individual Rpt proteins; all *rpt* mutant 19S complexes were defective in binding to the ER, suggesting that ATP-binding to the hexameric AAA-ATPase complex of the 19S base is important for this interaction. Using cells expressing epitope-tagged proteasomes we asked if we could isolate protein export channels by solubilising ER membranes and affinity-purification of proteasomes and associated proteins. In addition to the Sec61 channel, we found the ER membrane protein Sec63p, the ER-lumenal Hsp70 BiP, and several subunits of the Hrd1p complex, which is required for ER-associated degradation of misfolded proteins with defects in the ER lumen, associated with ER-bound proteasomes. We conclude that proteasome 19S subparticles are actively involved in protein export from the ER and during export physically interact with the protein export channel in the ER membrane.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA26

RIC-3: a chaperone for neuronal nicotinic acetylcholine receptors

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Nicotinic acetylcholine receptors (nAChRs) are pentameric neurotransmitter-gated ion channels which are expressed at the vertebrate neuromuscular junction (muscle-type nAChRs) and within the nervous system (neuronal nAChRs). Nicotinic receptors have been implicated in several neuromuscular and neurological disorders and are increasingly being seen as important

targets for therapeutic drug discovery. Nicotinic receptors are also expressed within the invertebrate nervous system and are major targets for insecticides which are used extensively in areas of animal health and crop protection. Nicotinic receptors display considerable heterogeneity in their subunit composition (17 different nAChRs subunits have been identified in vertebrates and about 10 subunits in insect species). As with other multi-subunit neurotransmitter receptors, heterologous expression studies have been used extensively to examine the influence of nAChR subunit composition upon the pharmacological and functional diversity of receptor subtypes. However, for several nAChRs subtypes, considerable difficulties have been encountered in efficient functional heterologous expression of recombinant receptors. This has been a particular problem for insect nAChRs and also for several mammalian subtypes such as homomeric α7 nAChRs when expressed in non-neuronal cultured cell lines (1). RIC-3 is a nAChR-associated transmembrane protein, originally identified in *C. elegans* (2), which is encoded by the gene *ric-3* (resistant to inhibitors of cholinesterase). It has also been cloned and characterised from other several other species, including humans. There is evidence that RIC-3 is required for nAChR maturation (2) and it has been shown to enhance levels of functional expression of nAChR subtypes such as α7 when expressed in *Xenopus* oocytes. More significantly, co-expression of human and *C. elegans* RIC-3 is able for facilitate functional expression nAChRs such as α7 in otherwise non-permissive cell lines (3-5). We have also found that RIC-3 can enhance levels of functional expression in cultured cell lines of several other nAChR subtypes, including heteromeric nAChRs such as α4β2 (4). In contrast, there have been reports that RIC-3 reduces or abolishes functional expression of some other nAChR subtypes. Recent studies appear to indicate that the effect of RIC-3 upon maturation and functional expression of nAChRs is influenced markedly by the host cell type.

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Funding provided by the BBSRC, MRC and Wellcome Trust.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C9

Rescue of V2R mutants in NDI by pharmacological chaperones

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Intracellular retention of functional vasopressin V2 receptors (V2R) is a major cause of congenital Nephrogenic Diabetes Inspidus (NDI). Rescue of V2R mutants by pharmacological chaperones may restore their basolateral membrane (BM) localization and function. However, the criteria for efficient rescue of V2R by pharmacological chaperones at clinically feasible concentrations are unknown.

The four non-peptide antagonists SR49059, OPC31260, OPC41061 and SR121463B induced maturation and rescued the BM expression of eight V2R mutants, stably expressed in polarized MDCK cells. The extent of maturation and BM localization correlated with the antagonists' concentration and affinity for the V2R. Displacement of the antagonists by AVP and the subsequent cAMP generation inversely correlated with the antagonists' affinities for the V2R, but is partially influenced by antagonist-specific effects. The low-affinity SR49059 optimally induced functional rescue at high concentrations, due to its easy displacement by AVP. At clinically feasible concentrations, however, only the high-affinity antagonists OPC31260 and OPC41061 induced functional rescue, as at these concentrations the extent of BM expression became limited. As OPC31260 and OPC41061 are clinically safe, they are promising candidates to relieve NDI.

Since receptor activation requires cell-permeable antagonists to be displaced by an agonist, the ability of non-peptide agonists to act as pharmacological chaperones was tested. Six ER-retained mutants responded to treatment with non-peptide agonists, whereas no effect was observed for the peptide agonist AVP. Therefore, non-peptide agonists represent a second class of promising therapeutics to relieve NDI in patients.

This work is supported by the Dutch Kidney Foundation and the Dutch Organisation for Scientific Research

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C10

Recovery of a maturation defective mutant of the α1b adrenoceptor by pharmacological chaperones: biochemical, cytological and pharmacological studies

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Recently, we have identified specific mutations in transmembrane domain I (TMI) and TMIV of the α1b adrenoceptor that have an effect on its quaternary organization as well as in its binding characteristics. In such studies, performed in transiently transfected HEK293 cells, we also detected that the TMI-TMIV mutations affected receptor localization. The mutant receptor was retained inside the cell rather than delivered to the plasma membrane as the wild type receptor and, therefore, failed to generate any intracellular signal. Mutations deriving to intracellular retention as well as the ability of some chemicals and/or specific ligands to recover cell surface expression have been described for various GPCRs(Ref.1). In order to evaluate the consequences of the ER retention as well as the possibility to regain cell surface delivery and function of the mutant receptor, we used cell lines stably expressing either the wild type or mutant α1b adrenoceptors. In this system we observed: 1-. α1b receptor antagonists are able to rescue the plasma membrane delivery of the mutant

receptor. 2-. Prazosin-induced relocation is related to the glycosylation pattern of the mutant receptor 3-. Antagonist-recovered receptor partially recovered the mutant receptor functionality 4-. The differing ligand binding properties between wild type and mutant receptor are due to the mutations per se. and 5-. Native gels show a different quaternary organization of the mutant receptor that shifts to the wild type one when treated with prazosin. These results therefore suggest a link between mutations affecting the molecular organization of some GPCRs and their consequences on receptor localization and function. In addition, we also provide another example of cell surface rescue and function recovery by a specific α1b -adrenoceptor antagonist.

Bernier V, Bichet DG, Bouvier M. Curr Opin Pharmacol. 4(5):528-33. (2004)

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA27

Molecular mechanisms of action and therapeutic potential of pharmacological chaperone on misfolded G protein-coupled receptors

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Conformational diseases are often caused by modest mutations in proteins that are unnecessarily recognized by the endoplasmic reticulum (ER) quality control system as misfolded. A well-characterized example is nephrogenic diabetese insipidus (NDI) that results from mutations within the V2-vasopressin receptor (V2R). To date, over 150 different V2R mutations occurring in unrelated families have been reported. From those tested, approximately 70% lead to trafficking-deficient V2Rs. Given that many of these mutations occurred outside of the predicted hormone binding or G protein-interaction sites, we predicted that rescuing cell surface expression of these receptor mutants could restore function. Based on the known stabilizing effects of ligand binding on receptor structure, we tested the ability of various V2R ligands to rescue the cell surface expression and function of 17 ER-retained NDI V2R mutants. We found that cell-permeant antagonists dramatically increase cell surface expression and rescue the function of 11 of these mutants by promoting their proper folding, maturation and cell surface trafficking. These compounds termed pharmacological chaperones, were found to act inside the cells most likely by binding to, and stabilizing folding intermediates of the mutant receptor thus favoring their proper folding and ER export. Their action in the ER is supported by the observation that sustained treatment with these compounds modulates the interactions of the receptor with ER-resident molecular chaperones such as calnexin and inhibits their ubiquitination and subsequent proteasome-mediated degradation. In a small scale clinical trial, the effect of one pharmacological chaperone, SR49059, was assessed in 5 patients harboring 3 different mutations. In all cases, treatment for 48 hours significantly decreased the urinary output and led to a corresponding elevation of urine osmolality without affecting plasma sodium concentrations. These data provide a proof of principle that pharmacological chaperones could represent valuable therapeutic agents in the treatment of NDI. Mutations in other G protein-coupled receptors have also been found to be involved in various human genetic diseases. For example mutations in the melanocortin type 4 recepto (MC4R) have been associated with congenital morbid obesity. Characterization of 14 of these mutations revealed that most lead to intracelluar retention and that treatments permitting escape from the quality control restore cell surface expression and function of many of these receptors systemt indicating that the development of pharmacological chaperones for the MC4R could represent a valid approach for the treatment of gongenital morbid obesity. In addition to their action on mutant receptors, pharmacological chaperones were found to markedly increase the maturation and the cell surface targeting of wild-type receptors. This indicates that pharmacological chaperoning maybe a general concept that could be applied to both normal and mutant proteins to modulate cell responsiveness to specific hormones thus offering a wide range of potential therapeutic applications.

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SA28

High throughput screening for pharmacological rescue of protein misfolding disorders

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"Pharmacological chaperones" are small molecules that are capable of binding to and stabilizing native structures of otherwise folding-defective mutant proteins and thus represent a promising therapeutic approach for many inherited diseases. To date successful pharmacological rescue has been achieved for a small number of targets using known high affinity agonists or antagonists of the protein products of the mutated genes. In most cases, however, the lack of high affinity ligands or structural information for the mutated gene product precludes the use of a rational approach to design ligands that are suitable for pharmacological rescue. Thus, pharmacological chaperone discovery for the majority of targets requires screening of large and diverse compound libraries using high-throughput screening (HTS) technology. We have developed a sensitive and rapid assay to detect folding of conditional mutants of genes encoding membrane or secreted proteins. This assay exploits enzymatic recombination of two fragments of pancreatic ribonuclease and the use of a hypersensitive fluorescent ribonuclease substrate and is well-suited to standard HTS platforms. We will present data demonstrating the efficacy of this approach for several transmembrane and secreted substrates including cystic fibrosis transmembrane conductance regulator (CFTR) and myocilin, a gene linked to hereditary glaucoma.

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SA29

Leukocyte transmigration in vivo: Role of endothelial cell junctional molecules and basement membrane remodelling

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The migration of leukocytes from the vascular lumen to the extravascular tissue is one of the most dramatic cellular responses observed at sites of inflammation and is a critical event in both innate and adaptive immunity. To achieve this, finely co-ordinated mechanisms must exist by which leukocytes are able to penetrate the vascular wall and migrate into sites of injury or infection without causing gross damage to the vessels from which they emigrate. Despite the tremendous interest in this response and its fundamental role in inflammation, details of the mechanisms by which leukocytes penetrate the vascular wall remain largely unknown. The slow progress in understanding this response is attributable to the complex nature of the vessel wall and the difficulties associated with modelling it in vitro. Specifically, venular walls have two distinct cellular components, endothelial cells (ECs) and pericytes, both of which appear to contribute to the generation of the matrix component of the vessel wall, its basement membrane.

Through formation of complex molecular organisations between adjacent cells, ECs form a confluent layer within the vessel wall and provide the first barrier for emigrating leukocytes. It is generally considered that migrating leukocytes penetrate this barrier by seeking and moving through these junctions and there is now a growing understanding of the mechanisms that mediate this process. Specifically, in addition to PECAM-1, the first EC junctional molecule shown to be involved in leukocyte transendothelial cell migration, there is now substantial in vitro and some in vivo evidence for the involvement of several other molecules including ICAM-2, members of the JAM family, CD99 & ESAM. Despite this growing knowledge, details of the precise involvement of such molecules is however currently unknown and is likely to be complex as the available data implies differential roles of endothelial cell junctional molecules in mediating different stages of leukocyte transmigration, transmigration of different leukocyte sub-types as well as mediating leukocytes transmigration in different vascular beds and in response to different inflammatory stimuli. In addition to migration through EC junctions (paracellular route), there is now a renewed interest in the significance and mechanisms by which leukocytes migrate through the body of the endothelium (transcellular route) though the mechanisms by which this occurs in vivo are not known. Furthermore, there are no indications of the inflammatory conditions that drive leukocytes to migrate via the transcellular route as opposed to the paracellular route.

The perivascular basement membrane (BM) provides a distinct and effective barrier to leukocytes and macromolecules, a barrier that has to be breached at sites of inflammation. Recent findings from our group have led to the identification of neutrophil permissive sites within the BM of mouse cremasteric venules. In these regions the expression of certain BM constituents (eg laminin 10 & collagen IV) were found to be lower than the average level (termed low-expression or LE sites) and the regions appeared to be preferential sites of neutrophil emigration. As these regions were directly co-localised with gaps between pericytes, neutrophil transmigration occurred specifically at sites of least resistance, ie gaps between adjacent pericytes and regions of low protein deposition within the BM. Of interest, neutrophil migration through LE regions resulted in a transient enlargement of these sites, a response that appeared to involve neutrophil elastase.

Collectively, leukocyte transmigration through venular walls involves complex mechanisms that guide leukocytes through the EC barrier and is also associated with elaborate remodelling of the vascular BM. These multiple barriers also appear to facilitate the transmigration process by eliciting distinct signals to transmigrating cells thus inducing an efficient and well-coordinated transmigration response.

This work is supported by the Wellcome Trust, UK, The British Heart Foundation and the European Commission.

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SA30

Defining a role for platelets in allergic inflammation

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There is now considerable evidence suggesting a role for platelets as inflammatory cells. These actions are distinct from their classically known actions performed during thrombosis and haemostasis; and include the expression of adhesion molecules and contact dependent activation of leukocytes, the release of a plethora of inflammatory mediators, activation of cells of the adaptive immune response, and the ability to migrate and undergo chemotaxis.

Chronic asthma is a disease characterised by a mixed inflammatory cell pulmonary infiltrate, airways hyper-responsiveness, and tissue remodelling. Clinical data from asthmatic patients reveals changes in platelet behaviour and function during or after allergen exposure (1). Furthermore, mouse models of allergic inflammation demonstrate a role for intact platelets in eosinophil and lymphocyte recruitment to the lungs, a mechanism that is platelet P-selectin dependent (2). Models of chronic inflammation reveal a participation in platelets in tissue remodelling events whereby platelet depletion was found to be more effective in suppressing airway remodelling processes then the administration of a glucocorticosteroid (3). This process of destruction and repair to the architecture of airway tissue is therefore perhaps enhanced by platelet activation and recent evidence demonstrates platelets can undergo chemotaxis towards allergen via an IgE dependent process and migrate through inflamed tissue, where they localise to specific tissue sites. Indeed, platelets have also been shown to localise to, and interact with airway dendritic cells, suggestive of a link between the innate and adaptive immune response. Thus, these actions may lead to alterations in lung function as platelet depletion suppresses airways hyper-responsiveness in allergic rabbits (4). Thus, further investigations into the

role of platelets in inflammation may be beneficial in the search for future therapeutic targets in the treatment of asthma.

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C11

Differential roles of IL-6 in modulating lymphocyte recruitment across endothelial cells conditioned by dermal or synovial fibroblasts

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The stromal microenvironment plays a pivotal role in the development and persistence of chronic inflammation. Here, we tested the hypothesis that stromal fibroblasts can modulate the ability of endothelial cells (EC) to recruit leukocytes in a site specific manner. We compared the abilities of fibroblasts isolated from either the synovium or skin of patients with rheumatoid arthritis to modify the recruitment of peripheral blood lymphocytes (PBL) by EC, with or without exposure to inflammatory cytokines. EC and fibroblasts were cultured on opposite sides of 0.4μm-pore filters for 24h; a combination of tumour necrosis factor-alpha and interferon-gamma was added for a further 24h when desired. Filters were incorporated into a flow chamber to assess the adhesive properties of PBL.

Co-culture of unstimulated EC with synovial, but not dermal fibroblasts, led to an increase in the capture of flowing PBL. This adhesion was inhibited by blockade of α4-integrin or CXCR4 on PBL, indicating that capture was supported by α4β1-VCAM-1 interaction and stabilised by activation through SDF-1α(CXCL12). Antibody neutralisation of IL-6 during co-culture effectively abolished the ability of EC to recruit PBL. Cytokine-stimulated EC supported high levels of PBL adhesion, through presentation of VCAM-1, E-selectin and chemokine(s) in this case acting through CXCR3. Interestingly, co-culture with dermal fibroblasts caused a marked reduction in cytokine-induced adhesion, whereas synovial fibroblasts induced a slight augmentation. In the dermal co-cultures, neutralisation of IL-6 caused a partial recovery of PBL adhesion, which was complete when both IL-6 and TGF-β were neutralised.

We conclude that resting stromal fibroblasts can regulate the cytokine-sensitivity of vascular endothelium, while fibroblasts isolated from sites of chronic inflammation bypass this and develop a directly inflammatory phenotype. Interestingly, we detected dual roles for IL-6 in regulating 'inflammation' depending on the surrounding milieu. Its action was stimulatory in otherwise unstimulated co-cultures with synovial fibroblasts, but in dermal co-cultures treated with cytokines, IL-6 combined with TGF-β to desensitise the endothelial cells.

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C12

Heparanase induces inflammatory cell recruitment to the peritoneal cavity of the rat

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The endoglycosidase heparanase1 (HPSE1)[1,2] is known to be involved in tumour cell migration.[3] HPSE1 selectively cleaves heparan sulphate proteoglycans, which are ubiquitously expressed and form a major component of sub-endothelial matrix, as well as being strongly expressed on the surface of endothelial cells and circulating blood elements. The metastatic potential of tumour cells correlates with HPSE1 expression[3] and inhibition of this enzyme has been demonstrated to reduce tumour cell adhesion, migration and colonisation.[3,4] Fewer studies have looked at the role of this enzyme in inflammation. However, leucocytes express heparanase, which is thought to facilitate their trafficking to sites of tissue inflammation and heparan sulphate influences the biological effects of an array of proteins, including chemokines and growth factors, involved in the inflammatory response.[5]

In the present study, we investigated the effects of recombinant HPSE1, pro-HPSE1 (inactive precursor) and enzymatically inactivated HPSE1, on inflammatory cell recruitment to the peritoneal cavity and leucocyte-endothelial interactions in the mesenteric microcirculation of the rat.

Intraperitoneal injection of HPSE1 (500 μg) induced a significant inflammatory cell infiltrate, assessed 4 h later by peritoneal lavage immediately post mortem ($1.05 \pm 0.24 \times 10^6$ *vs* $0.28 \pm 0.04 \times 10^6$ cells ml^{-1} saline control; n=6 per group, $P<0.05$). This was similar in magnitude to the response to IL1β (20 ng; $1.12 \pm 0.28 \times 10^6$ cells ml^{-1}) but co-administration of HPSE1 and IL-β did not elicit a greater response than either stimulus alone. Intravital microscopy of the mesenteric microcirculation, under terminal anaesthesia, 4 h post-HPSE1 injection, showed an increase in rolling cell flux (35 ± 2.9 *vs* 15 ± 2.1 rolling cells min^{-1} saline control; $P<0.05$) and adherent cells (30.3 ± 2.0 *vs* 3.5 ± 0.7 adherent cells per 100 μm saline control; $P<0.05$) in post-capillary venules (n=6 animals per group). Again, these responses were similar to those elicited by IL-1β (38 ± 1.8 rolling cells min^{-1}, 36.5 ± 2.9 adherent cells per 100 μm) and were sensitive to heparin, a non-selective inhibitor of HPSE1 activity. Pro-HPSE1 had similar effects to the active enzyme with respect to leucocyte accumulation in the peritoneal cavity, suggesting that the exogenously administered pro-enzyme is effectively processed to the active form *in vivo*. Finally, we further demonstrated a requirement for HPSE1 enzymatic activity in our model by assessing the effects of heat-inactivated enzyme in peritoneal lavage experiments, which had no effect on cell recruitment.

Our data further indicate a role for heparanase in leucocyte recruitment, suggesting this enzyme to be a potential therapeutic target in inflammatory disease.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA31

The role of PI3K in lymphocyte motility in vivo

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Class I phosphoinositide 3-kinase (PI3K) enzymes produce lipid second messengers that regulate diverse biological processes. In neutrophils, activation of class I PI3Ks is crucial for directed motility. However, the roles of class I PI3K isoforms in the basal and directed motility of lymphocytes are poorly understood. Using two-photon microscopy, we have identified PI3K as an important signaling molecule for maintaining basal T and B lymphocyte motility and homing in the intact lymph node. Pharmacological
inhibition of PI3K catalytic isoforms exerted broad effects on basal lymphocyte motility, including changes in homing kinetics, localization of B cells within the lymph node, and reduced cell velocities. Lymphocytes deficient in either or both of the Class IA PI3K regulatory subunits p85α and p85β also exhibited reduced velocities, to varying extents depending on cell-type and isoform-specificity. B cells deficient in p85α exhibited gross morphological abnormalities, which were not evident in cells treated with a PI3K inhibitor. Our results show, for the first time, that class IA PI3Ks play an important role in maintaining basal lymphocyte motility, and that p85α regulatory subunit expression is required to maintain B cell morphology in a manner independent of PI3K catalytic function. Moreover, we demonstrate distinct roles for catalytic domain function and class IA PI3K regulatory domain activity in lymphocyte motility, homing and homeostatic localization of resting B cells.

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SA32

Novel mechanisms of memory T cell trafficking

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Productive T cell immunity requires both the activation and the migration of specific T cells to the antigenic tissue. Naïve T cells are programmed to recirculate predominantly in secondary lymphoid tissue by non-specific cell:cell interactions and chemokines. In contrast, memory T lymphocytes must locate antigen location in non-lymphoid sites as their ability to respond is dependent on further antigen receptor triggering. The co-stimulatory molecule CD28 plays an essential role in the initiation of T cell-mediated immunity. We investigated the possibility that CD28 may also regulate migration of primed T cells to target tissue. *In vitro*, CD28-mediated signals enhanced T cell transendothelial migration, integrin clustering and integrin-mediated migration. *In vivo*, intact CD28 signalling – particularly PI3K activation - was required for efficient localization of primed T cell to non-lymphoid antigenic tissue. Importantly, antibody-mediated CD28 stimulation led to unregulated memory T cell migration to extra-lymphoid tissue, which occurred independently of TCR-derived signals and homing-receptor expression. Finally, we observed that CD28- and CTLA-4-mediated signals exert opposite effects on T cell trafficking *in vivo*. These findings highlight a novel physiological function of the balance of co-stimulatory signals in the regulation of memory T cell trafficking, which has crucial implications for the therapeutic manipulation of these and other co-stimulatory molecules.

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SA33

Imaging glutamate concentrations and new protein synthesis at synapses

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We have developed GluSnFRs, genetically encoded glutamate-sensing fluorescent reporters based on cyan and yellow fluorescent proteins surrounding a bacterial glutamate binding protein. Our newest GluSnFR, in which linker sequences and glutamate affinities have been systematically optimized, exhibits a 6.8-fold improvement in response magnitude over its predecessor and a dissociation constant of 11 μM. We demonstrate quantitative optical measurements of the timecourse of synaptic glutamate release, spillover and reuptake in cultured hippocampal neurons with millisecond resolution. These results indicate that significant glutamate spillover can occur between synapses. Active reuptake is shown to be the dominant mechanism of glutamate clearance from the dendritic surface. A simple kinetic model of GluSnFR measurements suggests that frequency of stimulation

modulates peak and average spillover glutamate concentrations, but does not change the proportion of glutamate recovered.

Because many forms of long-lasting learning and memory are associated with expansion or de novo formation of synapses, visualizing recently expanded or formed synapses may shed light on where memories are stored. Time-lapse microscopy can track synaptic growth in sparsely labeled neurons in superficial brain regions, but identifying growing synapses throughout a functional nervous system is currently not possible. One approach may be to label newly synthesized synaptic proteins as markers of synaptic birth or growth, analogous to labeling newly synthesized DNA to identify newly born cells. Existing methods for visualizing newly synthesized proteins that rely on sequential biarsenical labeling of tetracysteines or photoconversion of fluorescent proteins are not compatible with deep tissues or freely behaving animals, and suffer from problems with toxicity and sensitivity. Here we report TimeSTAMP, a time-specific tag for the age measurement of proteins, which allows small-molecule-triggered epitope tagging of newly synthesized proteins of interest. We use TimeSTAMP to relate new postsynaptic density protein accumulation to synaptic growth in cultured neurons and to visualize protein synthesis in living animals. Our results open up the possibility of retrospective identification of sites of synaptic plasticity anywhere in the nervous systems of freely behaving animals.

HHMI, NIH NS27177 to RYT, Jane Coffin Childs fellowship to MZL.

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SA34

Crosstalk between MAP kinase and cAMP during the formation of hippocampus-dependent memory

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The cAMP and Erk/MAP kinase (MAPK) signal transduction pathways are critical for hippocampus-dependent memory. However, the extent of coactivation and crosstalk between these pathways during fear conditioning has not been described. Here we report that PKA and MAPK are co-activated in a subset of hippocampal CA1 pyramidal neurons following contextual fear conditioning. Activation of MAPK was absolutely dependent on Ca^{2+}-stimulated adenylyl cyclase activity. Furthermore, there was a strong correlation between stimulation of MAPK and MSK-1, a downstream CREB kinase. These data indicate that activation of adenylyl cyclase is critical for memory formation because PKA stimulation supports the nuclear translocation and activation of MAPK following training.

This work was supported by National Institutes of Health grants NS 20498 and MH 073601.

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C13

Role of calcium-stimulated adenylyl cyclases in the modulation of the slow afterhyperpolarisation in hippocampal pyramidal neurons

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The slow afterhyperpolarisation (sAHP) follows bursts of action potentials, lasts 1-3 s and underlies the late phase of spike frequency adaptation in cortical neurons. The sAHP is mediated by a calcium-activated potassium current (sIAHP) that is inhibited by different monoaminergic neurotransmitters leading to activation of an adenylyl cyclase resulting in an increase in cAMP and the subsequent activation of protein kinase A (PKA) (1). Our aim was to investigate the role of calcium stimulated adenylyl cyclases (AC1, AC8) in the modulation of sIAHP in CA1 pyramidal neurons. We compared wildtype (WT) and double knockout mice for both AC1 and AC8 (DKO) using the whole cell patch clamp technique in voltage- and current-clamp modes on hippocampal slices. The monoaminergic inhibition of both sIAHP and sAHP was examined through bath application of isoproterenol, serotonin and dopamine. We found that inhibition of sAHP and sIAHP by these neurotransmitters remains intact in DKO mice. High frequency stimulation of the Schaffer collateral pathway results in a transient inhibition of sAHP in CA1 pyramidal neurons due to calcium influx through NMDA receptors and activation of PKA (2). Therefore we investigated whether this pathway was disrupted in DKO mice. Recordings were performed at 30°C in the presence of the GABA(A) receptor antagonist picrotoxin (50 μM). Following high frequency stimulation of the Schaffer collateral pathway, the sAHP was transiently suppressed by 23.5±2.6% (N=11, n=14, $p<0.0001$) in the first six minutes following stimulation. This suppression was dependent on the activation of NMDA receptors (NMDAR), since it was abolished by the NMDAR antagonist AP5 (100 μM) applied 30 mins prior to stimulation (% sAHP suppression in AP5: –8.8±4.9% N=5, n=5, $p<0.0001$). The suppression of sAHP following high frequency synaptic stimulation was significantly attenuated in DKO mice (% sAHP suppression in DKO: 6.1±2.7%, N=6, n=10, $p<0.0001$). Similar results were obtained with another stimulation paradigm known to activate PKA (3) consisting of pairing 5Hz stimulation of the Schaffer collaterals to postsynaptic depolarisation. Also under these conditions, the suppression of the sAHP was significantly attenuated in DKO compared to WT mice (% sAHP suppression in WT: 42.9±5.2%, N=9, n=10; in DKO: 13.2±4.4, N=9, n=11; $p<0.0001$). Our results suggest that specific patterns of synaptic stimulation can lead to the activation of AC1/AC8 by calcium influx through NMDAR resulting in generation of cAMP, activation of PKA and subsequent reduction in sAHP.

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This work was supported by grants from the MRC and the Wellcome Trust.

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C14

The L1 cell adhesion family and their interaction with the 4.1 superfamily

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The L1 immunoglobulin (Ig) subfamily of cell adhesion molecules includes L1, NrCAM, (Neuron glial-related cell adhesion molecule) and neurofascin. Their fundamental importance in mammalian development is highlighted by their constituting 1% of all membrane proteins in mature brains; their involvement in growth cone and synapse formation, and cancer development. Mutations can lead to human retardation and knockout studies show phenotypic changes. We have shown that these receptors bind to differing cytoplasmic proteins and so elicit differing signals. From these studies we found that both neurofascin and L1 but not NrCAM, can bind to the 4.1 superfamily protein member, Ezrin, but by different binding motifs (Gunn-Moore et al., 2006). Physiologically the interaction of Neurofascin and Ezrin appears to occur in the microvilli of interdigitating Schwann cells over the node of Ranvier, whilst L1 and Ezrin interaction is important for neuronal growth. This interaction between Neurofascin and Ezrin was via the FERM (4.1 Ezrin-Radixin-Moesin) domain of Ezrin and 28 amino acid sequence at the cytoplasmic C-terminus of Neurofascin. As part of these studies, we identified a novel FERM containing protein, "Willin". Willin has a recognizable N-terminal FERM domain, which is able to bind both phospholipids and proteins (Gunn-Moore et al., 2005). Recently Willin has been identified as the human homologue to the Drosophila protein, Expanded, which associates with Merlin, a tumour suppressor protein responsible for neurofibromatosis. We have shown that Willin is expressed in the peripheral nervous system in Schwann cells and we are currently investigating its association with Neurofascin and Merlin.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA35

Compartmentalization of functional plasticity: the basis for memory formation

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The main cellular mechanisms underlying memory formation are protein synthesis-dependent forms of long-term potentiation (late-LTP) and long-term depression (late-LTD). Recent data support the hypothesis that neurons store relevant information by processes of 'synaptic tagging' in dendritic, functional compartments during late-LTP and late-LTD, rather than in single synapses. Plasticity-related proteins (PRPs), partially non-specific to the locally induced process are synthesized in dendritic compartments and then captured by local, process-specific synaptic tags. We support these findings in two ways 1) late-LTP/LTD, locally induced in apical or basal dendrites of hippocampal CA1 neurons, normally does not spread to the basal or apical compartment, respectively; 2) the specificity of the synaptic plasticity event is achieved by the activation of process- and compartment-specific synaptic tag molecules. We have identified CaMKII as the first LTP-specific, and ERK1/2 as LTD-specific tag molecules in apical dendritic CA1-compartments, while PKA and PKMζ jointly mediate LTP-specific tags in basal dendrites. Under distinct circumstances however, functional compartmentalization can be overwritten by behavioral acts representing specific information. I will present findings supporting this hypothesis.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA36

Disrupted In Schizophrenia 1 and Phosphodiesterase 4B: Towards an understanding of psychiatric illness

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The Disrupted In Schizophrenia 1 (DISC1) gene was identified as a genetic risk factor for schizophrenia and affective disorders because it is disrupted by a balanced chromosomal translocation that co-segregates with major psychiatric illness (schizophrenia, bipolar affective disorder and severe recurrent depression) in a large family from Scotland (1). Several genetic studies have since provided further evidence for involvement of DISC1 in psychiatric illness, and in associated cognitive functions (1). DISC1 interacts with phosphodiesterase 4B (PDE4B), an independent risk factor identified due to disruption of the gene by a chromosomal translocation in cousins diagnosed with schizophrenia and psychosis (2). Subsequent genetic studies have confirmed the involvement of PDE4B in causing susceptibility to psychiatric illness, and intriguingly have also demonstrated a

likely involvement of the related PDE4D gene (3). PDE4B and PDE4D are members of the four gene PDE4 family (PDE4A-D), and DISC1 can bind protein isoforms expressed from each gene (2). PDE4s are homologues of the drosophila Dunce gene involved in learning and memory (4). Cyclic AMP hydrolysis activity of PDE4 isoforms is inhibited by the prototypic antidepressant drug rolipram (4), and mice deficient in PDE4B and PDE4D behave as if on antidepressants (5). Thus the interaction between DISC1 and PDE4s is likely to be of direct relevance to the pathology of major mental illness. DISC1 binding to PDE4s is cAMP-dependent, with specific isoforms dissociating in response to elevated cAMP levels, conditions which also lead to PDE4 activation (2). It is therefore possible that DISC1 sequesters PDE4 in an inactive state, releasing active PDE4 when cAMP signalling cascades are switched on, and thus modulating cAMP signalling. Two ENU-induced Disc1 missense mutations have been identified in mice, leading to phenotypes related to schizophrenia and depression that respond to antipsychotic and antidepressant (including rolipram) drug treatment. These mutations are located within PDE4B contact sites and result in significantly reduced binding between Disc1 and PDE4B isoforms. One mutation is also associated with significantly reduced PDE4B catalytic activity. Altered Disc1/PDE4B function is therefore critical to the phenotypes of these mice, suggesting that the same may be true of human psychiatric disorders.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA37

Homocysteine metabolism in diabetes

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Homocysteine (Hcy) arises as an intermediate in methionine metabolism. It is produced as a result of S-adenosylmethionine-dependent transmethylation reactions and is removed, either by remethylation (conversion to methionine) or transsulfuration (conversion to cysteine). These pathways are highly dependent on B-vitamin status and, consequently, Hcy levels are very sensitive to B-vitamin deficiency, particularly that of folate, B12 and pyridoxal. Elevated plasma Hcy is an independent risk factor for a number of chronic diseases, such as cardiovascular disease, Alzheimer's disease and osteoporosis. Elevated Hcy is associated with a higher risk of cardiovascular disease in diabetes mellitus than in the general population. Diabetes affects plasma Hcy in two quite different ways. In newly diagnosed Type 1 diabetes, plasma Hcy is often decreased whereas when diabetes is advanced, particularly when renal complications develop, plasma Hcy becomes elevated. Plasma Hcy is inversely related to glomerular filtration rate, regardless of the presence of diabetes. We have examined these phenomena in rats. We find that plasma Hcy is lower in rat models of both Type 1 (streptozotocin-induced) and Type 2 (ZDF fa/fa) diabetes. This decrease is accompanied by increased hepatic activities of two enzymes that remove Hcy, cystathionine beta-synthase and betaine:homocysteine methyltransferase. We have also found, in rat H4IIE cells, that insulin directly decreases the expression of these genes. Two consequences of the increased betaine:homocysteine methyltransferase activity in the diabetic rats are substantially reduced liver betaine levels and an enhanced ability of betaine to decrease Hcy production by isloated hepatocytes. Studies in rats have also revealed a major role for the kidney in the metabolism of plasma Hcy; we find a substantial arterial-renal venous difference for Hcy across the rat kidney. Since urinary Hcy is trivial, this implies a substantial intra-renal metabolism of Hcy. This occurs, via the transsulfuration pathway, in the cells of the proximal tubules. These observations are consistent with the increased Hcy that occurs in renal disease although data on renal Hcy metabolism in humans remain controversial.

Canadian Institutes of Health Research, Canadian Diabetes Association.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA38

Amino acid metabolism, insulin secretion and diabetes

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Specific amino acids are known to acutely and chronically regulate insulin secretion from pancreatic beta-cells in vivo and in vitro. Mitochondrial metabolism is crucial for the coupling of amino acid and glucose recognition to exocytosis of insulin granules. This is illustrated by in vitro and in vivo observations that mitochondrial dysfunction severely impairs insulin secretion. Mitochondria generate ATP, which is the main coupling messenger in insulin secretion, and other coupling factors, which serve as sensors for the control of the exocytotic process [1]. Numerous studies have sought to identify the factors that mediate the key amplifying pathway over the Ca2+ signal in nutrient-stimulated insulin secretion. Predominantly, these factors are nucleotides (ATP, GTP, cAMP, and NADPH), although metabolites have also been proposed, such as long-chain acyl-CoA derivatives and glutamate. This scenario further highlights the importance of the key enzymes or transporters, e.g., glutamate dehydrogenase, the aspartate and alanine aminotransferases and the malate-aspartate shuttle in the control of insulin secretion, see Figure 1 [2]. The coupling of cytosolic glycolytic NADH production with the mitochondrial electron transport chain is crucial for pancreatic beta-cell function and energy metabolism. The activity of lactate dehydrogenase in the beta-cell is low, thus glycolysis-derived electrons are transported towards the mitochondrial matrix by a NADH shuttle system, which in turn regenerates cytosolic NAD+. Mitochondrial electron transport then

produces ATP, the main coupling factor for insulin secretion. Aralar1, a Ca2+-sensitive member of the malate–aspartate shuttle expressed in beta-cells, has been found to play a significant role in nutrient-stimulated insulin secretion and beta-cell function. Increased capacity of Aralar1 enhances the responsiveness of the cell to glucose. Conversely, inhibition of the malate–aspartate shuttle results in impaired glucose metabolism and insulin secretion. Recent work has described potentiating or attenuating activities of various amino acids on insulin secretion, mitochondrial membrane potential and NADH production in Aralar1-overexpressing beta-cells. This work has provided evidence for a central role of Aralar1 in the regulation of nutrient metabolism in the beta-cell. Therefore, amino acids may play a direct or indirect (via generation of putative messengers of mitochondrial origin) role in insulin secretion and action. Furthermore in periods of fasting or starvation, amino acid release from skeletal muscle (primarily L-glutamine and L-alanine) may modulate glucagon release from pancreatic alpha-cells, which subsequently may influence insulin secretion from beta-cells. Dietary amino acids may also stimulate incretin release, e.g., GLP-1, from intestinal L-cells and therefore stimulate insulin secretion via indirect mechanisms. Work in my laboratory has demonstrated that amino acid metabolism in the beta cell is essential for acute stimulation of insulin secretion in the presence of stimulatory concentrations of glucose [3, 4]. The key roles of amino acid metabolism in the beta cell will be illustrated using the examples of L-alanine and L-glutamine.

In addition, after chronic exposure, amino acids may influence gene expression in the beta-cell, which subsequently alters levels of insulin secretion. Expression profiling of clonal BRIN-BD11 beta cells was performed using oligonucleotide microarray analysis. Culture for 24 hours with 10 mM L-glutamine compared to 1mM resulted in substantial changes in gene expression including many genes involved in cellular signaling, metabolism, gene regulation and the insulin secretory response. Subsequent functional experiments confirmed that L-glutamine increased the activity of the Ca2+ regulated phosphatase calcineurin. L-glutamate was released into the extracellular medium at high rates from beta cells exposed to L-glutamine [5]. These observations indicate important long-term effects of L-glutamine in regulating beta cell gene expression, signaling and secretory function.

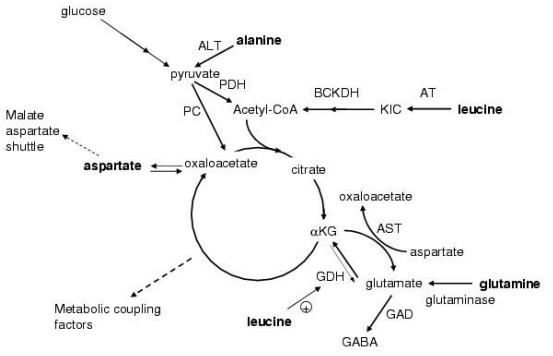

Fig. 1 Schematic describing metabolism of selected amino acids and the related production of metabolic stimulus-secretion coupling factors involved in insulin release. The pathway of glutamine metabolism via glutaminase, GDH, and entry into the TCA cycle (glutaminolysis) is illustrated along with essential points of amino acid interaction with glutamine and glucose metabolism.

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Health Research Board of Ireland, Enterprise Ireland and the Wellcome Trust.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C15

PERK is an intracellular ATP sensor in pancreatic b-cells

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PERK, an ER luminal eIF2alpha kinase, is essential for beta-cell function, as lack of functional PERK leads to beta-cell death and infancy onset diabetes, indicating a pro-survival role for the phosphorylation of eIF2alpha in beta-cells. PERK is activated in response to protein malfolding in the ER as part of the unfolded protein response (UPR) and it is widely believed that PERK is activated at high glucose due to an increased rate of protein synthesis, which exceeds the folding capacity of the ER. However, we demonstrate in pancreatic beta-cells that at high glucose concentration, neither PERK is activated or eIF2alpha phosphorylated. Whereas, in contrast, we show that at low glucose concentration, PERK is activated and eIF2alpha phosphorylated and that the over-expression of dominant-negative PERK inhibits this glucose dependent change in eIF2alpha phosphorylation. Indeed, by translational profiling we confirm that the eIF2alpha dependent integrated stress response is only activated at low glucose concentrations. By artificially altering cellular ATP levels, we were able to provide evidence that it is a decrease in ATP concentration activates PERK and stimulates the phosphorylation of eIF2alpha. Therefore, PERK can act as an intracellular ATP sensor in pancreatic beta-cells. The mechanism by which PERK is activated by glucose deprivation and the important physiological significance of these findings in will be discussed.

This work was supported by the Wellcome Trust

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C16

Glucose-dependent modulation of insulin secretion and intracellular calcium ions by GKA50 – a glucokinase activator

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Objective. As glucokinase (GK) is a metabolic sensor involved in the regulated release of insulin, we have investigated the acute actions of a novel GK activator – GKA50, on islet function. Research Design and Methods. Insulin secretion was determined by ELISA and microfluorimetry with fura-2 was used to examine intracellular Ca^{2+} homeostasis ($[Ca^{2+}]_i$) in isolated mouse, rat and human islets of Langerhans, and in the MIN6 insulin-secreting cell line. Results. In rodent islets and MIN6 cells, 1μM GKA50 was found to stimulate insulin secretion and raise $[Ca^{2+}]_i$ in the presence of glucose (2-10mM). Similar effects on insulin release were also seen in isolated human islets (n=2 donors). GKA50 (1μM) caused a leftwards shift in the glucose-concentration response profiles and the EC_{50} values for glucose were shifted by 3mM in rat islets (n=5) and approximately 10mM in MIN6 cells (n=7). There was no significant effect of GKA50 on the maximal rates of GSIS (n=7). In the absence of glucose GKA50 failed to elevate $[Ca^{2+}]_i$ (1μM GKA50) (n=4) or stimulate insulin release (30nM-10μM GKA50, n=3). At 5mM glucose the EC_{50} for GKA50 in MIN6 cells was approximately 0.3μM (n=7). Inhibition of GK with mannoheptulose or 5-thioglucose selectively inhibited the action of GKA50 on insulin release, but not the effects of tolbutamide (n=3). Similarly, 3-methoxyglucose prevented GKA50-induced rises in $[Ca^{2+}]_i$ but not the actions of tolbutamide (n=10). Finally, the K_{ATP} channel agonist diazoxide (200μM) inhibited GKA50-induced insulin release (n=4) and its elevation of $[Ca^{2+}]_i$ (n=8). Conclusions. We show that GKA50 is a glucose-like activator of insulin-secreting cell metabolism in rodent and human islets and a Ca^{2+}-dependent modulator of insulin secretion.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA39

Amino acids and mTOR signalling in anabolic function

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The mammalian target of rapamycin, mTOR, plays a key role in the control of a number of cell functions. The best-understood of these is protein synthesis, where mTOR regulates proteins involved in the initiation and elongation stages of mRNA translation. These proteins are regulated via changes in their states of phosphorylation. Although mTOR itself has protein kinase activity, it is clear that mTOR does not itself catalyze all these phosphorylation events.

mTOR forms complexes with several partner proteins to yield complexes termed mTOR complexes 1 and 2. Rapamycin inhibits many of the functions of mTORC1, but not mTORC2. One component of mTORC1 is raptor, which acts as a scaffold protein by interacting with proteins that are substrates for phosphorylation by mTOR via their TOR-signalling (TOS) motifs.

Signalling through mTORC1 is activated by amino acids and by anabolic or mitogenic stimuli (e.g., insulin, growth factors). The mechanism by which amino acids stimulate mTORC1 is unclear, and unanswered questions remain about its activation by hormones and growth factors. The latter is known to involve the small G-protein Rheb, which acts to stimulate signalling through mTORC1. It is clear that mTORC1 signalling is important for cell proliferation and cell growth. The latter is exemplified by the fact that rapamycin can prevent, or even reverse, cardiac hypertrophy, a condition that is characterized by elevated rates of protein synthesis and increased size of cardiomyocytes.

To test the role of Rheb and mTORC1 signaling in regulating protein synthesis in heart cells, we expressed Rheb in adult rat cardiomyocytes using an adenoviral vector. This increased the rate of protein synthesis and led to an increase in cell area and cell volume. Both effects were inhibited by rapamycin. This demonstrates that Rheb-induced activation of mTORC1-signalling is sufficient to stimulate the growth of cardiomyocytes. We are currently examining which mTORC1-activated steps in mRNA translation are important for the activation of protein synthesis in cardiomyocytes.

In yeast, 14-3-3 proteins play a key role in rapamycin-sensitive signalling. These proteins bind to phosphorylated proteins and may modify their functions in a variety of ways. PRAS40 (proline-rich Akt substrate, 40kDa) can interact with 14-3-3 proteins, and this requires both amino acids and insulin, a feature that suggested it might be regulated by mTORC1. Overexpression of Rheb overcomes the requirement for amino acids, consistent with the idea that PRAS40 is indeed controlled by mTORC1.

We have found that PRAS40 interacts with raptor and that this requires a TOS motif within PRAS40. In fact, PRAS40 associated stably with raptor/mTOR, indicating that it is a new component of mTORC1. PRAS40 is phosphorylated by mTOR in vitro, and this occurs at a novel site, distinct from the one phosphorylated by the insulin-stimulated kinase Akt (protein kinase B). To test the cellular role of PRAS40, we used siRNAs to knock down its expression. Surprisingly, this impaired signalling from mTORC1 to targets such as ribosomal protein S6. Thus, PRAS40 is a novel component of, and target for, mTORC1.

As mentioned above, mTORC1 controls the elongation phase of mRNA translation. It does so by regulating the kinase that phosphorylates and inactivates eukaryotic elongation factor 2 (eEF2). eEF2 mediates the movement of the ribosome along the mRNA. Signaling downstream of mTORC1 brings about the inactivation of eEF2 kinase and thus the dephosphorylation/activation of eEF2. This regulation of eEF2 kinase involves its mTORC1-dependent phosphorylation at 3 or more sites (Ser78/359/366). However, eEF2 kinase does not possess a TOS-motif and is not a substrate for mTORC1. This indicates that additional kinase(s) link mTORC1 to the control of eEF2 kinase.

We therefore developed assays to allow us to screen for kinases acting at regulatory sites in eEF2 kinase. Using this, we have purified and identified the kinase that phosphorylates Ser359 in eEF2

kinase. This enzyme is a cyclin-dependent kinase, whose activity is positively regulated by amino acids. Judging by several criteria, it is a target for control by mTORC1 signalling. Further data on its regulation will be presented.
In addition to providing one link between mTOR signalling and the control of eEF2 kinase, this discovery may have important implication for the control of the cell cycle by mTORC1. Such links are of particular interest given that hyperactivation of mTORC1 leads to dysregulation of cell proliferation and to cancer.

Work on mTOR signalling in the speaker's laboratory is supported by the Canadian Institutes for Health Research and the Ajinomoto Amino Acid Research Program.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA40

Muscle free amino acids in the intensive care unit

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The pattern of free amino acid in tissues is quite different from the pattern in plasma. The gradients between extracellular and intracellular space is highly variable and range from 1 to almost 1000. The amino acids with the highest ratio are taurine and glutamate. At the other end of the span we have amino acids with a small gradient, usually essential amino acids such as phenylalanine. Cysteine is a special case where we have the oxidised and reduced forms and the majority of the cysteine in blood is stored into the erythrocytes.
This normal pattern of free amino acids in muscle is a reflection of protein synthesis and degradation, interorgan transplantation of free amino acids as well as amino acids transported across the cellular membrane. It is also a reflection of denovo synthesis and break down or oxidation of the free amino acids. Taking into account the many inputs and outputs, the intracellular free amino acid concentrations is remarkable stable. Physiological changes in the normal state, such as defect of feeding, only gives marginal effects on the intracellular concentration although the plasma concentrations may change considerable.
In general terms there are two major differences between the healthy individuals and the ICU patients. 1) The low concentrations of glutamine, glutamate and the basic amino acids. 2) The comparatively high concentrations of the branched chain amino acids and the aromatic amino acids. In particular the concentration of glutamine is very low compared to healthy individuals, 20 – 25 % of normal value. Although these patients also have a low plasma glutamine concentration, the gradient is also lower than normal. This low value appears early in the course of ICU stay and is rather unaltered also during a long stay in the intensive care unit. There are evidence that the denovo synthesis of glutamine in muscle is not different from normal, this is reflected in the endogenous rate of appearance which is reported to be in the normal range also when a depletion in plasma or intracellular is muscle is seen. The effects of the low values of the basic amino acids, lysine, histidine and arginine, is less well known. It is highly unlikely that the depletion of arginine will have an effect upon NO-production. This is however a matter of controversy, in particular as iNO may respond to substrate availability.
The higher than normal levels of the branched chain and aromatic amino acids are thought to be a reflection of the increase in protein break down. These amino acids are not denovo produced in muscle, as they are essential. In particular the branched chain amino acids are usually oxidised to large extent in skeletal muscle. This seems to happen to a lesser degree in ICU patients. Among the aromatic amino acids in particular phenylalanine is often increasing over time. And the balance of phenylalanine across the leg is sometimes used as an indicator of muscle protein depletion.
The free amino acids in muscle in intensive care unit patients show a specific and reproducible pattern. The interpretation of this pattern is not yet fully understood. Availability of the different amino acids seems to have little impact on this pattern and the restoration back to normal takes a long time.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA41

GSK3β regulates synaptic plasticity in the hippocampus

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Glycogen synthase kinase-3 (GSK3) has been implicated in major neurological disorders but its role in normal neuronal function is largely unknown. Here we show that GSK3β mediates a novel interaction between the two major forms of synaptic plasticity in the brain, N-methyl-D-aspartate (NMDA) receptor-dependent long-term depression (LTD) and long-term potentiation (LTP). First we show that GSK3β is required for LTD; GSK3β inhibitors completely block the induction of LTD and the activity of GSK3β is enhanced during LTD. Next we show that LTP inhibits the activity of GSK3β and completely blocks the ability of synapses to undergo LTD for up to one hour, also via an NMDA receptor dependent mechanism. A key determinant of whether NMDA receptor activation leads to LTD or its inhibition may be the phosphorylation status of ser9 in GSK3β. During LTD, the activation of protein phosphatase 1 (PP1) leads to dephosphorylation of this residue, which equates to an increase in GSK3β activity. In contrast, during LTP, activation of the PI3K/Akt pathway leads to phosphorylation of ser9 and hence inhibition of GSK3β. We conclude that the regulation of GSK3β activity provides a powerful mechanism to preserve NMDA receptor encoded synaptic information from being erased by subsequent LTD, perhaps thereby permitting the consolidation of learnt information.

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SA42

Hippocampal long-term depression is critical for spatial memory

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Hippocampal long term potentiation (LTP) and depression (LTD) of glutamatergic transmission have been studied as cellular substrates for learning and memory. However, their exact roles in learning and memory are not well established, largely due to the lack of specific inhibitors for either LTP or LTD. It is generally accepted that the induction of both LTP and LTD at the CA1 synapse is postsynaptic and dependent upon Ca2+ influx through activated N-methyl-D-aspartate subtype glutamate receptors (NMDARs). However, the mechanisms underlying the expression of LTP and LTD remain hotly debated, and likely involve both a presynaptic component via alteration of transmitter release and a postsynaptic one through the modification of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid subtype glutamate receptors (AMPARs). Recent studies from many laboratories including our own have provided substantial evidence suggesting that AMPARs are continuously cycling between the plasma membrane and intracellular compartments via vesicle fusion mediated plasma membrane insertion and clathrin dependent endocytosis and that facilitated AMPAR insertion and endocytosis at postsynaptic membranes contributes to the expression of LTP and LTD, respectively. Using a combination of recombinant receptor expression systems and hippocampal brain slice preparations, we were able to demonstrate that facilitated endocytosis of postsynaptic AMPARs during LTD is AMPAR GluR2 subunit-specific (Man et al, 2000. These studies have lead us to develop a GluR2-derived interference peptide (GluR2-3Y) that, when delivered into neurons in the brain, can specifically block the expression of LTD without affecting the normal functioning of either NMDARs or AMPARs and hence basal synaptic transmission in many regions of the brain (Ahmadian et al, 2004). Importantly, we found that application of a membrane-permeant form of the GluR2 peptide (Tat-GluR2-3Y) specifically prevented certain LTD-dependent behaviours in rats in-vivo (Brebner et al, 2005). Availability of this systemic applicable, LTD-specific inhibitor allowed us for the first time to address specific roles of hippocampal CA1 LTD in hippocampus-dependent spatial learning and memory in the Morris water maze. We found that systemic administration of Tat-GluR23Y, but not a scrambled control peptide, 1h before the training phase of the task did not affect the rate of acquisition (learning) during the 8 training trials, but significantly impaired the hidden platform probing test (memory retrieval) performed on the second day. This impairment in memory retrieval was absent in the rat if peptide was given either immediately following training or before the probe test. Our findings strongly suggest that hippocampal LTD is induced during the learning phase or early phase of consolidation and that this LTD is necessary for the formation of long-term spatial memory. The present study also demonstrated the utility of peptides that disrupt AMPAR trafficking, the final step in the expression of synaptic plasticity, as tools to examine the critical role of LTD and/or LTP in specific aspects of learning and memory in conscious animals.

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The study is supported by Canadian Institute for Health Research, the NeuroScience Canada and the Howard Hughes Medical Institute

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA43

Intracellular machinery for the transport of neurotransmitter receptors at synapses

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Synaptic connections in the brain are continuously remodeled in response to neuronal activity. This process, known as synaptic plasticity, is widely accepted as the cellular process underlying learning and memory. We now know that an important contributor to synaptic plasticity in the hippocampus and other brain regions is the regulated addition and removal of glutamate receptors at excitatory synapses. In particular, AMPA type glutamate receptors (AMPARs) can be transported in and out of the postsynaptic membrane in a regulated manner, resulting in long-lasting changes in synaptic strength. Despite the importance of AMPAR trafficking for the regulation of synaptic function and plasticity, very little is known of the membrane trafficking machinery that mediates the intracellular sorting and targeting of AMPARs at synaptic terminals. During this presentation, I will describe our latest results that have led us to identify an intricate network of distinct endosomal compartments mediating the bidirectional movement of AMPARs in and out of dendritic spines and the synaptic membrane during plasticity.

The work presented in this conference is supported by the National Institute of Mental Health (grant MH070417).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA44

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) -type glutamate receptors: regulation by interacting proteins and alternative splicing

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Glutamate receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type mediate the fast component of excitatory synaptic transmission in the central nervous

system, play an important role in synaptic plasticity and contribute to cell death under excitotoxic conditions. AMPA receptors are tetramers of variable combinations of the receptor subunits GluR1, GluR2, GluR3 and GluR4. These subunits bind to cytoplasmic and transmembrane proteins that control their subcellular distribution. We have used proteomic approaches in order to identify proteins that interact with the GluR4 AMPA receptor subunit. These approaches have led to the identification of five candidate proteins as interactors for GluR4, some of which also interact with the other AMPA receptor subunits. We have biochemically confirmed the interactions, and found evidence of the binding partners colocalization with AMPA receptors in cultured hippocampal neurons. Moreover, some of these interactors modulate cell surface expression of AMPA receptors. AMPA receptor subunits exist in two functionally different isoforms, flip and flop, generated by alternative splicing. We identified transcripts for alternatively spliced isoforms of AMPA receptor subunits which lack both the flip and the flop exons, in hippocampal and retinal cultures. These transcripts originate AMPA receptor subunits lacking the flip/flop cassette, the fourth transmembrane domain and the intracellular C-terminus. Truncated GluR1 associates with full-length GluR1 and exerts a dominant negative effect, giving rise to non-functional receptors. Moreover, truncated GluR1 reaches the cell surface, but is not efficiently targeted to the synapse. Hippocampal neuronal transfection with truncated GluR1 resulted in a significant reduction in apoptotic neuronal death triggered by toxic concentrations of glutamate. Furthermore, mRNA coding for the truncated subunits is increased in some regions of the brain in epileptic rats and in hippocampal neurons submitted to toxic concentrations of glutamate. The existence of truncated AMPA receptor subunits and their upregulation under hyperexcitability conditions may constitute an intrinsic neuroprotective mechanism.

Supported by Fundação para a Ciência e a Tecnologia, Portugal (SFRH/BD/1286/2000, POCTI/BCI/39127/2001 and POCI/SAU-NEU/58955/2004).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C17

Regulation of AMPA receptor trafficking by leptin

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Evidence is growing that the hormone leptin plays an important role in the synaptic plastic mechanisms underlying learning and memory. Indeed leptin-insensitive rodents display impaired hippocampal LTP and LTD (1), whereas leptin converts STP into LTP (2) and it evokes a novel form of LTD (3). AMPA receptor trafficking plays a pivotal role in activity-dependent synaptic plasticity (4). However, it is unclear if changes in AMPA receptor trafficking underlie the effects of leptin on hippocampal synaptic plasticity. Thus, we examined the effects of leptin on GluR1 and GluR2 cell-surface expression on hippocampal neurons using immunocytochemical approaches and confocal microscopy. Primary hippocampal cultures were prepared from neonatal rats using standard procedures. To label surface AMPA receptors, live neurons were incubated with antibodies directed against N-terminal epitopes of GluR1 or GluR2, followed by fixation with 2% paraformaldehyde and subsequent incubation with a fluorescently-conjugated secondary antibody. A confocal microscope was used for image acquisition and analysis. Application of leptin (1-100nM) resulted in a dose dependent increase in surface GluR1 labelling relative to control, such that 1nM and 50nM leptin evoked 14.5 ± 5.1% (n=27; $P<0.05$) and 43.7 ± 5.7% (n=27; $P<0.01$) increases in GluR1 staining, respectively. In contrast, leptin was much less potent at increasing GluR2 surface expression as at 1nM (n=27) and 10nM (n=29) leptin had no significant effect on GluR2 staining. However, at higher concentrations leptin increased GluR2 surface staining such that 50nM and 100nM leptin increased GluR2 surface labeling by 20.6 ± 5.4% (n=27; $P<0.01$) and 41.0 ± 4.7% (n=27; $P<0.01$), respectively. We also compared the temporal profile of the effects of leptin (50 nM) on GluR1 versus GluR2 surface labeling. Exposure of neurons to leptin (50 nM) for 15-180 min resulted in a time-dependent increase in GluR1 staining such that increases in GluR1 were evident after only 15 min exposure to leptin (11.1 ± 5.5%; n=36) and this was increased further after application of leptin for 90 min (35.8 ± 6.4%; n=27). Increases in GluR2 surface staining were also observed following 15-60 min exposure to leptin (50nM). However, longer duration applications markedly reduced GluR2 surface staining indicating that leptin also internalizes AMPA receptors. Indeed, after 180 min exposure to leptin GluR2 surface expression was reduced to 85.8 ± 4.6% of control (n=27; $P<0.05$). In conclusion, these data indicate that leptin differentially modulates the surface expression of GluR1 and GluR2 AMPA receptor subunits in hippocampal neurons. These findings have important implications for the role of leptin in modulating hippocampal synaptic plasticity.

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This work is supported by The Wellcome Trust (grant no. 075821).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA45

Novel anti-inflammatory and pro-resolving lipid mediators: resolvins and protectins

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A well-integrated inflammatory response and its ending, i.e. resolution, is essential in health and disease. Our research focuses

on understanding the cellular and molecular events that govern resolution. This lecture will give an update and overview of recent advances from studies by the author and colleagues on the biosynthesis and actions of the novel anti-inflammatory lipid mediators, resolvins, and protectins that are generated from the omega-3 fatty acids (EPA and DHA). These previously unappreciated families of lipid-derived mediators were originally isolated from experimental murine models of acute inflammation captured during the natural spontaneous resolution phase. They possess anti-inflammatory, proresolving, and protective properties. The resolvins and protectins, in animal models, control the duration and magnitude of inflammation. Defective resolution mechanism(s) may underlie our current appreciation of the inflammatory phenotype(s) that characterize some prevalent human diseases. Hence, mapping of these resolution circuits provides new avenues for appreciating the molecular basis of many inflammatory diseases.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA46

Driving resolution of inflammation as a therapeutic strategy

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Leukocytes such as neutrophils and macrophages play a fundamental role in host defence and likely contribute to tissue damage associated with chronic inflammatory diseases (e.g., emphysema, bronchitis, rheumatoid arthritis, inflammatory bowel disease, etc). Apoptosis or programmed cell death is a key process regulating inflammatory cell survival providing an efficient non-inflammatory mechanism for removal of potentially histotoxic cells from inflamed sites by resident or recruited macrophages and is critically involved in the successful resolution of inflammation. Recent findings have demonstrated how neutrophil apoptosis as well as macrophage phagocytosis of apoptotic cells can be regulated pharmacologically suggesting that selective interference of these fundamental processes may be harnessed for therapeutic gain. Signalling pathways, including those involving transcription factors (e.g., NF-κB) and kinases (e.g., MAPK and PI3K) have been shown to be key regulators of inflammatory cell survival and apoptosis *in vitro*. In addition, manipulation of such pathways *in vivo* has indicated that they are also involved in the resolution of inflammation. Furthermore, manipulation of proteins directly involved in the control of apoptosis such as Bcl-2 family members and caspases can also be targeted *in vivo* to influence inflammatory resolution. Recently, it has been shown that cyclin-dependent kinase (CDK) inhibitor drugs, under development for the treatment of cancer, induce caspase-dependent human neutrophil apoptosis possibly by altering levels of the anti-apoptotic Bcl-2 family member, Mcl-1. Importantly, the CDK inhibitor drugs augment the resolution of established 'neutrophil dominant' models of inflammation (including pleurisy, pulmonary fibrosis and arthritis) by promoting apoptosis of neutrophils. Thus in this presentation I will discuss how manipulation of apoptotic pathways together with ensuring macrophage clearance of apoptotic cells appear to be viable pharmacological targets for driving the resolution of inflammation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C18

Characterization of novel phosphoinositide 3-kinase gamma inhibitors: ex vivo selectivity profiles and cellular functionality

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Phosphoinositide 3-kinases (PI3Ks) generate lipid second messenger molecules. Class IB PI3K p110gamma (PI3Kgamma) is an attractive drug target for inflammatory disease, for which extensive target validation data from genetically engineered mice are available (1). One of the key issues for PI3Kgamma inhibitors in drug discovery, is their selectivity over other family members of the lipid kinase family (2) and, in fact, protein kinases.

We have developed novel and selective inhibitors for PI3Kgamma through the use of a proteomics assay for both screening and selectivity profiling. With this assay, we can monitor quantitatively the interaction of compounds with their kinase targets and off-targets directly from cultured cells or even tissue samples from patients.

In a quantitative experiment, the compound is applied over a range of concentrations to cell or tissue lysate, which is subsequently captured on KinobeadsTM, a proprietary affinity matrix which specifically binds hundreds of kinases,not only protein- but also lipid kinases. The unbiased, target profile of the compound is then determined by differential analysis of the captured proteins from treated and untreated samples, by quantitative mass spectrometry using stable isotope labeling. For screening a set of 10 000 compounds against PI3Kgamma, we used as a source for the target the lysate of a PI3Kgamma expressing cell line and quantified the amount of PI3Kgamma captured on the beads by antibody detection. In this way, several hit series were identified and optimized for potency and selectivity.

Here we show quantitative proteomic protein and lipid kinase profiles of some of our novel PI3Kgamma inhibitors and how these compare to a range of PI3K inhibitors reported in the literature. We will show an example of potencies of our compound against a set of lipid kinases from primary human leukoctes and how this translates into cellular activity in a neutrophil migration assay.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C19

Annexin-1: a novel target for the therapy of autoimmune diseases

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Introduction
Annexin-1 (Anx-A1) is an anti-inflammatory protein that plays an important homeostatic role in innate immunity (1-2), however its potential actions in the modulation of adaptive immunity have never been explored. In this study we investigated the role of exogenous and endogenous Anx-A1 in T cell activation and differentiation and its role in the development of Th1/Th2-driven diseases.
Results
Addition of human recombinant (hr)Anx-A1 to stimulated T cells augmented anti-CD3/CD28-mediated CD25 and CD69 expression and cell proliferation. This effect was paralleled by increased NF-κB, AP-1 and NFAT activation and preceded by a rapid T cell receptor (TCR)-induced externalization of the Anx-A1 receptor. Conversely, analysis of T cell responses in Anx-A1 knock-out cells showed a decrease in cell activation and proliferation as well as in the activation of the above mentioned transcription factors. Interestingly, differentiation of naïve T cells in presence of hrAnx-A1 increased skewing in Th1 cells whereas Anx-A1 deficient T cells demonstrated an increased Th2 phenotype compared to cells from control littermates. In the collagen induced arthritis model (Th1-driven model) treatment of mice with hrAnx-A1 during the immunization phase exacerbated signs and symptoms at disease onset. Consistent with these findings blood CD4+ cells from patients with rheumatoid arthritis showed a marked upregulation of Anx-A1 expression (3). Finally, analysis of allergic response in Anx-A1 knock-out mice using a mouse model of ovalbumin-induced allergic peritonitis (Th2-driven model) demonstrated an exacerbated Th2 response i.e. increased number of T cells and eosinophil at site of inflammation compared to the wild type littermates.
Conclusions
Together these results demonstrate that Anx-A1 acts as a molecular "tuner" of TCR signalling and suggest this protein might represent a new target for the development of drugs directed to pathologies where an unbalanced Th1/Th2 response or an aberrant activation of T cells is the major etiological factor.

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This work is supported by the Medical Research Council (New Investigator Award to FDA) and by the Arthritis Research Campaign (PhD studentship to NP).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA47

Complementary action of the prostaglandin D2 receptors DP and CRTH2 in promoting allergic responses

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Prostaglandin D2 (PGD2) is one of the most abundant arachidonic acid metabolites produced by mast cell cells and Th2 lymphocytes and has been detected in elevated concentrations in a number of allergic conditions. PGD2 has the ability to mimic a number of the key features of allergic disease including the vascular effects leading to erythema and oedema and effects on Th2 lymphocytes, eosinophils and basophils leading to their recruitment and activation. It is well established that the vasodilator of properties of PGD2 are mediated by DP1 while the ability of PGD2 to promote chemotaxis of Th2 lymphocytes, eosinophils and basophils has more recently to been discovered to be due to an action on chemoattractant-homologous receptor expressed on Th2 cells (CRTH2, also known as DP2)(Hirai et al 2001).
Mast cells play a central role in orchestrating the pathophysiological changes that occur in allergic disease including influencing the characteristic pattern of Th2 lymphocyte recruitment and activation that is associated with the late phase allergic response. It is of interest, therefore, that the ability of supernatants from immunologically activated mast cells to promote chemotaxis of Th2 lymphocytes is mediated by CRTH2(Gyles et al, 2006) as is the production of the Th2 cytokines interleukin 4, 5 and 13 in response to PGD2(Xue et al, 2005). CRTH2 may also contribute to mast cell independent activation of Th2 cells since CRTH2 antagonists inhibit the ability of supernatants from activated Th2 cells to stimulate chemotactic activation of naïve Th2 cells (Vinall et al, 2007). Interestingly, PGD2 can also inhibit the production of cytokines such as interleukin 12 by dendritic cells, an effect mediated by DP1 which in some settings may promote an environment where polarisation of Th0 to Th2 cells is favoured. Therefore, based on these observations it is proposed that the concerted action of DP1 and CRTH2 plays a fundamental role in the polarization of T cells to the Th2 phenotype and their subsequent recruitment and activation at sites of allergic inflammation. In vivo pharmacological studies with recently discovered antagonists combined with genetic analysis support the view that these receptors play a pivotal role in mediating these aspects of allergic disease that are resistant to current therapy.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA48

Targeting the stromal microenvironment in chronic inflammation

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One of the most important but as yet unanswered questions in inflammation research is not why chronic inflammation occurs but why is does not resolve. Current models of inflammation stress the role of antigen-specific lymphocyte responses and attempt to address the causative agent. However recent studies have begun to challenge the primacy of the lymphocyte and have begun to focus on an extended immune system in which stromal cells, such as macrophages and fibroblasts play a role in the persistence of the inflammatory lesion.

In this lecture I will illustrate how fibroblasts play an important role in regulating the switch from acute resolving to chronic persistent inflammation associated with the pathology of diseases such as rheumatoid arthritis [1]. In chronic inflammation the normal physiological process of the death and emigration of unwanted inflammatory cells becomes disordered leading to accumulation of leucocytes [2-4] within lymphoid aggregates that resemble those seen in lymphoid tissue [5]. I will describe how fibroblasts provide survival and retention signals for leucocytes leading to their inappropriate and persistent accumulation within inflamed tissue [6]. Our work suggests that targeting the stromal microenvironment is likely to be an important strategy for future anti-inflammatory therapies [7].

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SA49

Synaptic memory mechanisms: Alzheimer disease amyloid β protein-induced dysfunction

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Alzheimer's disease can be viewed as a protein misfolding disease. Inappropriate processing of the proteolytic fragment of amyloid precursor protein, amyloid β-protein (Aβ), in early stages of the disease may lead to the release of stable small oligomers that are highly mobile with the potential to be more toxic than larger fibrillar assemblies. Recently, the importance of such soluble species of Aβ in triggering synaptic dysfunction, long before neuronal loss occurs, has become apparent. Animal models have revealed that plasticity of hippocampal excitatory synaptic transmission is relatively selectively vulnerable to Aβ both in vitro and in vivo. This presentation focuses on the mechanisms of Aβ inhibition of long-term potentiation (LTP) at synapses in the rodent hippocampus from two complimentary perspectives. First, we will examine evidence that the synaptic plasticity disrupting effect of this peptide resides primarily in oligomeric rather than monomeric or fibrillar Aβ species. Cell-derived Aβ oligomers can be purified with size-exclusion chromatography and shown to inhibit LTP at subnanomolar concentrations. Exogenously applied and endogenously generated antibodies that can avidly bind Aβ oligomers can protect against the inhibition of LTP by directly neutralizing them in the brain. Second, the importance of different oxidative/nitrosative stress-linked cascades including JNK, p38 MAPK and NADPH oxidase/iNOS-generated reactive oxygen/nitrogen free radicals in mediating the inhibition of LTP by Aβ will be evaluated. Selective inhibitors of these cascades can abrogate the inhibition of LTP by Aβ. Remarkably, agents that reduce the levels of the cytokine Tumor Necrosis Factor (TNF)α are also protective and mice deficient in type 1 TNF receptors are resistant to the inhibion of LTP by Aβ. Such mechanistic studies provide a plausible explanation for the sensitivity of hippocampus-dependent memory to impairment in early Alzheimer's disease patients. Mechanism-based therapeutic strategies targeting Aβ oligomers and pro-inflammatory synaptic stress provide an attractive strategy in the control of early Alzheimer's disease.

Supported by Science Foundation Ireland and Programme for Research in Third Level Institutes in Ireland.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA50

Control of neuronal survival by the glycolytic pathway

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We previously reported that, following inhibition of cytochrome c oxidase activity by endogenous nitric oxide (1), astrocytes can maintain energy homeostasis and survival by reactivating the

glycolytic pathway; however, this metabolic and survival response was not invoked by neurons (2). Recently, we elucidated the signalling mechanism responsible for those metabolic effects found in astrocytes, and identified that the AMP-activated protein kinase (AMPK) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (Pfkfb or Pfk2) pathway played an essential role (3). Pfkfb catalyzes the formation and degradation of fructose-2,6-bisphosphate, i.e. the most potent positive alosteric effector of 6-phosphofructo-1-kinase (Pfk1) –a master regulator of the glycolytic pathway (4). Here, we sought to investigate whether the control of glycolysis and survival of cortical neurons in primary culture would lie on the regulation of Pfkfb. Western blot analyses, using a specific antibody that we raised against the brain Pfkfb isoform (Pfkfb3) revealed that the Pfkfb3 protein was profusely expressed in rat cortical astrocytes in primary culture. In contrast, the Pfkfb protein was undetectable in terminally differentiated rat cortical neurons in primary culture. Interestingly, we found that the Pfkfb3 protein was expressed in undifferentiated neuronal precursors, but it progressively disappeared along with the differentiation process. In contrast, Pfkfb3 mRNA, as assessed by Northern blotting, was found to be present at any differentiation stages. Furthermore, inhibition of the proteasome activity using MG132 rapidly induced the accumulation of the Pfkfb3 protein in fully differentiated, post-mitotic neurons. Finally, over-expression of Pfkfb3 full-length cDNA altered neuronal survival during terminal differentiation.

Together, these results strongly suggest that neuronal glycolysis and survival can be controlled by the stability of Pfkfb3 through the ubiquitin-proteasome pathway. This may have implications for our understanding of the mechanisms of neurodegeneration by oxidative and nitrosative stress.

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This work was supported by the Junta de Castilla y León, Ministerio de Educación Ciencia and Fondo de Investigación Sanitaria (Spain).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C20

Neurotoxicity of tryptophan metabolites in cultured cerebellar granule neurones

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There is increasing interest in the effects on neuronal viability of several kynurenine metabolites of tryptophan, including the NMDA receptor agonist quinolinic acid (possibly implicated in several neurodegenerative disorders) and the glutamate antagonist kynurenic acid, a possible neuroprotectant. This study examines the ability of several other metabolites to affect neuronal viability by inducing oxidative stress. Cerebellar granule neurone cultures were prepared from 8-day neonatal Sprague-Dawley rats, and effects on neuronal viability (measured using fluorescein diacetate) of selected kynurenine pathway compounds studied, including 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA) and 5-hydroxyanthranilic acid (5-HAA), applied for 1 to 9 hours. Alterations to neurotoxicity due to variation in glucose concentration (5.5 vs. 25mM), and the presence of exogenously applied antioxidant enzymes were examined. Application of 3-HK, 3-HAA, and 5-HAA at concentrations from 10 micromolar to 1 millimolar caused dose-dependant neurotoxicity, potentiated by prolonged exposure. The remaining compounds caused neurotoxicity, intensified by increased exposure times but not dose. Tryptophan, kynurenine, anthranilic acid, quinolinic acid and picolinic acid were significantly more neurotoxic in the presence of a higher glucose concentration ($p<0.05$). The co-application of anthranilic acid with 3-HAA did not potentiate the neurotoxic effect of either metabolite. Catalase (200U/ml) reduced 3-HK, 3-HAA, and 5-HAA toxicity significantly ($p<0.05$), whereas superoxide dismutase (SOD) at 200U/ml did not. It is demonstrated that certain tryptophan metabolites cause neurotoxic effects due to oxidative stress, and that the toxicity of some metabolites can be exacerbated by increased glucose levels.

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C21

Translation in human mitochondria: what recycles the ribosomes?

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Despite many years of research translation in human mitochondria is still poorly understood. Up to now only a few factors involved in this process have been identified. The aim of this research is to characterise the protein involved in mitochondrial ribosome recycling after translation termination has been completed. Although a candidate protein was identified bioinformatically on the basis of sequence similarity, no further investigations had been performed.

We have subsequently cloned this gene, namely mitochondrial ribosome recycling factor (mtRRF). A series of experiments have been designed to determine whether mtRRF is truly mitochondrial in vivo. i) A GFP fusion construct was generated and HeLa cells transiently transfected. Mitochondria were visualised by Mitotracker staining and positive co-localisation was identified microscopically. ii) To confirm that this co-localisation truly reflected internalisation into mitochondria we performed an in vitro import assay into isolated organelles.

Bacterial RRF has been described as an essential protein. To determine if the same is true for the mitochondrial counterpart, the steady-state levels have been downregulated by the application

of siRNA in HeLa cells. Transfected cells develop morphological changes and loss of mtRRF for more than 6 days appears lethal. Thus is appears that mtRRF is also an essential gene for higher eukaryotic cells, consistent with a critical role in mitochondrial function.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA51

Mitochondrial function in neurodegenerative disorders

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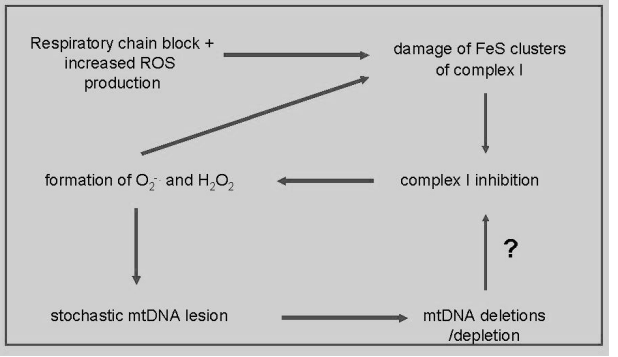
There is compelling evidence for the direct mitochondrial involvement in certain neurodegenerative disorders, like Morbus Parkinson, Friedreichs ataxia, amyotrophic lateral sclerosis, and temporal lobe epilepsy with Ammon's horn sclerosis. This includes the direct genetic evidence of pathogenic mutations in mitochondrial proteins in certain forms of inherited parkinsonism (like PARK2 - with mutations in parkin, and PARK6 – with mutations in the mitochondrial PTEN-induced kinase 1 (1)) and in Friedreichs ataxia (with mutations in the mitochondrial protein frataxin (2)). Moreover, there is functional evidence for the impairment of mitochondrial respiratory chain in sporadic forms of parkinsonism, amyotrophic lateral sclerosis, and temporal lobe epilepsy with Ammon's horn sclerosis. In the sporadic forms of the above mentioned neurodegenerative disorders increased oxidative stress appears to be the crucial initiating event which affects respiratory chain function and starts a vicious cycle leading finally to neuronal cell death (cf. Figure). A critical factor which determines the survival of neurons in neurodegenerative disorders is in particular the degree of mitochondrial DNA damage and the maintenance of an appropriate mitochondrial DNA copy number. Clear evidence for a depletion of intact copies of the mitochondrial genome has been provided in all above mentioned neurodegenerative disorders, including amyotrophic lateral sclerosis (3), and temporal lobe epilepsy with Ammon's horn sclerosis.(4). In the present review we provide a summary and a critical discussion of recently available data.

Hypothetical vicious cycle, explaining mitochondrial dysfunction in neurodegenerative diseases



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This study was supported by the Deutsche Forschungsgemeinschaft (KU-911/15-1 and SCHR-562/4-3).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA52

MtDNA mutations in neurodegenerative disease

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Mutations in mitochondrial DNA (mtDNA) can cause a wide range of well-characterised clinical disorders. However, the relationship between genotype and phenotype is not always straightforward. Mitochondria are predominantly involved in providing energy necessary for normal cell function and maintenance. For this reason high energy-requiring tissues are often affected such as heart, muscle and brain. MtDNA mutations have been found to increase with age in a variety of tissues. Mitochondrial dysfunction has also been observed in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (PD). We have recently shown that substantia nigra neurons from aged and PD patients have mtDNA deletions levels of ~50% (ref 1). Whether these mtDNA mutations have a role in the neurodegeneration observed in this brain region, or are just a secondary consequence of other factors is still to be established.

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SA53

SNARE proteins and lipid rafts

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SNAREs are small, mostly membrane associated proteins that are essential for all intracellular membrane fusion steps (except for mitochondrial fusion). SNAREs share as a common feature

a conserved stretch of 60-70 aa, the so called SNARE-motif. For each fusion step, a specific set of SNARE proteins is required. For instance, in regulated exocytosis the neuronal SNAREs syntaxin 1A and SNAP-25 at the plasma membrane form a complex with synaptobrevin 2 at the vesicle membrane, leading to fusion of the opposed membranes.

In 2001 two groups independently from each other found evidence that the neuronal SNAREs are not uniformly distributed but spatially organized. Chamberlain et al. tested if SNAREs co-float with membrane raft domain markers. At that time, proteins were defined to be associated with membrane rafts when they enriched in so-called detergent resistant membranes (DRMs). After cell solubilisation, DRMs can be readily isolated from lysed cells by density gradient centrifugation: As they are composed of proteins and associated lipids, they are of lower density than e.g. cytosolic proteins and float up to lower densities during gradient centrifugation. Chamberlain et al. showed that indeed a fraction of the neuronal SNAREs co-floated with detergent resistant membrane markers. It was also shown that the protein is not floating after cholesterol depletion, consistent with the idea of raft association and documenting that cholesterol is important for the spatial organisation of SNAREs. Interestingly, block of cholesterol synthesis by lovastatin led to a block in exocytotic activity indicating that raft association is of functional importance. At the same time a microscopic analysis of the spatial distribution of syntaxin 1 in plasma membrane sheets showed that syntaxin 1 was concentrated in cholesterol-dependent clusters that defined docking and fusion sites for exocytosis (Lang et al., 2001). Evidence for cholesterol association was provided by photolabeling of synatxin by photoactivatable cholesterol. Cholesterol depletion led to disintegration of syntaxin clusters and to a loss of exocytosis. Hence, both studies in principle provided evidence for the idea that SNAREs form plasmalemmal stuctures that depend on cholesterol and are important for exocytosis. However, the DRMs isolated by Lang et al. were devoid of SNAREs. Additionally it was shown on membrane sheets that syntaxin clusters did not overlap with raft markers (see also Ohara-Imaizumi et al., 2004). These conflicting results led to a disagreement regarding the interpretation of the nature of these structures, which from then on were called rafts by some groups and clusters by others. The controversy went on as in the following years DRMs were prepared also from other cell types, and depending on how stringent the solubilization criteria were applied, SNAREs were partially associated with or not present in DRMs. When investigated in model membranes, SNAREs were usually found to prefer the disorderd (non-raft) phase.

However, despite of the controversy if SNAREs are associated with rafts, it is undebated that they form plasmalemmal structures that are important for membrane fusion, and the different affinities of SNAP-25 and SNAP-23 (involved in constitutive exocytosis) to rafts may even fine tune the different exocytotic pathways in which these proteins operate (Salaun et al., 2005).

In any case, a recent new definition of rafts will probably make the debate on DRMs association less crucial. On the Keystone Symposim on "Lipid rafts and cell function" membrane rafts have now been defined (Pike L.J. (2006). J. Lipid Res. 47, 1597-1598) to be "small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions." Interestingly, the "raftologists" have disregarded detergent resistance as criteria for raft association. According to this new definition, all characteristics so far determined for SNAREs fulfill the raft criteria: they contain cholesterol, compartmentalize cellular dynamics, and a recent study (Sieber et al., 2006) has shown that their size is in the required range. Hence, it may turn out in the future that SNAREs are bona fide membrane rafts.

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T.L. was supported by a grant from the Deutsche Forschungsgemeinschaft (LA1272/2-2).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA54

Tomosyn negatively regulates release at the *C. elegans* neuromuscular junction

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Synaptic transmission is a highly orchestrated process in which calcium triggers neurotransmitter release on a microsecond timescale. Exocytosis requires the assembly of SNARE complexes between the vesicle SNARE synaptobrevin and the plasma membrane SNAREs, syntaxin and SNAP-25. Thus the regulation of SNARE complex assembly is a potentially important mechanism by which proteins can adjust synaptic strength.

Tomosyn is a syntaxin-binding protein capable of regulating SNARE complex assembly in vitro through its SNARE binding domain. Tomosyn forms a stable complex with syntaxin and SNAP-25 that is virtually indistinguishable from SNARE complexes, based on X-ray crystallography. Tomosyn overexpression in endocrine cells leads to inhibition of dense core vesicle (DCV) fusion, suggesting tomosyn inhibits endocrine release. To test whether tomosyn regulates synaptic transmission we examined C. elegans tomosyn mutants.

We have obtained two mutants in C. elegans tomosyn (tom-1). tom-1 mutants have mild behavioral defects indicating that TOM-1 is not an essential protein, however, pharmacological assays suggest that ACh transmission is enhanced. To elucidate the physiological basis of this phenotype we measured release at NMJs of dissected tom-1 mutants. Evoked responses in these

mutants were significantly broader than wild-type, resulting in a two-fold increase in evoked charge integral. We attributed this effect to a change in presynaptic ACh release for the following reasons. 1) Neither the amplitude nor the kinetics of minis was altered, suggesting that post-synaptic reception is normal. 2) TOM-1 is enriched in presynaptic terminals based on immunohistochemistry. 3) We could reverse the tom-1 mutant synaptic phenotype, by expressing TOM-1 in cholinergic neurons. How might tomosyn regulate presynaptic release? Like its mammalian orthologs, the SNARE domain of TOM-1 complexes with syntaxin and SNAP-25, an interaction predicted to inhibit synaptic vesicle priming. We therefore, examined the responses of tom-1 mutants to hyperosmotic saline to measure the primed vesicle pool. The two-fold increase in hyperosmotic responses observed at tom-1 mutant synapses suggests that the number of primed vesicles was increased. We have recently shown that plasma membrane contacting vesicles represent a morphological correlate of priming, based on analysis of priming-defective unc-13 mutants. When we examined tom-1 mutant synaptic profiles, we observed a two-fold increase in morphologically contacting vesicles consistent with the enhanced release observed electrophysiologically. Conversely, overexpression of TOM-1 reduced both evoked responses and the number of morphologically docked vesicles. Based on these data we propose that tomosyn acts as a negative regulator of synaptic vesicle priming. Consistent with this conclusion, we found that tom-1 mutants partial suppressed unc-13 mutant priming defects, suggesting that TOM-1 antagonizes UNC-13-dependent priming, possibly by competing with synaptobrevin in SNARE complex assembly, thereby restricting vesicle fusion-competence.

We next asked whether TOM-1 similarly regulates DCV release. DCV fusion requires CAPS, encoded by unc-31 in C. elegans. The paralysis of unc-31 mutants can be rescued by expressing UNC-31 in cholinergic neurons. To determine the physiological basis for the behavioral defects in unc-31 mutants we examined their NMJs. unc-31 mutants exhibited a 50% reduction in evoked release and a three-fold accumulation of DCVs, consistent with the proposed requirement of UNC-31 in DCV release. Conversely the number of DCVs in tom-1 mutants was reduced by 50%, where as TOM-1 overexpression caused DCV accumulations similar to unc-31 mutants. We attributed the loss of DCVs in tom-1 mutants to exuberant release, the replenishment of DCVs failing to keep pace. To examine whether there is a genetic interaction between unc-31 and tom-1 we generated double mutants. The behavioral defects of tom-1;unc-31 mutants were less pronounced than unc-31 alone. Consistent with this observation, NMJ evoked responses improved in the double mutants and the accumulation of DCVs observed in unc-31 mutants was partially suppressed. These data indicate that tom-1 negatively regulates CAPS-dependent DCV release.

In summary we have demonstrated that both UNC-13-dependent synaptic vesicle priming and CAPS-dependent DCV release are negatively-regulated by tomosyn. On the basis of these results we propose that tomosyn, through its interactions with the SNARE protein syntaxin, restricts the priming processes of both secretory organelles.

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We would like to thank the Caenorhabditis Genetics Center (CGC) for providing strains. This research was supported by NIH grants R01 MH073156-01A2 and RO1 NS041477.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA55

Regulation of SNARE function by post-translational modification

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Exocytosis is generally triggered by a rise in the intracellular free calcium concentration. This calcium-induced secretion is also subject to modulation by a variety of signalling mechanisms. The most widely studied and best understood of these is protein phosphorylation, which has been recognised for many years to be a near universal modulator of regulated exocytosis. More recently, nitric oxide-induced protein S-nitrosylation has emerged as an important post-translational modification affecting exocytosis in a variety of cell types, including neurons. Nevertheless, there is relatively little mechanistic understanding of how post-translational modification of the protein machinery for exocytosis can lead to the observed cellular effects.

Formation of a ternary SNARE complex between syntaxin, SNAP-25 and VAMP is essential for neuroexocytosis and is believed to directly drive the membrane fusion process. Complex formation occurs sequentially, proceeding via an initial syntaxin-SNAP-25 heterodimer intermediate, to which VAMP subsequently interacts. The ability of syntaxin to form such a binary complex is in turn dependent on its SNARE motif being accessible, thus requiring the adoption of a so-called 'open' conformation. In contrast, in the 'closed' conformation, the SNARE motif of syntaxin is sequestered in an intramolecular helical bundle that prevents binding to SNAP-25, but allows binding to Munc18. As syntaxin is thought to exist in equilibrium between open and closed conformations, and as the formation of the initial syntaxin/SNAP-25 heterodimer is the rate-limiting step in assembly of the ternary SNARE complex, this represents a potential point of regulation by signalling mechanisms.

Here we report that post-translational modification of syntaxin can regulate this process. In protein interaction assays in vitro,

nitrosylation of a single cysteine residue in syntaxin inhibits binding to Munc18-1, but facilitates binding to SNAP-25 and VAMP to form the ternary SNARE complex. This effect is apparently mediated by a conformational change in syntaxin, as it induces alterations in protease sensitivity that mimic those seen in the previously characterised open mutant syntaxin. Consistent with this notion, expression of a cysteine mutant syntaxin construct designed to mimic the nitrosylated state alters the release kinetics of single vesicle exocytosis events in a similar manner to the open mutant. Taken together, our data suggest that nitrosylation of syntaxin may be a regulatory switch that helps the protein to adopt a more 'open' conformation, thereby facilitating SNARE complex formation and hence membrane fusion.

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SA56

Regulation of SNARE fusion machinery by fatty acids

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Vesicle fusion is a ubiquitous biological process involved in membrane trafficking and a variety of specialised events such as exocytosis and neurite outgrowth. The energy to drive biological membrane fusion is provided by fusion proteins called SNAREs. Indeed, SNARE proteins play critical roles in neuronal development, neurotransmitter and hormone release. SNARE proteins form a very tight alpha-helical bundle which can pull two membranes together thereby initiating fusion. Whereas a great deal of attention has been paid to partner proteins which can affect SNARE function, recent genetic and biochemical evidence suggests that local lipid environment may be as important in SNARE regulation. I will discuss in detail regulation of the pivotal SNARE protein - syntaxin.

Syntaxin together with its chaperone Munc18 constitute a molecular tandem essential for exocytosis in all eukaryotes. We previously showed that Munc18 inhibition of neuronal syntaxin1 can be overcome by arachidonic acid, suggesting that this common second messenger acts to disrupt the syntaxin/Munc18 interaction. Our recent data indicate that arachidonic acid can stimulate syntaxin1 alone indicating that it is syntaxin1 that undergoes a structural change in the syntaxin1/Munc18 complex. The same principle operates in the case of the ubiquitous syntaxin3 isoform highlighting the conserved nature of the mechanism of arachidonic acid action. We also show that syntaxin3 plays an important role in the growth of neurites and serves as a direct target for the dietary omega-3 fatty acids. Our findings provide a molecular basis for the previously established action of omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) in cell membrane expansion and identify the first single effector molecule for these essential nutrients.

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Rickman, C. and Davletov, B. Arachidonic acid allows SNARE complex formation in the presence of Munc18 Chem Biol 2005, v.12, pp. 545-553

I thank Colin Rickman, Frederic Darios and Emma Connell for contributions to this work.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C22

Fusion pore opens to subnanometer diameters in resting neuroendocrine cells

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Introduction. Lactotrophs, neuroendocrine cells of the anterior pituitary, release peptide hormone prolactin from secretory vesicles through a fusion pore that is formed upon the fusion of the vesicle membrane with the plasma membrane. Spontaneous hormone discharge from a single vesicle is slower than under stimulation likely due to the kinetic constrains of the fusion pore openings (**1**). In the present work, we tested whether slow hormone release at rest could also be a consequence of a relatively narrow fusion pore.

Methods. We monitored the permeation of fluorescent FM 4-64 dye molecules (diameter = ~1 nm) (**1**,**2**) and HEPES molecules (diameter = ~0.5 nm) through spontaneously forming fusion pores of prolactin vesicles by time-lapse confocal microscopy in resting lactotrophs, expressing synaptopHluorin (spH), a pH sensitive green fluorescent protein (**3**).

Results. Exocytosis of prolactin vesicles (n = 36) in 10 mM HEPES solution resulted in a rapid increase in spH fluorescence, indicating an efflux of protons from acidic vesicles. We distinguished two populations of spontaneous fusion events, type I (Fig. 1A) and type II events (Fig. 1B). Type I events were devoid of FM 4-64 loading (n = 19, 53%), on the contrary in type II events vesicles loaded the FM 4-64 (n = 17, 47%). In both types of events, following the peak in spH signal, the signal either exponentially decayed (transient events; n = 23, Fig. 1), implying reacidification of the vesicle lumen due to the resealing of the fusion pore, or it remained at an elevated level during the monitoring (persistent events; n = 13), indicating that the vesicle lumen remained in contact with the extracellular space.

Conclusions. The absence of vesicle entry by FM 4-64 dye in type I events and the absence of time-course modulation of these events in the presence of increased concentration of HEPES (100 mM), suggest that FM 4-64 and HEPES molecules did not pass the fusion pore in type I events (pore diameter <0.5 nm). In contrast, the fusion pore diameter in type II events appears to be wider than 1 nm. These results show that at rest >50% of the exocytic vesicles undergo fusion yielding subnanometer fusion pore diameters, precluding discharge of the hormone prolactin (diameter = ~5.2 nm).

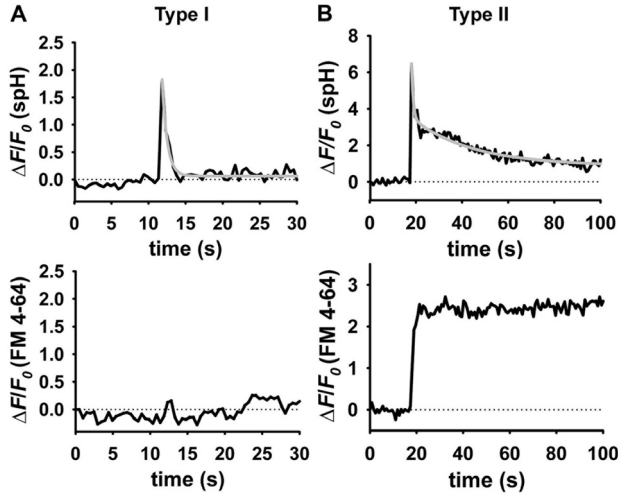

Fig. 1. Spontaneous exocytotic events of type I (A) and type II (B). Time-dependent fluorescence changes at the vesicle site, separately for spH and FM 4-64. In type II events (B), FM 4-64 fluorescence simultaneously increased with the spH fluorescence signal.

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This work was supported by grants P3310381 from the MVZT of the Republic of Slovenia, NS36665 from the NIH, EC grant #QLG3 2001-2004, EC support DECG, CLG3-CT-2001-02004, and EC GROWBETA QLG1-CT-2001-02233.

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C23

Tumour protein D52: expression in pancreatic β-cell lines and its role in glucose-stimulated insulin secretion

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Regulated secretion is crucial to many cellular functions and is the pathway that neurotransmitters, digestive enzymes and hormones utilise to leave the cell, often in response to a specific stimulus. Insulin secretion from pancreatic β cells is a biphasic response induced by increased extracellular glucose levels. The first rapid phase of secretion requires the opening of voltage-dependent calcium channels resulting in increased intracellular calcium concentrations. This triggers exocytosis from a pool of previously docked and primed insulin-containing granules. The second phase, defined by prolonged, gradually increasing insulin secretion, requires recruitment of granules from reserve pools and is dependent on granules being made competent for exocytosis. Uncertainty exists over what the primary sensor of increased calcium levels is and what the molecular mechanisms responsible for vesicle priming are. Understanding these mechanisms is crucial to our understanding of the secretory defect in Type II diabetes, where there is a loss of the first phase and a reduction in the second phase of glucose-stimulated insulin secretion.

Tumour protein D52 (TPD52) interacts with several proteins involved in membrane trafficking, e.g. annexin VI (1) and MAL2 (2), and is involved in the regulated secretion of digestive enzymes from pancreatic acinar cells after secretagogue stimulation (3). We found expression of TPD52 in both the rat and mouse pancreatic β cell lines, INS-1 and MIN6, and hypothesise that TPD52 is involved in regulated secretion of insulin from the β cell. To determine the effect of TPD52 on regulated secretion, we have knocked down TPD52 RNA levels using a vector based siRNA approach. The siRNA constructs were checked for efficacy against an HA-tagged TPD52 transgene and then a stable knockdown cell line was created by transfecting the TPD52-siRNA vector into INS-1 cells and selecting G418 resistant colonies. Two clonal lines were created that showed a 50 % and an 80 % reduction in TPD52 RNA levels. Western blot analysis confirmed a marked reduction in TPD52 protein levels in the latter cell line. Initial data showed that this reduction in TPD52 protein levels did not affect glucose-stimulated insulin secretion over a 3 hour stimulation period. Isoelectric focusing gels, however, showed that in normal INS-1 cells TPD52 was rapidly phosphorylated into three phosphoisoforms only two minutes after glucose stimulation. Phosphorylation was transient, peaking at 15 min post-stimulation and returning to basal levels within 1 hour. This suggests that TPD52 may be involved only in the initial rapid phase of insulin secretion and work is currently underway to determine the effects of TPD52 knockdown on the first phase of glucose-stimulated insulin secretion.

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Y. Manning is in receipt of a BBSRC funded studentship; funded by BBSRC

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SA57

Xtreme-Everest: a field study of human adaptation to hypoxia

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The Xtreme-Everest program of investigation is based on 2 ideas:
1. "Investigating human adaptation to hypoxia is an important element of understanding the pathophysiology of critical illness and has the potential to lead to the development of new treatments"
2. "Healthy volunteers exposed to hypobaric hypoxia in field studies are a useful model for the study human adaptation to hypoxia"

Hypoxia in critical illness

Cellular hypoxia is a fundamental mechanism of injury in the critically ill.1 Hypoxia may occur as either a cause or consequence of, a variety of critical illnesses. For example hypoxia-mediated

cell death may lead to the generation of an inflammatory response. Systemic inflammation is associated with microcirculatory dysfunction resulting in reduced oxygen delivery and thus the development of cellular hypoxia. Furthermore, critical illness is associated with deranged cellular oxygen utilisation.

Investigating the physiology and pathophysiology of hypoxia

Xtreme-Everest is a human healthy volunteer study investigating hypoxic adaptation. Hypoxic adaptive processes are likely to be common to tissue hypoxia whatever the cause, and studying healthy individuals progressively exposed to hypoxia through ascent to high altitude may inform the nature of the adaptive processes to hypoxia occurring in the critically ill. This approach offers the advantages of a relatively homogenous study population and environmental challenge, in contrast to those observed on Critical Care Units, as well as the availability of "pre-morbid" information and levels of function. It also offers an ethical alternative to hypoxia experimentation in patients; all involved are willing participants in climbing or trekking ventures, as a consequence of which they expose themselves to a hypoxic environment.

Human physiology at altitude

Xtreme-Everest is an observational cohort study with nested interventional sub-studies. A large cohort of healthy volunteers will be progressively exposed to hypoxia at a standardised rate expected to result in minimal altitude illness and provide time for acclimation (n=224). During the ascent, the adaptive response to hypoxia will be investigated, beneficial phenotypes will be identified and these will be related to underlying genotype. Baseline studies have been performed at sea level and these will be repeated at four laboratories in Nepal at altitudes of 1350m, 3400m, 4200m and 5300m (Everest Base Camp). A broad range of experimental protocols will be carried out investigating cellular oxygen utilisation, oxygen delivery, microcirculatory function, cerebral blood flow and oxygenation, neurocognitive function, sleep disturbance and metabolism. 15 climbers will ascend Mount Everest and a subset of these investigations will be performed at extreme altitudes, observing physiology at the limits of human endurance. I will present early data from the Xtreme-Everest Expedition including, I hope, an arterial blood gas from the summit.

M Grocott, H Montgomery, A Vercueil; Crit Care. 2007 Feb 1;11(1):203 High-altitude physiology and pathophysiology: implications and relevance for intensive care medicine.

Dr Mike Grocott on Behalf of the Caudwell Xtreme Everest Research Group

http://www.caudwell-xtreme-everest.co.uk/team

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SA58

Neuroscience research at extreme altitude

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Hypobaric hypoxia results in a a number of neurophysiological adaptations. The Caudwell Xtreme Everest project involves a series of experiments designed to investigate these changes and elucidate any underlying relationship with concurrent neurocognitive (functional) changes. 224 subjects will be studied at altitudes of up to 5300m during a standardised ascent to Everest Base Camp. More in-depth studies will be performed on 24 researchers and the summit team of 10 at altitudes up to 8848 metres.

Brain Oxygenation at Altitude (measured using Near Infra Red Spectroscopy) – Our pilot work from Tibet has demonstrated that some subjects have an ability to maintain brain oxygenation during exercise to a greater extent than others. The hypothesis that this ability would correlate to a smaller rise in neural markers (S100) and an ability to maintain neurocognitive function will be investigated.

Brain Blood flow and Inferred Intracranial Pressure (measured using Transcranial Doppler) –

It has been hypothesised that high altitude headache and acute mountain sickness are related to the formation of cerebral oedema resulting in a rise in intracranial pressure. To investigate this, transcranial Doppler of the middle cerebral artery is being used to calculate blood flow velocity, vessel calibre and to infer intracranial pressure using the calculated value of pulsitility index.

Reflex and Neurocognitive Changes at Altitude:

Hypoxia causes a deterioration in neural function. This is being been investigated on a number of levels. At the basic reflex level, the velocity of the papillary light reaction will be studied in all 224 subjects. At the next level, saccadic eye movements will be studied in the 24 researchers. On a more global functional level, neurocognitive and coordination tests are being carried out on all 224 subjects. These results will be related to headache scores and physiological changes.

Neural Markers and MRI evidence of hypoxic brain injury

Clinically, episodes of brain hypoxia are known to result in the release of S100 and other neuronal markers. Plasma S100 levels are being measured in all 224 subjects. The summiteers are also having MRI scans to investigate microanatomical changes in hippocampal and basal ganglia structure. All of these facets will be correlated with AMS symptoms and neurocognitive changes.

Anthropermororphic Predispotision to Acute Mountain Sickness:

The "Tight Fit" Hypothesis was proposed by Ross in the 70's to account for individual predisposition to AMS. He suggests that those with a large brain in a small skull are more susceptible. Skull size and volume has been measured in all 224 subjects to assess cranial size and symptoms scores will be recorded during the ascent. Subjects with the highest and lowest symptom scores will undergo MRI scans to calculate the ratio brain:skull volume.

Retinal Imaging and Intraoccular Pressure:

It is known that many people ascending to altitude develop retinal haemorrhages. Retinal photography will be used to investigate the nature and incidence of retinal haemmorhages in all 224 subjects. The development of retinal haemorrhages will be correlated with headache, cerebral oxygenation and cerebral blood flow. In the research group intraocular pressure will be correlated with the presence of haemorrhages.

Many aspects of neurophysiology and neurocognitive changes are being investigated during the Xtreme Everest expedition. A summary of the preliminary findings will be presented here.

Dr Mark Wilson on behalf of the Caudwell Xtreme Everest Research Group

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C24

Free radical exchange kinetics and pulmonary artery pressure response in high-altitude pulmonary oedema

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The present study examined if high-altitude (HA) exposure altered the vascular generation of free radicals across the human lungs and subsequent implications for the pathophysiology of high-altitude pulmonary oedema (HAPE). Since HAPE is preceded by a marked elevation in pulmonary artery pressure (PAP), we hypothesised that HAPE would be characterised by a net outflow or release of free radicals that would directly correlate with the rise in PAP.

Thirty four subjects were examined at sea-level (SL) and 20h following active ascent to HA (4559m) following an overnight stay. Resting plasma samples were obtained from a central venous (superior vena cava) and radial arterial catheter for direct detection of the ascorbate free radical ($A^{\bullet-}$) by EPR spectroscopy. Haemoglobin and haematocrit were also measured to correct for plasma volume shifts. Pulmonary blood flow (PBF) was determined by a re-breathing technique and pulmonary $A^{\bullet-}$ exchange kinetics calculated via the Fick method [arterio-venous concentration difference (a-v_{diff}) x (plasma) PBF]. Systolic PAP (PASP) was estimated by Doppler echocardiography and chest radiography confirmed HAPE. Clinical acute mountain sickness (AMS) was diagnosed as previously described (Bailey *et al.*, 2006). Due primarily to an increase in the arterial concentration, the a-v_{diff} of $A^{\bullet-}$ increased from 18 (mean) ± 195 (SD) at SL to 206 ± 215 arbitrary units (AU)√Gauss (G) at HA ($P < 0.05$, Wilcoxon Matched Pairs Signed Ranks Test) resulting in a marked increase in $A^{\bullet-}$ outflow (SL: 56 ± 736 *vs.* HA: 750 ± 834 AU√G/min, $P < 0.05$). Outflow correlated ($r = 0.35$, $P < 0.05$, Pearson Product Moment Correlation) with the increase in PASP also observed at HA (SL: 23 ± 4 *vs.* HA: 38 ± 9 mmHg, $P < 0.05$). Four subjects developed HAPE and twenty subjects developed mild to severe AMS. Compared to healthy controls ($n = 10$), HAPE subjects presented with a greater increase in PASP (+27 ± 9 *vs.* +13 ± 6 mmHg, $P < 0.05$) and corresponding outflow of $A^{\bullet-}$ (+2379 ± 652 *vs.* 0 ± 1020 AU√G/min, $P < 0.05$) that were linearly related ($r = 0.80$, $P = 0.05$).

These findings provide the first direct evidence for a net outflow or release of free radicals across the lungs at HA that may have implications for the pathophysiology of HAPE. A superoxide-mediated reduction in endothelium-derived nitric oxide bioavailability may prove the underlying mechanism responsible for the hypoxic pulmonary vasoconstriction observed in HAPE.

Bailey DM *et al.* (2006). J Cereb Blood Flow Metab 26, 99-111.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C25

Exercise-induced arterial hypoxaemia is associated with a reduction in the systemic bioavailability of nitric oxide

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Exercise and hypoxia can independently generate free radicals subsequent to a decrease in intracellular oxygenation (Bailey *et al.*, 2007). The oxidative stress typically associated with exercise-induced arterial hypoxaemia (Bailey *et al.*, 2001) may have important implications for vascular homeostasis since the superoxide anion can directly scavenge endothelium-derived nitric oxide (NO) (Gryglewsky *et al.*, 1986). Thus, the present study tested the hypothesis that exercise-induced arterial hypoxaemia would be associated with a "downstream" reduction in the systemic bioavailability of NO.

Eighteen healthy male volunteers aged 26 ± 6 (mean ± SD) years were examined at rest in normoxia, after 6h passive exposure to normobaric hypoxia that required volunteers to inspire 12% oxygen (HYP-REST) and immediately following a maximal cycling test to volitional exhaustion (HYP-EX). The venous plasma (200μL injection) and red blood cell (RBC-100μL injection) concentrations of total NO were assayed using a modified ozone-based chemiluminescence technique (Rogers *et al.*, 2005) and not subject to plasma volume correction. Arterial haemoglobin oxygen saturation (SaO2) was measured using pulse-oximetry calibrated at the respective inspirates. Data were not normally distributed (Shapiro-Wilk-W tests) and thus differences relative to the normoxic control values were assessed using Wilcoxon Matched Pairs Signed Ranks Tests.

Despite a marked decrease in SaO2 during HYP-REST (-12 ± 4%, $P < 0.05$ *vs.* normoxia), no changes were observed in the plasma (-6.4 ± 201.4 nmol/L, $P > 0.05$) or RBC (+15.6 ± 121.6 nmol/L, $P > 0.05$) concentration of total NO. In contrast, HYP-EX was associated with a more marked reduction in SaO2 (-18 ± 6%, $P < 0.05$) and concomitant decrease in plasma (-155.5 ± 228.1 nmol/L, $P < 0.05$) and RBC (-45.0 ± 119.2 nmol/L, $P < 0.05$) total NO.

The present findings indicate that active but not passive exposure to inspiratory hypoxia caused a reduction in the systemic bioavailability of NO. We suggest that this may be due to the more severe arterial hypoxaemia encountered when exercise is superimposed on inspiratory hypoxia and its subsequent ability to compound reductive stress (Bailey *et al.*, 2001).

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA59

Cardiopulmonary exercise testing at extreme altitude

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The factors limiting exercise performance at extreme altitude remain unclear. Maximal exercise tests have been performed up to 7400 metres on small numbers of climbers using Douglas bag expired gas analysis. However, to our knowledge a fully validated breath by breath cardiopulmonary exercise testing system has not been previously used at extreme altitude.
Using hypobaric chambers, cold chambers and field testing, we have validated a portable breath by breath expired gas analysis system which we plan to use on Mount Everest in May 2007. The system has been shown to be reliable up to altitudes of 9000 metres and at temperatures of -20 degrees celcius.
On Everest we will measure changes in anaerobic threshold, ventilatory equivalents for oxygen and carbon dioxide and maximal oxygen consumption in a group of 15 climbers during a standardised ascent from sea level to 8000 metres. Pilot data from a field expedition to Tibet demonstrate that the anaerobic threshold can be reliably identified at altitude. We hope to present this pilot data along with early results from Everest.

Dr Denny Levett on behalf of the Caudwell Xtreme Everest Research Group

http://www.caudwell-xtreme-everest.co.uk/team

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA60

Oxygen delivery at high altitude

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By studying the delivery of oxygen from the air we breathe to its cellular destination in a high altitude environment we may learn more about the physiological adaptations which hypoxic critically ill patients need to develop in order to survive. We believe that the hypobaric hypoxia experienced by lowland residents when they venture to high altitude is an acceptable model for the investigation of possible sea level mechanisms attributable to pathological normobaric hypoxia.
Exercise provokes the cardio-respiratory system due to the increased oxygen demand of active skeletal muscles. This stimulus can serve as a tool with which to manipulate cardio-respiratory physiology in the laboratory setting in order to observe the effects of hypoxia at varying levels of oxygen consumption. There remain some important unanswered questions regarding systemic oxygen delivery at high altitude. The possible explanations for a reduced maximal cardiac output during exercise at altitude are numerous and no one hypothesis has yet been proven. Using pulse contour analysis technology and lithium dilution calibration (the LiDCO system, LiDCO Ltd, UK) we will look closely at changes in stroke volume, cardiac output and oxygen delivery during steady state and ramped exercise protocols. The LiDCO system will allow us to plot changes on a beat-by-beat basis throughout the studies. We also hope to return from Everest in the summer of 2007 with the results of the first arterial blood gas to be taken from a climber on the summit (8848m). This may provide some insight into the extraordinary physiological adaptation necessary to perform at such an altitude.
We have previously investigated the effect of exercise on gastric perfusion under conditions of normoxia and hypoxia using gastric tonometry to calculate the gastric-end tidal partial pressure of carbon dioxide gap. We have shown that when healthy subjects exercise to maximum exertion in normoxic environment an increase in the gastric-end tidal carbon dioxide gap suggesting a reduction in gastric perfusion (1). Subjects undergoing submaximal exercise at an altitude of 5000m also had a reduction in gastric perfusion by the same criterion (2). We are testing the hypothesis that abnormal gastric perfusion occurs at a lower exertional level at high altitude by studying the effects of various peri-anaerobic threshold work rates on gastric perfusion at sea level and high altitude.
Even if systemic and regional oxygen delivery is maintained there remains a vital stage of the oxygen cascade which, if defective, will result in inadequate transfer of oxygen to metabolising cells: the microcirculation. This part of the vascular system comprises the arterioles, capillaries and venules; if flow is abnormal in these vessels there may be shunting of oxygenated blood resulting in tissue hypoxia. Using a specialised camera system known as Sidestream Darkfield (SDF) imaging we have been able to study the microcirculation of mucous membranes. In a small pilot study we have demonstrated abnormal blood flow in the sublingual microcirculation at an altitude of 6,400m. Sluggish flow was noted in vessels of small and large calibre. We do not currently know whether this is due to hypoxia or simply due to the raised haematocrit that occurs as a result of acclimatisation to high altitude. We now intend to observe changes in the sublingual microcirculation at greater altitudes, as well as conducting studies to investigate the effect of normalising certain physiological variables on the microcirculation (e.g. barometric pressure in a portable altitude chamber, inspired oxygen partial pressure, haematocrit).
By looking at systemic, regional and microcirculatory components of the oxygen delivery process at altitude it is possible that we may answer questions otherwise difficult to address in the critically ill population. We hope to be able to share exciting new data with you when we return from our expedition to Mount Everest this summer.

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The Xtreme Everest Investigators Team.

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C26

On the launch of UK PubMed Central

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Since launch of the service at ukpmc.ac.uk in January 2007, scientists working in many areas of biomedical and health research have submitted hundreds of manuscripts UK PubMed Central. This talk provides an introduction to UK PubMed Central. In addition to providing free access to the PubMed Central collection of life science literature, UK PubMed Central currently offers a manuscript submission system at ukpmc.ac.uk/ukmss that enables scientists to make their scientific publications freely available in a new national online repository. UK PubMed Central is run by the British Library and will be developed together with the University of Manchester and the European Bioinformatics Institute to offer further UK-specific services and enhanced content such as links into molecular and medical datasets. The development of UKPMC is supported by principal sponsors of biomedical and health research in the UK.

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C27

Embedding ethics into the undergraduate and postgraduate curricula in biological sciences

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Biological Sciences covers an enormous range of disciplines, many of which incorporate sensitive and contentious areas which have major ethical implications. The QAA Biosciences benchmark statement (QAA, 2002) also requires students to be provided with a training in ethics.

Whilst the Faculty of Biological Sciences, University of Leeds provides some ethics training for its students, it is not available to all students, nor is it provided in an integrated way. The award of a Centre for Excellence in Teaching and Learning in Interdisciplinary Ethics (IDEA CETL) to the University has provided a unique opportunity for biological scientists and ethicists grounded in philosophy to work in partnership to address this problem. Our aim is not to replicate case studies or teaching material in areas where these already exist, but rather, to produce subject-specific materials in areas where these are not available in order to fulfill our objectives.

Our strategy has been to provide a comprehensive and progressive training in ethics throughout individual degree programmes. Within our undergraduate programmes, we have introduced students to ethics and ethical thinking at Level 1, with topics covering both non-discipline specific issues (e.g. plagiarism) and discipline-specific issues which require limited subject-specific knowledge (e.g. drugs in the third world). This training continues at Level 2 where we introduce discipline-specific issues such as the use of animals in scientific research; these seminars covering both the law and the wider ethical implications of such studies. At level 3, we have developed an optional ethics module providing advanced training in ethics and ethical thinking for Biomedical Sciences students. Given that a significant number of biological sciences graduates do not subsequently undertake a career in research, we are developing ethics-based "teaching" or "public understanding of science" projects as an alternative to laboratory-based final year projects. This development of ethics provision extends to all of our postgraduate courses, with both taught and research students being provided with training in generic and discipline-specific issues throughout their programmes. Overall, we seek to equip our graduates with the ability to think through ethical issues which they may encounter in future employment, regardless of career chosen.

Having developed these teaching materials, we also seek to disseminate them to other interested subject practicioners. Thus, all the materials required for delivery of the individual topic by a non-ethicist are available via the IDEA CETL website.

The Quality Assurance Agency for Higher Education (2002) http://www.qaa.ac.uk/academicinfrastructure/benchmark/honours/biosciences.asp#1

Interdisciplinary Ethics Applied Centre of Excellence in Teaching & Learning http://www.idea.leeds.ac.uk

The contribution made by other members of the FBS ethics theme team is acknowledged

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SA61

Plasma membrane calcium pumps and hereditary deafness

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Ca^{2+} plays an essential role in the hearing process. It enters stereocilia of hair cells through mechanoelectrical transduction (MET) channels opened by the deflection of the hair bundle, and is exported back to endolymph that bathes the apical portion of apical cells by an unusual splicing isoform (w/a) of the PMCA2 pump. The w/a isoform carries inserts at sites A and C. The C insert induces premature truncation of the pump. The w/a isoform of PMCA2 is the only means available to stereocilia to export Ca^{2+}. Ablation or missense mutations of the pump cause deafness, as described for the first time in G283S mutation of the deafwaddler (dfw) mouse. A novel deafness-inducing missense mutation of PMCA2 (G293S), has been identified in a human family. The wild type PMCA2 w/a isoform and its G283S and G293S mutants were overexpressed in CHO cells. The other splice variants of PMCA2 (w/b, z/a, z/b) were also expressed as control. Recombinant aequorin was used to monitor Ca^{2+}. At

variance with the other PMCA2 isoforms the w/a variant became activated only marginally when exposed to a Ca^{2+} pulse induced in CHO cells by InsP3. The G293S and G283S mutations did not compromised firther the already poor ability of the w/a variant to became rapidly activated by Ca^{2+} pulse, but delayed the longer term dissipation of the Ca^{2+} transients. In organotypic cultures, Ca^{2+} imaging of vestibular hair cells showed that the dissipation of stereociliary Ca^{2+} transients induced by Ca^{2+} uncaging was compromised in the dfw and also in the PMCA2 KO mice. A digenic mechanism was operational in the case of the human family with impaired hearing described here. The family was screened for mutations in cadherin 23, which accentuated hearing loss in a previously described human family with a PMCA2 mutation. A T1999S substitution was detected in the cadherin 23 gene of the healthy father and affected son but not in that of the unaffected mother, who presented instead the PMCA2 mutation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA62

Plasma membrane calcium pumps: structural diversity as basis for functional versatility

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Precise control of Ca^{2+} movement across the cellular membrane requires specialized transporters such as ion channels, exchangers, and ATP-driven pumps. Plasma membrane calcium pumps (PMCAs) are essential for expulsion of Ca^{2+} from the cell to maintain overall calcium homeostasis and to provide local control of intracellular calcium signaling. Recent work has shown the functional versatility of PMCAs, with specific pumps being required for sperm motility, cochlear hair cell function, signaling feedback in cardiac muscle, and pre- and post-synaptic Ca^{2+} regulation in neurons. The functional versatility of PMCAs is due to differences in their regulation by calmodulin, kinases and other signaling proteins, as well as to their targeting and retention in defined plasma membrane domains. The basis for this is the structural diversity of different PMCAs. In mammals, four genes encode PMCA isoforms 1-4, and each of these has multiple variants due to alternative RNA splicing. The alternatively spliced regions are intimately involved in different regulatory interactions and differential membrane localization of the pumps. For example, alternative splicing generates two major variants of each PMCA (named "a" and "b") that differ in their 50-100 C-terminal residues. The C-terminal tail acts as autoinhibitory domain by interacting with the catalytic core of the pump. Binding of calmodulin to this tail releases the inhibition and activates the pump. The degree of inhibition and the kinetics of interaction with calmodulin differ between PMCA splice variants such as PMCA4a and PMCA4b. This translates into functional differences as illustrated by simulations showing how PMCA4a and PMCA4b handle agonist-evoked Ca^{2+} signals. The simulation replicates experimental data obtained in PMCA4a- and PMCA4b-expressing cells [1] and thereby demonstrates how structural diversity provides functional versatility in PMCAs.

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Supported by NIH grants RO1-GM28835 and RO1-NS51769.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C28

Pre-synaptic plasma membrane Ca^{2+} ATPase isoform 2a regulates excitatory synaptic transmission in rat hippocampal CA3

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Plasma membrane calcium ATPase isoforms (PMCAs) are expressed in a wide variety of tissues where cell-specific expression provides ample opportunity for functional diversity amongst these transporters. The PMCAs use energy derived from ATP to extrude sub-micromolar concentrations of intracellular Ca^{2+} ($[Ca^{2+}]i$) out of the cell. Their high affinity for Ca^{2+} and the speed with which they remove $[Ca^{2+}]i$ depends upon splicing at their carboxy (c) -terminal site.
Here we provide biochemical and functional evidence that a brain-specific,
C-terminal truncated and therefore fast variant of PMCA2, PMCA2a, has a role at hippocampal CA3 synapses. PMCA2a was enriched in forebrain synaptosomes and in hippocampal CA3 it co-localised with the pre-synaptic marker proteins synaptophysin and the vesicular glutamate transporter 1, VGLUT1, but not with the post-synaptic density protein PSD-95. PMCA2a also did not co-localise with glutamic acid decarboxylase-65, GAD-65, a marker of GABA-ergic terminals, although it did localise to a small extent with parvalbumin-positive presumed inhibitory terminals. Pharmacological inhibition of PMCA increased the frequency but not the amplitude of mEPSCs with little effect on mIPSCs or paired pulse depression of evoked IPSCs. However, inhibition of PMCA activity did enhance the amplitude and slowed the recovery of paired pulse facilitation (PPF) of evoked EPSCs. These results indicated that fast PMCA2a mediated clearance of $[Ca^{2+}]i$ from pre-synaptic excitatory terminals regulated excitatory synaptic transmission within hippocampal CA3.

This work was supported by the Epilepsy Research Foundation, UK and BBSRC Grant BBS/B/05338.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C29

The plasma membrane calcium ATPase isoform 4 modulates tumour necrosis factor α-induced apoptosis

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The plasma membrane calcium ATPase (PMCA) is a pump that extrudes calcium from the cytosol. It has four major isoforms of which 1 and 4 are ubiquitously expressed. In recent years, it has been reported that PMCA4 is involved in apoptosis via regulation of intracellular calcium levels [Ca2+]i. However, it is not clear whether PMCA4 promotes or prevents apoptosis. Since PMCA4 has been reported to modulate the activity of neuronal nitric oxide synthase (nNOS) via physical interaction; and because nitric oxide (NO) has both pro and antiapoptotic potentials, it is possible that the PMCA4-nNOS interaction is involved in PMCA4-mediated modulation of apoptosis.

To study the role of PMCA4 in apoptotic signalling, the effect of PMCA4 deletion on tumour necrosis factor α (TNFα)-induced apoptosis was examined and the relevance of the PMCA4-nNOS interaction was assessed.

Mouse embryonic fibroblasts (MEF) were derived from PMCA4 knockout (PMCA4-/-) and wild-type (PMCA4+/+) embryos. Apoptosis was induced in MEF by TNFα and quantified by measuring the activity of the executioner caspases 3 and 7 and by flow cytometric analysis of annexin V/propidium iodide labelled cells. Activation of signal mediators in TNFα signalling pathways was assessed by measuring the activated (phosphorylated) forms relative to total expression by immunoblotting. [Ca]i was measured by fluorometry using the Ca2+ fluorophore fluo-3.

In non-stimulated cells, growth rates, [Ca2+]i and apoptosis levels were similar in PMCA4+/+ and -/- MEFs. Following stimulation with TNFα (10ng/ml) for 24 hours, PMCA4-/- MEF had significantly lower (by 46%) caspase 3/7 activation compared to PMCA4+/+ MEF ($p<0.01$, $n=14$). This was confirmed by flow cytometry.

In pursuing the mechanism underlying this phenotype, the activation patterns of TNFα signal mediators were assessed. The proapoptotic caspase-8, the antiapoptotic inhibitor of kappa kinase and extracellular regulated kinase1/2, as well as c-Jun N-terminal kinase which has pro- and antiapoptotic potentials were similarly activated in PMCA4/+/+ and -/- MEF. Interestingly, [Ca2+]i did not change in either group up to 4 hours after TNFα treatment.

To investigate whether modulation of nNOS by PMCA4 was responsible for this phenotype, the effects of the nNOS inhibitor S-methyl thiocitrulline (SMTC) and the NO donor S-nitroso-acetyl penicillamine (SNAP)were assessed. Treatment with SMTC or SNAP prior to induction of apoptosis by TNFα eliminated differences in apoptosis levels between PMCA4+/+ and -/- MEF, either by decreasing apoptosis in the PMCA4+/+ MEF (SMTC effect) or increasing it in the PMCA4-/- MEF(SNAP effect).

In conclusion, this work has shown that PMCA4 mediates susceptibility to TNFα-induced apoptosis, likely through modulation of nNOS signalling.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA63

Role of plasma membrane calcium ATPases in neuronal dysfunction

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Plasma membrane calcium ATPases (PMCAs) are essential ion pumps, which extrude calcium from cells. Although two isoforms, PMCA2 and PMCA3, are primarily expressed in neurons, their contribution to neuronal pathology is not well defined. Earlier studies in our laboratory indicated that the expression of PMCA2 is significantly decreased in spinal cord neurons at onset of experimental autoimmune encephalomyelitis (EAE)[1,2]. EAE is an animal model of Multiple Sclerosis (MS), an inflammatory, demyelinating and neurodegenerative disease of the central nervous system. The aforementioned finding was of particular interest as it raised the possibility of an involvement of PMCA2 in neuronal injury and suggested a putative novel mechanism underlying cellular dysfunction in EAE. Subsequent studies established a causal relationship between inhibition of PMCA activity and neuronal pathology. Treatment of spinal cord neuronal cultures with carboxyeosin (CE), a PMCA inhibitor, delayed the clearance of depolarization induced calcium transients which was followed by abnormal expression of non-phosphorylated neurofilament H (NFH), neuritic beading, an increase in the number of activated caspase-3 positive cells and decreased survival[3].

To further unravel the mechanisms leading to neuronal injury in response to inhibition of PMCA, we evaluated activation of calpain by quantifying the breakdown of its substrate α-spectrin. We focused on calpain because it is a calcium dependent protease which has been implicated in MS pathology. There was a two-fold increase in the ~ 145 KDa α-spectrin breakdown product in cultures treated with CE as compared to controls. The augment in the α-spectrin degradation product preceded induction of caspase-3 and neuronal loss.

The triggers that modulate PMCA2 levels in spinal cord neurons are not well defined. As activated microglia are abundant in the spinal cord during EAE and have been implicated in neuronal injury, we determined whether these cells secrete soluble factors that suppress PMCA2 expression. We found a significant but transient decrease in PMCA2 protein levels in neurons co-cultured with microglia. The reduction in PMCA2 correlated with an increase in the number of neurons expressing non-phosphorylated NFH, a change that persisted despite the return of PMCA2 levels to control values.

Additional investigations were undertaken to determine whether factors which are believed to be present in the inflammatory milieu during EAE, modulate PMCA2 levels. One of these agents,

glutamate, is released by activated microglia and damaged neurons. It has been reported that glutamate mediates EAE pathology by acting via the AMPA/kainate receptors[3,4]. Treatment of pure spinal cord neuronal cultures with 4 μM kainic acid (KA) for 36 hours decreased PMCA2 protein levels without affecting cell viability. We concluded that continuous exposure to KA for extended periods can suppress PMCA2 protein expression even when the concentration is low. Higher KA concentrations (20 μM) decreased PMCA2 levels within 12 hours without inducing cell death. A further reduction in PMCA2 was observed after 24 hours but this was in part due to neuronal loss.

To further analyze the role of PMCA2 in spinal cord neurons, in vivo, the consequences of a null mutation in the PMCA2 gene were examined in heterozygous and knockout mice. Our earlier investigations had shown a significant reduction in the number of motor neurons in the lumbar spinal cord of PMCA2-null mice. Motor unit number estimation (MUNE), a non-invasive, electrophysiological method that quantifies the approximate number of motor neurons innervating a single muscle or a small group of muscles, indicated a 60% decrease in the knockout as compared to the wild type mice. Although heterozygotes exhibit a phenotype which roughly appears similar to that of the wild type, a 30% reduction in MUNE was observed in these mice as compared to the wild type littermates. In contrast, sensory function was not affected in either $PMCA2^{-/-}$ or $PMCA2^{+/-}$ mice. The performance of heterozygotes in the rotarod test was significantly inferior to that of the wild type. Thus, even partial reductions in PMCA2 may be sufficient to induce motor deficits.

In sum, PMCAs and in particular PMCA2 appear to play a critical role in the integrity and function of spinal cord neurons. In pathological conditions involving inflammation, a number of triggers, including those produced by microglia, may suppress PMCA activity or expression, leading to neuronal pathology and, in some cases, to cell death.

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This work was supported by grants NS 046363 from NIH/NINDS and 01-3008-SCR-S-0 from NJCSCR to SE.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA64

Structural views of the gating process for inwardly rectifying K^+ channels

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Ion channels play pivotal roles in many of the body's physiological processes such as the regulation of the heart beat, release of insulin, and the initiation and modulation of all nerve impulses. They perform these functions through a combination of ion selection and tight regulation of the ion movement across the membrane which is known as gating. For inwardly rectifying K^+ (Kir) channels the KirBac1.1 crystal structure revealed the closed state in which ion movement is blocked through a hydrophobic gate. Presentation of two new crystal structures of the related KirBac3.1 channel that were captured in intermediate gating states between the closed and open conformations illustrates the conformational changes that occur during gating. These structures uncover how for this family of K^+ channels gating involves a coordinated change in salt bridge connections between the cytoplasmic domains, reveals the location of a spermine binding site which is important in the rectification process and shows an unforeseen flexibility between the transmembrane sections and the cytoplasmic domains.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA65

Structure of the inositol 1,4,5-trisphosphate receptor

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Inositol 1,4,5-trisphosphate receptors (IP_3R) are large (~1.2MDa) tetrameric intracellular Ca^{2+} channels predominantly located in the endoplasmic reticulum. They are responsible for initiating and propagating the Ca^{2+} signals evoked by the diverse extracellular stimuli that initiate the phosphatidylinositol signalling cascade. Overall each monomeric subunit comprises an inositol 1,4,5-trisphosphate (IP_3) binding core at its N-terminus, a transmembrane region comprising six transmembrane segments towards its C-terminus and an intervening regulatory domain. It is still not clear how these different domains are structurally arranged and how they interact during channel activation.

We have used electron microscopy and single particle analysis to calculate the structure of the type I IP_3R in the absence of IP_3 and Ca^{2+}, which are its native regulators (1). By relating the organisation of the structural domains in the resulting 3D map to secondary structure predictions and biochemical data we have developed a structural model in which the amino acid sequence was mapped onto the domains formed by the densities of the 3D reconstruction. Similar methods have now been used to investigate the effect of IP_3 binding upon the structure of IP_3R. A direct comparison between the maps obtained for the unliganded and IP_3 bound IP_3R reveals substantial rearrangements of the structural domains. Furthermore, the location of the binding site of IP_3 within IP_3R has been inferred by calculating a 3D map of the IP_3R bound to a novel higly electron dense undecagold derivative of IP_3 coupled via a reactive amine attached to the 2-position of IP_3.

Although the new 3D maps of the IP_3R allowed a direct observation of the extent of the conformational reorganisation associated with channel activation, at the present resolution levels no direct information can be gained from these maps on the molecular mechanisms underlying channel gating and conductance properties. However, information on these mechanisms

can be obtained using high resolution structural information already available for related families of channel proteins. This involves a careful aminoacid sequence alignment between members of the different protein families and the constructions of homology models, followed by a validating analysis of the model by its consistence with available biochemical data. Here the structural basis by which Ca^{2+} conductivity of IP_3R is regulated has been investigated using aminoacid sequence analysis to produce models for the transmembrane region of IP_3R in its closed and open states, using the x-ray crystallographic structures of two bacterial K^+ channels KirBac (2) and MtHK (3) as templates, respectively. From these models we proposed a common mechanism for channel opening for tetrametic K+ channels and the IP_3R. Furthermore, it was possible to infer onto the molecular basis of cation selectivity between the highly selective K^+ channels and the Ca^{2+} channels, IP_3R and ryanodine receptor.

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This work has been funded by grants from the Wellcome Trust and Cancer Research UK

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA66

Single-molecule FRET measurements of MscL conformational changes

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The mechanosensitive channel of large conductance (MscL) is a non-selective ion channel which opens in response to changes in the transmembrane pressure profile, providing an important mechanism by which prokaryotes can respond to osmotic changes in their environment. The structure of this homo-pentameric ion channel has been well characterized in its closed state, but because it has not been crystallized in its open state, the details of its open structure remain somewhat unclear. The large distance involved prevents an accurate measurement of the size of the open pore using electron paramagnetic resonance. Ensemble fluorescence resonance energy transfer (FRET) measurements are not definitive because energy transfer between multiple donors and acceptors within the pentamer and between neighboring channels precludes direct interpretation of the data. We present a single-molecule FRET approach to measuring the diameter of the MscL open pore. A single cysteine mutation in the channel was labeled with an equal ratio of donor (AlexaFluor 488) to acceptor (Alexa 747) maleimides, but with a low overall ratio of fluorophores to monomers. The channel was reconstituted into small unilamellar vesicles at a protein to lipid ratio such that roughly one channel was inserted into each liposome. Measurements were made on the channels in the closed state, or while trapped in the open state by the introduction of lysophospholipids. Histograms were generated including only fluorescence traces in which both the donor and the acceptor each photobleach in a single-stepping pattern. These FRET histograms are used to determine changes in pore radius as the channel moves from closed to open states.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA67

Activation and inactivation gating in potassium channels: insights from structure and dynamics

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Ion channel "gating" refers to the conformational changes involved in the opening and closing of the ion conducting pathway. In KcsA potassium channel, gating involves a conformational change between the closed and open conformation in response to intracellular pH. Recently, it was found that channel opening is followed by an inactivation process which resembles the C-type inactivation observed in voltage-gated eukaryotic channels (Gao et al 2005, Cordero-Morales et al 2006). This inactivation is suppressed by mutation E71A. The crystal structure of E71A at 2.5 Å suggested that interactions between residues E71, D80, W67 and R64 might be involved in the distortion of the selectivity filter responsible for the inactivation process.
Using patch clamp, EPR spectroscopy, X-ray crystallography and MD simulations, we have characterized the effect of a wide range of side chain substitutions at position 71, in an attempt to establish the role of the selectivity filter in steady-state gating. Single channel analysis demonstrated that at position 71, side-chain polarity and charge affect activation and inactivation gating in KcsA. We classified these mutants in three kinetic groups; the first with Po $\approx$ 0.9 and mean open times $>$ 70 ms, the second group, represented by the WT KcsA with an apparent Po $\approx$ 0.1 and mean open time $<$ 30 ms and the last group with Po $\approx$ 0.5 and a mean open times of less than 6 ms. Interestingly, all the substitutions had a mean closed times of $<$ 7 ms, suggesting that inactivation was affected in all mutations (in contrast, the WT shows MCT $>$ 500 ms). Moreover, macroscopic currents from these mutants are similar to the non-inactivating E71A mutant than WT KcsA. We solved the crystal structure of mutants in each representative group and no significant difference was observed compared to the WT structure, suggesting that the conformational changes at the selectivity filter might occur only when the inner helix bundle is in the open conformation.
We have carried out molecular dynamics simulations of E71A and wild-type KcsA channel, to address the basic principles underlying the changes that take place near the selectivity filter

and their relationship with the inactivation mechanism. In the wild-type channel, the presence of hydrogen bond network behind the selectivity filter formed by E71, D80 and W67 residues acts like a molecular "spring" that modulates the conformation of the filter, leading to inactivation. In E71A mutant, the absence of this molecular spring due to E71 mutation, the filter is unchanged and hence the channel is constitutively active. Even though the analysis is carried out on the closed state of the channel (in regards to the conformation of the inner helix bundle), we believe that interactions behind the filter also play a crucial role in the open state.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C30

A novel rapid inactivation process in cardiac Kv1.5 channels revealed by voltage clamp fluorimetry

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Voltage-gated Kv1.5 channels contribute to the ultra-rapid cardiac K^+ current, I_{Kur}, and have been implicated in atrial fibrillation. We used voltage clamp fluorimetry to directly observe the conformational changes associated with Kv1.5 channel gating by attaching a tetramethylrhodamine-5-maleimide (TMRM) fluorescent probe to substituted cysteine residues in the voltage sensor domain (M394C to R400C). We reveal that the fluorescence report of voltage sensor movement in Kv1.5 is uniquely different from that in the archetypical voltage-gated K^+ channel, *Shaker*. Whereas the fluorescence report of voltage sensor movement (from TMRM attached at A359C in the S3-S4 linker) in *Shaker* channels was mono-exponential and occurred with a similar time course (τ=2.4 ± 0.5 ms at +60 mV; n=4) to ionic current activation (τ=4.5 ± 1.0 ms at +60 mV; n=4), the report of Kv1.5 voltage sensor movement reflected complex conformational changes. In these first reports of Kv1 family channel fluorescence, we show that upon depolarization, TMRM at M394C and A397C in the S3-S4 linker of Kv1.5 channels reported a transient rapidly activating fluorescence deflection that was followed by a prominent rapidly decaying component that represented 27 ± 4 % of the total signal and occurred with a τ of 3.7 ± 0.4 ms at +60 mV (n=4). Using 4-aminopyridine and the ILT triple mutation (V407I, I410L and S414T) to dissociate channel opening from voltage sensor movement, we show that the decaying component of fluorescence was associated with channel opening. Furthermore, inhibition of inactivation (by raising external K^+ from 3 to 99 mM, or the mutation R487V) abolished the decaying component of fluorescence. Interestingly, raising external K^+ also increased macroscopic conductance and this was reduced by the mutation R487V. These data suggest that the rapidly decaying component of fluorescence reflects channel inactivation. Furthermore, this inactivation may be responsible for the reduced conductance with 3 mM external K^+ since raising external K^+ both abolished the rapidly decaying fluorescence component and increased channel conductance. This implies that with physiological K^+ conditions, a considerable proportion of Kv1.5 channels become rapidly inactivated upon depolarization and that local changes in external K^+ may act to critically regulate the availability of Kv1.5 channels and therefore the amplitude of I_{Kur} current in the heart.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C31

In silico study of putative calcium-binding domains in hBK and hBest1

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Human big conductance Ca^{2+} and voltage gated potassium channels[1] (hBK) play an important role in the repolarization of the membranes and are putative drug targets for cardiovascular, respiratory and urological diseases. Human Bestrophins1[2] (hBest1) are suggested to function as Cl^- channels, having Ca^{2+} sensitivity and are associated with age-related macular degeneration, particularly with Best vitelliform macular distrophy.
The crystal structures for both channels are unknown. However they share the known calcium-binding motif pattern DDD-x-[D,E]. We have used bioinformatics and molecular simulation approaches to construct models of putative calcium-binding domains in hBK and hBest1 channels. Models could help to rationalize the available experimental data and to plan new experiments in order to understand the physiological role of these classes of channels. The search of this calcium-binding motif in the Swiss-Prot database by means of Prosite produced a high number of results. However, only a few of them correspond to proteins, which are actually known to bind calcium. Among them just the human Thrombospondin-1[3] (hTSP-1) has the three-dimensional (3-D) structure available. Therefore human TSP-1 calcium-binding domain was used as a template for the modeling the putative calcium-binding domains of hBK and hBest1. Molecular dynamics simulations were then carried out on both models to evaluate the thermodynamic stability and to study the dynamical properties of both calcium-binding domains.

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SA68

Oxidative stress and cerebrovascular dysfunction in neurodegenerative disease

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Several factors have been involved in Alzheimer's disease (AD) but there is no definite conclusion as to the main pathogenic agents. Mutations in the amyloid precursor protein (APP) that lead to increased production of amyloid beta (Aβ) peptide are associated with the early onset, familial forms of AD. However, in addition to aging, the most common risk factors for the sporadic, prevalent form of AD are hypertension, hypercholesterolemia, ischemic stroke, the ApoE4 allele and diabetes, all characterized by a vascular pathology. In AD, the vascular pathology is essential to the diagnostic to the same extent as senile plaques, neurofibrillary tangles and neurodegenerative changes. Recently, chronic cerebral hypoperfusion has been proposed as an important factor in the cognitive deficits of AD.

The vascular pathology in AD is characterized by the accumulation of Aβ in the vessel wall, atherosclerosis, vascular fibrosis, and structural changes of the blood vessels. Here, we used two transgenic mouse models that reproduce different but complementary aspects of the cerebrovascular pathology of AD to investigate the consequences on brain vascular functions. Mice overexpressed a mutated form of APP (APP mice) or an active form of the cytokine transforming growth factor-β1 (TGF-β1, TGF mice), and both have been shown previously to display cerebral hypoperfusion and hypometabolism.

Using aged APP (>12 month-old) and TGF (>18 months) mice, we characterized their cerebrovascular responsiveness to vasoactive agonists, investigated alterations in proteins involved in vascular oxidative stress or vascular fibrosis, and compared changes in cortical microvessels and markers of oxidative stress to those found in brain and brain vessels from neuropathologically confirmed cases of AD. Additionally, we tested the effects of antioxidant therapy *in vivo* on the cerebrovascular dysfunctions induced by Aβ and TGF-β1. Aged mice were or not treated, and cerebrovascular functions (online videomicroscopy), vascular and neuronal protein alterations and, for APP mice, Aβ plaque load and soluble Aβ levels (Western blot and immunocytochemistry) were determined. The age was selected so that at the end of treatment, cerebrovascular dysfunctions in untreated mice would be fully manifest in order to best mimic the conditions seen in AD patients at the time of diagnostic.

Aged APP mice displayed impaired dilatations to acetylcholine (ACh) and calcitonin gene-related peptide (CGRP), and a decreased contractile response to inhibition of nitric oxide synthase (NOS) as compared to wild-type controls. These alterations were associated with increased cerebrovascular levels of superoxide dismutase 2 (SOD2 or MnSOD), an antioxidant enzyme that is upregulated by its substrate superoxide, further supporting that Aβ exerts its deleterious effects on vascular functions through oxidative stress. Treatment with antioxidants Tempol or NAC (N-acetylcysteine) fully restored cerebrovascular functions in APP mice and normalized cerebrovascular SOD2 levels. Aβ plaque load and soluble levels of Aβ1-42 were unaltered by antioxidant therapy.

Aged TGF mice exhibited impaired dilatations to ACh and CGRP, and decreased contractile responses to NOS inhibition or to endothelin-1 (ET-1) as compared to wild-type controls. These dysfunctions were accompanied by increased levels of collagen-IV, connective tissue growth factor (CTGF) and endothelin receptors type B (ETB receptors), which have all been associated with vascular fibrosis. The vascular alterations in these mice compared exquisitely well to that found in brain vessels of neuropathologically confirmed cases of AD. Antioxidant therapy was devoid of any beneficial effects on either cerebrovascular functions or protein alterations in TGF mice.

These experiments show that oxidative stress is the major mechanism via which Aβ alters cerebrovascular functions, and this even at a very advanced stage of the pathology. Further, the results demonstrate that it is possible to fully reverse the Aβ-induced cerebrovascular dysfunctions in aged APP mice. However, as shown in the TGF mice, antioxidant therapy did not improve the TGF-β1-induced functional and structural alterations. These results indicate that antioxidants will have limited benefits in the cerebrovascular pathology of AD, in which both Aβ and TGF-β1 have been shown to be increased.

Supported by CIHR grant (MOP#53334) and studentship (NN), FRSQ (BO) and MNI-Jeanne Timmins Costello (TA) fellowships.

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SA69

Genes, endothelial function and cerebral small vessel disease

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Cerebral small vessel disease (SVD) accounts for 20% of ischaemic stroke and is the major cause of vascular dementia. Disease of the small perforating arteries supplying white matter and the deep grey matter nuclei results in small focal areas of complete infarction (lacunar infarction) and/or areas of diffuse incomplete infarction (leukoaraiosis) which neuropathologically show ischaemic demyelination, axonal loss and gliosis. The pathogenesis is incompletely understood. It has been suggested there may be two pathological types of cerebral SVD.(1) Microatheroma at the origins or in the proximal perforating arteries resulting in single larger lacunar infarcts without leukoaraiosis, and a diffuse small vessel arteriopathy affecting the smaller perforating arteries resulting in multiple smaller lacunar infarcts and leukoaraiosis. The latter subtype has been referred to as ischaemic leukoaraiosis.(2) In this subtype hypertension is the major risk factor, and impaired autoregulation and reduced cerebral blood flow appear important, resulting in leukoaraiosis, initially in those internal watershed areas in which arterial perfusion pressure is lowest. Both cerebral blood flow and cerebral autoregulation are dependant on normal endothelial function and nitric oxide release.(3) A number of lines of evidence implicate impaired endothelial function in cerebral

SVD pathogenesis including pathological data, circulating endothelial markers, and imaging findings.(2) Genetic factors appear important, with the heritability of MRI determined cerebral SVD in community populations estimated to be as high as 60%.(4) Candidate gene association studies have implicated genes affecting endothelial function including eNOS and components of the renin-angiotensin system. However, larger studies have been unable to consistently confirm these findings.(5) Almost all candidate gene studies have been underpowered to date. Larger multicentre collections are now being performed to evaluate the responsible genetic variants.

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SA70

Hypoxia and hypercapnia: changes in cerebrovascular regulation during sleep

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During wakefulness, cerebral blood flow (CBF) is closely coupled to regional cerebral metabolism; however, CBF is also strongly modulated by breathing, increasing in response to both hypercapnia and hypoxia. During slow wave sleep (SWS), cerebral metabolism, CBF and arterial blood pressure decrease whilst the partial pressure of arterial CO_2 increases due to a reduction in alveolar ventilation. The reduction in CBF during SWS therefore occurs despite a relative state of hypercapnia and could be explained, at least in part, by changes in the cerebral vascular response to CO_2. As sleep is a state in which pathophysiological challenges to the cardio-respiratory system are most marked, it is important to understand the regulation of cerebral blood flow during sleep.

In a series of studies in our laboratory, we have determined changes in CBF and the cerebral vascular responses to hypercapnia ($CVR\text{-}CO_2$) and hypoxia using transcranial Doppler ultrasound to measure middle cerebral artery blood flow velocity (MVAV) as a non-invasive index of cerebral perfusion in humans. We confirmed the observation of others that CBF during SWS is reduced; further, we determined that $CVR\text{-}CO_2$ is reduced by 70% compared to the waking state – a change that would permissively allow CBF to fall at a time when arterial PCO2 is increased (Meadows et al. 2003). Strikingly, cerebral vasodilation to mild hypoxia (arterial oxygen saturation -10%) is completely abolished during slow wave sleep (Meadows et al. 2004); indeed these observations are consistent with a cerebral vasoconstriction in response to hypoxia during SWS.

Rapid eye movement sleep (REM) is associated with notable fluctuations in cardiovascular and respiratory variables; overall, CBF and cerebral metabolism are reported to be similar to, or slightly raised, compared to the waking state. To test the hypothesis that $CVR\text{-}CO_2$ would also be reduced during REM sleep, MCAV was measured in 12 normal healthy male subjects (age 26.2 ± 4.2 years, body mass index 23.8 ± 3.0 $kg.m^{-2}$). Minute ventilation decreased (Wake: 8.2 ± 2.4; REM: 6.0 ± 1.7 $l.min^{-1}$; $p<0.05$) and $PETCO_2$ increased during REM compared to Wake (41 ± 3 vs. 44 ± 3 mmHg; $p<0.05$). Overall, MCAV did not change (53.3 ± 13.9; 54.2 ± 15.1 $cm.sec^{-1}$, $p>0.05$). However, MCAV during phasic REM was significantly higher than tonic REM (56.5 ± 13.3 vs. 51.7 ± 9.2 $cm.sec^{-1}$, $p<0.05$). REM sleep may be divided into 2 phases – tonic (absence of eye movements) and phasic (bursts of eye movements) REM. The proportion of tonic and phasic REM was not changed by hypercapnia and $CVR\text{-}CO_2$ during REM was not significantly different from that during wake (Wake: 2.2 ± 0.9; REM: 1.7 ± 1.0 $cm.sec^{-1}.mmHg^{-1}$, $p>0.05$).

Upon waking in the morning, $CVR\text{-}CO_2$ is reduced compared to measurements made during wakefulness in the previous evening (e.g. Meadows et al. 2005); this observation has been linked to the increased risk of stroke in the early morning. The mechanism for this change is uncertain but might, in part, reflect the sleep-related changes in $CVR\text{-}CO_2$ reported above. It is of interest that CVR to hypoxia, upon waking in the morning, is no different from that measured on the previous evening (Meadows et al. 2005) and suggests that state-related changes in CVR to hypercapnia and hypoxia may not be mediated by the same mechanisms.

In conclusion, the changes in CVR to hypercapnia and hypoxia, that we report across the sleep-wake cycle, indicate that the regulation of these factors is modulated by changes in neural state. As these changes in $CVR\text{-}CO_2$ mirror the changes in CBF and cerebral metabolism across these states, the regulation of both CBF and $CVR\text{-}CO_2$ may be directly linked to cerebral metabolism. The mechanism for any such linkage remains to be established. These changes in cerebral vascular regulation during sleep may make the brain particularly susceptible to vascular insults associated with sleep-related breathing disorders and stroke.

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Supported by the Wellcome Trust

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA71

Central fatigue: muscles work but the brain gets tired

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Central fatigue describes circumstances under which strength appears to be limited by the ability of the central nervous system to recruit motoneurones. Central fatigue manifests when the effort to contract skeletal muscles is intense, and thus increases when exercise is performed under stress, as provoked by an unusual environment, and is attenuated following training. Central fatigue is associated not only with reduced strength but also with an inability to maintain contraction. Generation of force, thereby, resembles that developed under the influence of a competitive neuromuscular blocking agent, which mainly affects slow twitch muscle fibres. Central fatigue has not been explained, but the cerebral metabolic response to intense exercise, as to other modalities of cerebral activation, is a reduction in its metabolic ratio, i.e. the ratio between the brain's uptake of oxygen relative to that of carbohydrate. At rest the cerebral metabolic ratio is close to 6 but during intense whole body exercise it decreases to less than 3, with the uptake of lactate being as important as that of glucose. This apparent inability of the brain to oxidise carbohydrate taken up during activation remains debated with the concomitant uptake of ammonium suggesting that less than 10% of the up to 10 mmol "extra" carbohydrate taken up by the brain is used for formation of amino acids. Accumulation of metabolic intermediates and compartmentalisation of substrate between astrocytes and neurones are avenues that need to be explored considering that breakdown of glycogen in the astrocytes may account for the additional 10 mmol glycosyl units.

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C32

Cerebral vascular dilator responses are reduced in Zucker obese rats with insulin resistance

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Dilator responsiveness of cerebral arteries is selectively deranged in insulin resistance (Erdos et al., 2004; 2006). While the mechanisms involved are not fully explored, abnormal mitochondrial function may be involved (Katakam et al., 2007). Diazoxide (DZ), a mitochondrial ATP sensitive potassium channel activator, elicits arterial vasodilation by increasing the generation of mitochondria-derived reactive oxygen species (ROS) that stimulate Ca2+ sparks and Ca2+ activated K+ channels. We evaluated DZ induced vasodilation in isolated, pressurized cerebral arteries from Zucker obese (ZO) and lean (ZL) rats by videomicroscopy. The ZO rats were hyperinsulinemic but were not hyperglycemic or hypertensive. DZ induced vasodilation was diminished in ZO compared to ZL [Figure; 5±5% (n=12) versus 43±6%(n=12)]. 3-nitropropionic acid, a succinate dehydrogenase inhibitor and a promoter of mitochondrial ROS generation, had no effect on cerebral vasodilation in ZL (43± 17%; n=4), but enhanced DZ induced vasodilation to 71±11% (n=5) in ZO. DZ induced vasodilation was partially inhibited by iberiotoxin in ZL while it was totally abolished in ZO (Figure). ZO arteries exhibited reduced pressure-induced constriction compared to ZL. However, vasodilation to nitroprusside was similar in both ZO and ZL [87±4% (n=17) versus 80±6% (n=14]. Thus, cerebral arteries from ZO exhibit abnormal DZ-induced vasodilation, which is likely mediated by reduced generation of mitochondrial ROS and/or Ca2+ sparks. Impaired cerebrovascular dilator responses in insulin resistance may chronically impair blood flow/metabolism coupling in the brain and lead to neurological diseases such as Alzheimer's Disease.

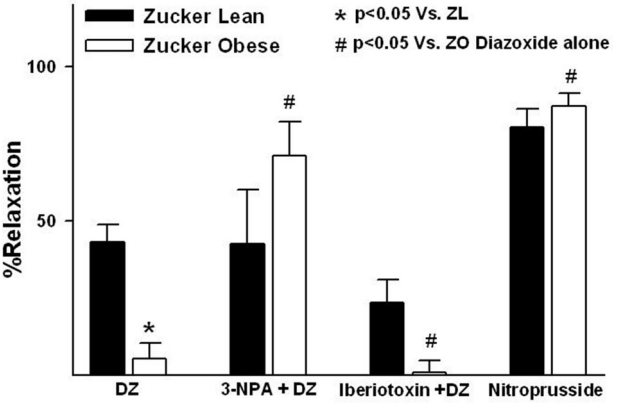

Figure. Adverse Effects of Insulin Resistance on Cerebral Arterial Responses

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Supported by NIH grants HL-07731, HL-030260, DK-062372, and HL-065380.

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SA72

Phospholipase Cβ as a neuronal coincidence detector

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Endogenous cannabinoids (endocannabinoids) mediate retrograde signals at various regions of the brain. Endocannabinoids are produced on demand in activity-dependent manners and

released from postsynaptic neurons. The released endocannabinoids travel backward across the synapse, activate presynaptic CB1 cannabinoid receptors, and cause transient or long-lasting suppression of neurotransmitter release. Endocannabinoid release can be triggered by membrane depolarization that elevates intracellular calcium concentration ($[Ca^{2+}]_i$) to a micromolar range or by activation of the $G_{q/11}$ protein-coupled receptors such as group I metabotropic glutamate receptors (mGluRs) and M_1/M_3 muscarinic acetylcholine receptors. Furthermore, coincidence of $[Ca^{2+}]_i$ elevation and $G_{q/11}$ protein-coupled receptor activation cooperatively induces endocannabinoid release. $G_{q/11}$ protein-coupled receptors activate phospholipase Cβ (PLCβ) leading to diacylglycerol (DAG) production. DAG is then broken down to the major endocannabinoid 2-arachidonoylglycerol (2-AG) by the action of DAG lipase. To determine the role of PLCβ in endocannabinoid release, we used cultured hippocampal neurons and monitored the endocannabinoid release by measuring cannabinoid-sensitive synaptic currents (Reference 1). The PLCβ family consists of 4 isoforms (PLCβ1-4) of which PLCβ1 is the predominant isoform in the forebrain including hippocampus. We found that the receptor-driven endocannabinoid release was absent in mutant mice lacking PLCβ1. This PLCβ1-mediated endocannabinoid release was dependent on physiological levels of $[Ca^{2+}]_i$. We measured PLCβ1 activity in intact neurons by using exogenous TRPC6 channel as a biosensor for the PLC product DAG. The TRPC6 channel is a member of canonical transient receptor potential family and is activated by intracellular DAG. In hippocampal neurons expressing exogenous TRPC6 channels, large inward currents were induced by application of a membrane-permeable DAG analogue or by activation of M_1/M_3 muscarinic acetylcholine receptors or group I mGluRs. These currents were negligible in control neurons, indicating that exogenous TRPC6 channels mediate these currents. In hippocampal neurons from PLCβ1-knockout mice expressing exogenous TRPC6 channels, activation of $G_{q/11}$ protein-coupled receptors caused negligible inward currents. The receptor-driven TRPC6-mediated currents showed a similar $[Ca^{2+}]_i$ dependence to that of the receptor-driven endocannabinoid release. These results indicate that PLCβ1 serves as a coincidence detector for triggering endocannabinoid release in the hippocampus.

We then examined the roles of PLCβ in endocannabinoid release triggered by synaptic activity. For this purpose, we made whole-cell recordings from Purkinje cells in mouse cerebellar slices and examined their excitatory synapses arising from climbing fibers and parallel fibers (Reference 2). We sampled Purkinje cells from the rostral half of the cerebellum where PLCβ4 is the predominant isoform in Purkinje cells. We first characterized three distinct modes to induce endocannabinoid release by analyzing climbing fiber to Purkinje cell synapses. The first mode is strong activation of mGluR subtype 1 (mGluR1) - PLCβ4 cascade without detectable Ca^{2+} elevation. The second mode is Ca^{2+} elevation to a micromolar range without activation of mGluR1 - PLCβ4 cascade. The third mode is Ca^{2+}-assisted mGluR1 - PLCβ4 cascade that requires weak mGluR1 activation and Ca^{2+} elevation to a sub-micromolar range. By analyzing parallel fiber to Purkinje cell synapses, we found that the third mode is essential for effective endocannabinoid release from Purkinje cells by excitatory synaptic activity. Furthermore, we demonstrated by biochemical analysis that combined weak mGluR1 activation and mild depolarization in Purkinje cells effectively produces 2-AG, whereas either stimulus alone does not produce detectable 2-AG. Our results strongly suggest that under physiological conditions, excitatory synaptic inputs to Purkinje cells activate the Ca^{2+}-assisted mGluR1-PLCβ4 cascade, and thereby produces 2-AG that retrogradely modulates synaptic transmission to Purkinje cells.

Our results obtained from hippocampal neurons and cerebellar Purkinje cells indicate that PLCβ functions as a neuronal coincidence detector through its Ca^{2+} dependency and may play important roles in various synaptic modulations and plasticity.

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SA73

Dynamic regulation of PIP2 and its role in ion channel modulation: regulation of M-type potassium channels

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A wide variety of ion channels are subject to regulation by membrane PIP2 (phosphatidylinositol-4,5-bisphosphate) (Suh & Hille, 2005). We have concentrated our attention on a species of K+ channel, the M-channel. This is a low-threshold, voltage-gated K+ channel that is widely distributed in central and peripheral neurons, where is serves to regulate neuronal excitability. Native M-channels are composed (primarily) of a heteromeric assembly of Kv7.2 and Kv7.3 subunits. The channels are closed by activating many different receptors that couple to Gq/11 G proteins, most prominently M1 muscarinic acetylcholine receptors (mAChRs), leading to an increased neuronal excitability. Channel opening requires the presence of PIP2 and available evidence suggests that closure following M1-mAChR stimulation results from Gq / phospholipase-C (PLC) mediated PIP2 hydrolysis and consequent PIP2 depletion (Delmas & Brown, 2005; Suh & Hille, 2005).

We have tried to follow the dynamics of mAChR-induced PIP2 hydrolysis and depletion in single living sympathetic neurons using (initially) the fluorescently-tagged PH domain of PLCδ, GFP-PLCδPH, in combination with voltage-clamp membrane current recording (Winks et al., 2005). This probe binds to membrane PIP2 then translocates to the cytosol following PIP2 hydrolysis. Since the probe also binds to the cytosolic hydrolysis product inositol-4,5-bisphosphate (IP3), we used an intracellular IP3 displacement assay to calculate changes in membrane PIP2 from the fluorescence signals. Fluorescence changes showed a close temporal and concentration-dependent correlation with mAChR-induced M-current inhibition, and inhibition could be satisfactorily accounted for from the calculated changes in membrane PIP2, with a maximal depletion of ~83%.

However, these calculations break down if PIP2 synthesis is accelerated during receptor activation. This has been suggested to occur following activation of another Gq/11-coupled receptor, the B2-bradykinin (BK) receptor, for which M-current inhibition seems to result from the IP3-induced release of Ca2+ rather than PIP2 depletion (see Delmas & Brown, 2005). Hence, we have now used another PIP2-binding probe that does not bind to products of PIP2 hydrolysis, the C-terminus of the transcription factor tubby (Santanaga et al., 2001; Quinn & Tinker, 2004), mutated to reduce affinity for PIP2 (YFP-tubby-R332H). This co-localized with the PLCδ-PH probe and the two translocated with a similar time course following mAChR stimulation. In contrast, BK induced much less translocation of tubby-R332H than PLCδ-PH. BK, but not mAChR-induced translocation was then increased when PIP2 synthesis was inhibited by wortmannin. Thus, BK produced less PIP2 depletion than mAChR stimulation because it accelerated PIP2 synthesis but could produce equivalent depletion when synthesis was inhibited.
We further observed that, when the Ca2+-mediated component of M-current inhibition by BK was reduced by depleting Ca2+ stores with thapsigargin, inhibition was restored by wortmannin. In this way we could switch the mechanism of BK-induced inhibition from that mediated by the action of a product of PIP2 hydrolysis (IP3 / Ca2+) to one mediated by PIP2 depletion.
Thus, these K+ channels can be regulated by both PIP2 and by products of PIP2 hydrolysis. While the contributions of these two may vary with different agonists (and probably in different cell types), they are not mutually exclusive, and indeed may interact (see Delmas & Brown, 2005).

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This work has been supported by an MRC studentship (to SH), and by grants from the Wellcome Trust and European Union (to DAB) and the British Heart Foundation (to AWT).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C33

Independent detection of inositol 1,4,5-trisphosphate and phosphatidylinositol 4,5-bisphosphate in live cells using fluorescent biosensors

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Tubby protein binds with high affinity and selectivity to phosphatidylinositol 4,5-bisphosphate (PIP_2), and GFP-Tubby has been shown to enrich to the plasma membrane and translocate to the cytosol on activation of $G\alpha_q$-coupled GPCRs (Santagata et al., 2001), making it a good candidate for a PIP_2-selective biosensor. Here, we have investigated the relative contributions of inositol 1,4,5-trisphosphate (IP_3) production and PIP_2 depletion to the translocation of GFP-Tubby and eGFP-PH-PLCδ1 in response to $G\alpha_q$-coupled GPCR activation.
Both eGFP-PH-PLCδ1 and GFP-Tubby are predominantly localized to the plasma membrane when transiently expressed in SH-SY5Y neuroblastoma cells. Activation of the endogenous M_3 mACh receptor population (with methacholine (MCh)) caused both probes to translocate from plasma membrane to cytosol in a reversible (on agonist washout) and concentration-dependent manner (pEC_{50} values: 5.19 ± 0.11 (n = 9) for eGFP-PH-PLCδ1 and 4.53 ± 0.21 (n = 5) for GFP-Tubby). For both eGFP-PH-PLCδ1 and GFP-Tubby, cytosolic fluorescence returned to baseline within 4 min of agonist washout in control cells while, in the presence of a high concentration of wortmannin (10 μM), both eGFP-PH-PLCδ1 and GFP-Tubby responses to MCh (1 mM) were prolonged (responses maintained at significantly elevated plateau levels of 44 ± 8% (n=12) and 79 ± 6% (n=10), respectively ($p<0.001$ in each case)). In the presence of a lower concentration of wortmannin (1 μM), responses were not significantly different from controls, indicating that the wortmannin effect is due to inhibition of phosphatidylinositol 4-kinase, not phosphoinositide 3-kinase activity.
Co-expression of a dsRed2-tagged IP_3 3-kinase construct significantly attenuated MCh-mediated translocation of eGFP-PH-PLCδ1 (27 ± 8% in cells co-expressing 3-kinase, versus 129 ± 17% in controls ($n\geq10$; $p<0.001$)). In contrast, GFP-Tubby translocation in response to a similar stimulus was unaffected by co-expression with the IP_3 3-kinase construct (53 ± 11% in cells co-expressing 3-kinase, versus 47 ± 13% in controls ($n\geq12$)). Therefore, unlike eGFP-PH-PLCδ1, agonist-induced translocation of GFP-Tubby does not report changes in cytosolic IP_3.
These data indicate that eGFP-PH-PLCδ1 translocation in response to $G\alpha_q$-PCR activation is primarily driven by changes in cytosolic IP_3, in agreement with our previous findings (Nash et al., 2002, 2004), while GFP-Tubby translocation reflects dynamic changes in plasma membrane PIP_2 levels and is independent of changes in cytosolic IP_3. GFP-Tubby therefore represents a selective fluorescent biosensor for investigating the dynamic regulation of cellular [PIP_2].

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We gratefully acknowledge the Wellcome Trust for financial support

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SA74

Pro-death and pro-survival NMDA receptor signalling require different subcellular pools of calcium

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It is long-established that high levels of NMDA receptor activity kill neurons. Acute excitotoxic events such ischemia, mechan-

ical trauma or epileptic seizure can also lead to excessive extracellular glutamate accumulation and cell death. Even chronic neurodegenerative diseases have been associated with toxic levels of NMDA receptor activity. The potentially destructive effects of excessive NMDA receptor activity are in marked contrast to the more recent findings that synaptic NMDA receptor activity can promote neuronal survival and resistance to trauma (Hardingham, 2006). Thus, neuronal responses to glutamate or NMDA follow a bell-shaped curve: both too much and too little NMDA receptor activity is potentially harmful (Hardingham and Bading, 2003). In treating neurological or neurodegenerative disorders associated with pro-death NMDA receptor signalling, it would be desirable to be able to interfere with pro-death signalling from the NMDA receptor, while not affecting pro-survival signalling, or signalling to synaptic plasticity. While death and survival signaling pathways are both activated by calcium influx, a greater knowledge as to the spatial calcium requirements to activate these pathways may aid in their selective inhibition, and enhance our understanding of the dichotomous nature of NMDA receptor signalling. If calcium effectors of survival and death were located in different parts of the cell, potentially requiring different protein-protein interactions, this may point to more specific ways of selectively targetting pro-death events. Analysis of NMDA receptor-dependent activation of the pro-death stress-activated kinases (JNK and p38) revealed that they require spatially distinct subcellular pools of calcium from those required to activate survival signalling to CREB and Akt. Moreover, neuroprotective disruption of p38 signalling could be achieved by uncoupling the NR2B PDZ ligand from downstream proteins by using intracellular peptides mimicking the NR2B PDZ ligand. The effectiveness of this peptide in reducing NMDA receptor dependent excitotoxicity in vitro can be achieved without inhibiting NMDA receptor dependent neuroprotection or synaptic plasticity. The in vivo effectiveness of this peptide compared to other therapeutic anti-stroke strategies will also be discussed. In conclusion, the dichotomous death/survival signals that emanate from the NMDA receptor have spatially distinct calcium requirements and are amenable to selective inhibition.

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This work is funded by the European Commission, the Royal Society and the Wellcome Trust.

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SA75

Spatially specified signaling networks in hippocampal neurons

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Signal flow within intracellular signaling pathways in neurons is known to be compartmentalized. Signals are dynamically controlled in local regions (microdomains) within the dendrite and cell body, although many signaling components can diffuse through the cell body and dendrites. The mechanisms underlying the formation and dynamics of microdomains are not well understood. In addition, it is not clear how spatial information is transmitted from upstream to downstream components within a signaling network. We have computationally studied the various factors responsible for local signaling and the flow of spatial information in neurons. For this we have used the β-adrenergic receptor cAMP/protein-kinase A/ b-Raf/MAP-kinase signaling network that consists of a negative feedback loop stacked on top of a feedforward loop when signal flows from the receptor to MAP-kinase 1, 2. Numerical simulations of partial differential equation models of this network using realistic cell shapes were conducted in the *Virtual Cell*. Cell shape and surface-to-volume ratios play important roles in the origin of microdomains of upstream signaling components of this network. For transmission of spatial information, cell shape serves as a constraint for local activity of negative regulators, such as phosphodiesterases and protein phosphatases to control information from cAMP to MAP-kinase. While information regarding the activation state is transmitted through the stimulatory arm of the feedforward motif, the spatial information is transmitted through control of the negative regulator of MAPK1,2. Predictions that an upstream negative regulator controls the propagation of spatial information to downstream components were verified experimentally in rat hippocampal slices. We conclude that cell shape-constrained local reaction balance within stacked regulatory loops propagates spatial information through signaling networks.

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SA76

Visualisation of receptors in native tissue

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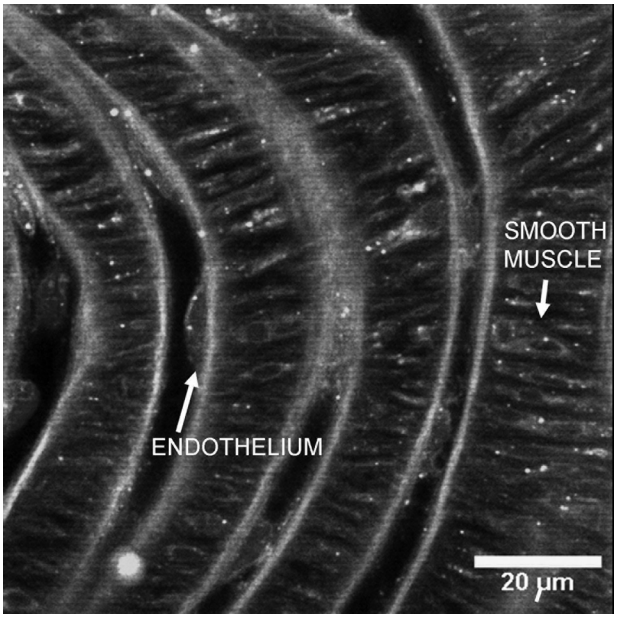
In native tissues, relatively low protein expression levels and overlapping sensitivities of antibodies within families of GPCRs present challenges to transfer of knowledge of receptor mechanisms from cell culture. In contrast, in recombinant systems epitope tags and fluorescent proteins provide powerful and sensitive means of tracking the concentration, location and movement of receptors. Two approaches have now started to close this gap.
First is the use of fluorescent ligands, which can be tested and validated in recombinant systems [1] and then employed in intact, live native tissue using confocal microscopy [2-4]. Agonist and antagonist ligands can be employed, each conferring advantages and disadvantages. The second approach is the creation of mouse strains expressing transgenic receptors labeled with fluorescent proteins [5].
Examples will be given illustrating the current state of play with these approaches on alpha-1, alpha-2 and beta-adrenoceptors, and angiotensin receptors, mainly using rat and mouse

blood vessels, in which fluorescent ligand binding shows that a range of cell types express adrenoceptors, e.g. nerves, adventitial fibroblasts, smooth muscle, endothelial and fat cells. The distribution of receptors is shown to be both in intracellular organelles and the cell surface, the consequences of dimerisation can be explored in receptor KOs and phenomena such as binding kinetics and receptor internalization and trafficking can be followed in real time in native tissues. These approaches are steadily closing the gap between cell culture and native tissues.

In the particular example of blood vessels, these approaches have shown that receptors exist on types of cells where they were previously not known to exist. Receptors for vasoactive agents such as catecholamines and angiotensin II have been found on adventitial and endothelial cells as well as where they were expected, on smooth muscle. This has led to new avenues of research into vascular control. An example is shown in figure 1, where adrenoceptors are shown on the endothelial cells of mouse carotid artery as well as on smooth muscle.



A confocal "slice" through the ridged luminal surface of a mouse carotid artery. The alternating vertical "bands" show the repeating sequence of (from left) luminal space, endothelium, internal elastic lamina and medial smooth muscle. The continuous, curving vertical white lines show the natural fluorescence of the elastic lamina. All other staining indicates the binding of a fluorescent ligand to α_1-adrenoceptors, which can be seen on both endothelial and smooth muscle cells.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA77

Imaging the vascular wall with confocal microscopy

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Blood vessels are capable of continuous structural changes in response to varying conditions and functional demands. In physiological situations, these structural modifications -known as "vascular remodelling"- are adaptive and aimed to maintain a relative constant shear and wall stress. Vascular remodelling is also common to several pathological conditions, such as hypertension and atherosclerosis, where the structural changes are no longer adaptive and contribute to the progression of the disease. Vascular remodelling is a dynamic process involving cell growth, death, as well as extracellular matrix synthesis and degradation. However, knowledge of vascular remodelling has been largely based on a long-standing, static view from two-dimensional histology and the 3-dimensional (3-D) organization of the different types of vascular cells and their relationship with the extracellular matrix and innervation has not been thoroughly explored. In addition, important questions related to the active process of vascular remodelling, such as which cells undergo proliferation, apoptosis, phenotypic change or migration, are still unsolved.

An integrated view of the interrelationships of the different elements of the arterial wall is made possible by fluorescence Laser Scanning Confocal microscopy (LSCM). Confocal microscopes allow obtaining serial optical sections of relatively thick specimens without the need to cut them as with conventional histology. With the aid of image analysis software, these serial sections can be further reconstructed to obtain 3-D images, where the structures of interest are localized and quantified. LSCM can be combined with pressure myography to obtain simultaneously information on vascular function and 3-D structure at near-to-physiological conditions. The vessel is firstly pressurized at physiological pressure to study vascular function and mechanics. Thereafter, it is fixed and stained with the fluorescent dyes of the structures of interest. The fixed vessel is mounted intact on a slide provided with a small well to avoid 3-D distortion and it is visualized with a LSCM. The vascular wall is scanned from the adventitial to the endothelial layer and serial optical sections are acquired with the microscope. These serial images can be processed with an image analysis software that allows for reconstruction of 3-D models and for quantification of the stained structures. There are a vast number of fluorescent compounds useful to study vessel structure. For example, fluorescent dyes that bind with DNA, such as propidium iodide, DAPI or Hoescht 3332, are useful to identify the different types of vascular cells -adventitial, smooth muscle and endothelial cells- by the shape and orientation of their nuclei. With the aid of morphometric methods, which involve segmentation and object extraction, it is possible to identify and quantify cell type, number, shape, orientation and density in the different layers of the vascular wall. The use of fluorescent antibodies and kits allows locating, within an intact vascular wall, the distribution of nerves, specific enzymes and extracellular matrix elements and to study their relationship with the vascular cells. LSCM enables to scan and process relatively large amounts of tissue in short time. This

makes possible to locate rather infrequent events in the vascular wall, such as cell apoptosis or proliferation, cells undergoing phenotypic change or migration. LSCM is not only useful to image vascular wall structure, but also to visualize and quantify by the intensity of fluorescence, the generation of vascular cell products, such as nitric oxide or superoxide anion. In conclusion, confocal microscopy and image analysis software permits to image vascular wall structure and function and to assess the active process of vascular remodelling in physiological and pathological situations.

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C34

Morphological analysis of the rat ureteric terminal arterioles

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Morphological technique that allows a detailed descriptive and quantitative analysis of vascular and endothelial cells of micro vessels *in situ* is of importance in studying the effects of vascular diseases or ageing could have on vascular structure. Confocal imaging of Fluo-4-loaded ureter used in the present study allowed us to perform morphological analysis of the microvessels: terminal arterioles with an inner diameter < 50 μm in intact rat ureter. Optical sectioning showed that muscle coat (media) of the terminal arterioles consisted of a monolayer of highly curved smooth muscle cells which run circumferentially around endothelium. This technique allowed us to measure the inner diameter of the terminal arterioles, define an orientation and a number of revolutions an individual smooth muscle cell made around endothelium and measure thickness, width, and morphological profile of the myocyte. By multiplying circumference of the arteriole by a number of revolutions we calculated the average length of the smooth muscle cell which in the terminal arterioles with the inner diameter of 15 μm was 100.4±4.2 μm (n=29 cells, 17 vessels). The average thickness and width of the smooth muscle cell in it's central part were 3.04±0.05 μm and 6.7±0.2 μm, respectively (n=14 cells, 5 vessels). Endothelial cells of the ureteric terminal arteriole were arranged as a monolayer of slightly overlapping longitudinally oriented asymmetrical cells with the central nucleated part projecting markedly into the lumen of the arteriole. The width and the height of the endothelial cells in their central part were 3.2±0.1 μm and 5.06±0.44 μm (n=21, 10 vessels), respectively and the length was 61.8±27 μm. Some of our morphological data can be compared to that from electron microscopy, electron scanning microscopy and silver nitrate staining technique. Our technique allowed us not only to measure all the dimensions and orientation of the smooth muscle and endothelial cells of the terminal arterioles in their natural environment but also study mechanisms controlling Ca signalling and vasomotion in these microvessels.

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C35

Effects of mechanical stretch on inter- and intracellular Ca^{2+} signals in ultra-thin slices of human myometrium

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Intercellular communications through gap-junctions are crucial for synchronising myometrial activity but the mechanisms of these communications in native tissue remain poorly elucidated. Cultured uterine myocytes, often used in research are a convenient, but not ideal model of human myometrium. Studying intercellular communications between uterine myocytes in ultra-thin myometrial slices presents the advantage of preserving the intercellular contacts within the tissue in its native state. In this study, we investigated the effect of mechanical stretch on evolution of intercellular communications in human myometrium. Changes in cytoplasmic Ca2+ elicited by spontaneous action potentials and applications of oxytocin (OT, 100 nM) were recorded daily from slices maintained in organotypic culture for one week. Myometrial biopsies were taken from patients undergoing Caesarean Section with informed written consent and approval from the Local Ethics Committee (REC-05/Q2802/107). The slices, 200 μm thick, were cut using a vibroslicer in modified Krebs solution (4°C) and cultured in SmGM-2 medium (Cambrex) with 5% foetal calf serum. Ca2+ signalling events were recorded from Fluo-4 loaded slices using a Zeiss LSM 510 META confocal microscope at 1 frame/s. Myometrial slices were cultured under two different conditions, unstretched, free floating slices and stretched slices(slices were pinned to the bottom of the Sylgard-filled Petri dish at 150% of their slack length). Spontaneous activity was observed in all freshly cut slices. After one day in culture, spontaneous activity was observed only in 1 out of 11 unstretched slices and in 2 out of 10 stretched slices. No spontaneous activity was observed after two or more days of culturing. In freshly cut slices, application of OT triggered synchronous Ca2+ transient in all cells within the field of view. The synchronicity of the Ca2+ transients was quantified as standard deviation of time delays between the oxytocin application and peaks of Ca2+ transients in different cells within the field. During culturing, there was a marked decrease in synchrony in both stretched and unstretched slices. That is, the standard deviations of the Ca2+ transient time delays in unstretched and stretched slices increased, respectively, from 8.2 s and 7.4 s in freshly cut slices to 30.6 s and 41.9 s after 7 days in culture. Upon culturing and loss of synchrony, some cells demonstrated spontaneous calcium oscillations that were unaffected by OT and did not propagate to neighbouring cells. In conclusion, our data show that

the loss of spontaneous activity and intercellular coupling in human myometrium under culture conditions could be delayed but not prevented by mechanical stretch.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA78

Gap junctional communication in retinal development

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Neuroprogenitor cells in the developing retina extend processes, which stretch from the basal vitread surface to the apical ventricular surface. During the cell cycle, the nucleus undergoes interkinetic migration, moving in a vitread direction during G1, passing through S-phase at its peak and then, on entering G2, returning towards the ventricular surface where it will enter M-phase and divide. We have previously shown that individual saltatory movements of the nucleus correlate with calcium events within the progenitor cells and that these events spread to neighbouring progenitor cells through connexin43 (Cx43) gap junction channels, thereby coordinating the migration of coupled clusters of cells. Disrupting coupling by pharmacological agents or molecular means with Cx43-specific antisense oligodeoxynucleotides (asODNs) or dominant negative Cx43 (dnCx43) inhibits the sharing of calcium events, reducing the number that each cell experiences, and significantly slows their interkinetic migration. Monitoring the location within the retina of progenitor cells that were transfected with GFP-dnCx43 24 hours previously, we find 70% of them to be located, at the ventricular surface of the retina, where mitosis normally takes place. A similar effect was produced in eGFP-transfected progenitor cells in retinae that had been treated with Cx43-specific asODNs or cultured in the presence of pharmacological blockers of gap junctional communication. These findings potentially imply either that more progenitor cells were undergoing mitosis in conditions of reduced communication or that they were taking longer to go about it.

Closer examination of the GFP-transfected cells at the ventricular surface revealed that they had begun to grow neurites, implying that they had differentiated ectopically, having failed to migrate away from the ventricular surface towards the vitread surface of the retina as differentiating neurons should. Immunostaining of untransfected retinae with the neuronal marker TuJ1 after overnight treatment with Cx43asODNs or pharmacological gap junctional blockers revealed strong staining at the ventricular surface which was not seen in control retinae. These findings imply that blocking communication prevents cells from migrating away from the ventricular zone, either by interfering with the migration mechanism prior to neuritogenesis or by causing the precocious differentiation of neurites which prevent subsequent migration.

Clearly, appropriate levels of gap junctional communication between retinal neuroprogenitor cells are essential for their normal cell cycle progression, differentiiaton and migration to appropriate terminal sites. Other parts of the CNS, such as the cerebral cortex, undergo similar processes during development, so it is likely that gap junctional communication has a fundamental role in the proliferation, differentiation and migration of neurons throughout the CNS, coordinating the formation of its normal architecture.

Acknowledgements: This work is funded by the BBSRC. The following members of our lab have contributed in some part to this study as project students or researchers: Ms Niki Tsakiri, Mr Kayur Patel, Ms Luciana Sowole, Mr Daniel Ciantar, Dr Jeremy Cook, Dr Chris Thrasivoulou, Dr Kevin Webb.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA79

Spatially resolved biochemistry in individual cells by fluorescence lifetime imaging

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To advance our understanding of cellular processes, it would be desirable to obtain information beyond mere localisation of a labelled protein. Recent advances in microscopy have made it possible to visualise in both space and time the biochemistry of individual cells. Fluorescence lifetime imaging (FLIM) is a powerful technique for cellular imaging and is widely regarded as the most robust method of measuring Förster Resonance Energy Transfer (FRET) to study protein-protein interactions or protein conformational changes. We visualise the supramolecular organization of receptor phosphorylation using FLIM to report FRET (Treanor et al. (2006) J Cell Biol 174:1, 153-161). This broadly applicable technique can be used to image protein phosphorylation of any green fluorescent protein (GFP)-tagged receptor at an intercellular contact. Specifically, we examine the phosphorylation of Killer Ig-like Receptor (KIR) by measuring FRET between GFP-tagged KIR2DL1 and a Cy3-tagged generic anti-phosphotyrosine mAb. Visualization of KIR phosphorylation in Natural Killer (NK) cells contacting target cells expressing cognate Major Histocompatibility Complex (MHC) class I proteins revealed that KIR signalling is spatially restricted to the intercellular contact. This explains how NK cells can respond appropriately when simultaneously surveying susceptible and resistant target cells. Surprisingly, contrary to an expected homogeneous distribution of KIR signalling across the intercellular contact, phosphorylated KIR was confined to microclusters within the large aggregate of KIR. Thus, the spatial confinement of receptor phosphorylation within the intercellular contact may influence how NK cells integrate activating and inhibitory signals.

In addition, developing new bio-imaging methods that can probe the microenvironment of proteins in live cells may help in our understanding of diverse aspects of cell biology such as membrane architecture, receptor trafficking, and endocytosis.

Recently, we reported on the ability of FLIM to report the local refractive index of GFP in solution (Suhling et al. (2002) Biophys J 83:6, 3589-95). We further examined the application of FLIM to probe the microenvironment of GFP-tagged proteins at the cell surface and at the intercellular contact between NK cells and target cells, where a complex molecular architecture known as the immunological synapse (IS) forms. Following a novel quantitative analysis of fluorescence lifetime images, we report that the variation of observed fluorescence lifetime of GFP-tagged proteins at the cell surface is within the expected statistical range (Treanor et al. (2005) J Microscopy 217:1, 36-43). However, the lifetime of GFP-tagged proteins in cells is shorter than recombinant GFP in solution. Intriguingly, the lifetime of GFP-tagged MHC class I protein is shortened at the inhibitory NK cell IS compared to the unconjugated membrane. This likely reveals a distinct local refractive index for MHC protein clustered at the IS. This first example of the use of FLIM to probe the local environment of an IS indicates how FLIM may be broadly useful in imaging membrane heterogeneity or complexes of proteins and lipids such as those involved in IS formation. One future challenge will be to understand how to interpret refractive index changes within live cells and whether such changes can be correlated to specific membrane microdomains.

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SA80

The AMP-activated protein kinase system - protecting cellular and whole body energy balance

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The AMP-activated protein kinase (AMPK) system is a key regulator of energy balance. AMPKs are heterotrimeric complexes composed of a catalytic alpha subunit and regulatory beta and gamma subunits. Any metabolic stress that increases ATP consumption (e.g. muscle contraction) or that inhibits ATP production (e.g. hypoxia, glucose deprivation, or mitochondrial inhibitors) will cause a fall in the ATP:ADP ratio, which is amplified by adenylate kinase into a much larger rise in the AMP:ATP ratio. Binding of AMP to two tandem "Bateman domains" on the gamma subunit of AMPK causes activation of the kinase, an effect antagonized by high concentrations of ATP. Intriguingly, point mutations in the Bateman domains of the gamma-2 isoform that interfere with binding of AMP and ATP cause heart disease associated with excessive storage of glycogen. AMPK is only active after phosphorylation of a conserved threonine within the kinase domain (Thr-172 in the human) by upstream kinases. The principal upstream kinase in mammals is the tumour suppressor, LKB1, in complex with two accessory subunits, STRAD and MO25. The LKB1 complex appears to phosphorylate AMPK at Thr-172 constitutively, but in basal conditions the phosphate is immediately removed by phosphatases, most likely forms of protein phosphatase-2C. Binding of AMP to the Bateman domain inhibits dephosphorylation of Thr-172, thus switching the kinase into the active, phosphorylated form. In mammals, the phosphorylated kinase is also allosterically activated by AMP. We have recently shown [1] that AMP binding relieves inhibition of the kinase domain by a pseudosubstrate sequence that occurs within the N-terminal Bateman domain on the gamma subunit.

In certain cells such as neurones, endothelial cells and T lymphocytes, AMPK can also be activated by a rise in cytosolic calcium, which binds to calmodulin and triggers phosphorylation of Thr-172 by calmodulin-dependent protein kinase kinase-beta. This can occur in the absence of an increase in AMP, and may represent a mechanism to anticipate the demand for ATP that often accompanies elevation of cell calcium.

Once activated, AMPK switches on catabolic processes such as the uptake and oxidation of glucose and fatty acids, and mitochondrial biogenesis. Conversely, it switches off anabolic processes such as fatty acid and cholesterol synthesis, gluconeogenesis, and glycogen and protein synthesis. It achieves these effects both by direct phosphorylation of metabolic enzymes and via phosphorylation of transcription factors and co-activators that regulate gene expression. In proliferating cells, AMPK activation also inhibits cell growth by inhibition of the mTOR pathway, as well as progress through the cell cycle.

AMPK appears to be an ancient system that evolved in single-celled eukaryotes to mediate the response to starvation for a carbon source. However, it has recently become clear that its role became adapted during development of multicellular organisms, where it is also involved in the regulation of energy balance at the whole body level. Thus, in muscle AMPK is activated by adipokines and cytokines such as leptin, adiponectin and interleukin-6, where it increases energy expenditure by stimulating glucose and fatty acid oxidation. In the liver, AMPK is activated by adiponectin, stimulating fatty acid oxidation and repressing the expression of enzymes of gluconeogenesis. Finally, in the hypothalamus it is modulated by agents that regulate appetite and food intake, being inhibited by leptin, and activated by ghrelin, cannabinoids and hypoglycaemia. Activation of AMPK in the hypothalamus also increases food intake in rodents. Thus, a system that appears to have evolved for the response to starvation in single-celled eukaryotes now controls a complex behavioural response in multicellular eukaryotes, i.e. feeding.

Given the downstream effects of AMPK, i.e. its ability to stimulate oxidation of glucose and fatty acids in the periphery, and to repress synthesis of lipids and glucose in the liver, activators of the kinase have great potential as drugs to treat type 2 diabetes and the metabolic syndrome, and possibly even obesity [2]. Consistent with this, AMPK appears to be the therapeutic target of metformin, a drug now used to treat 120 million people with type 2 diabetes worldwide. However, metformin activates AMPK indirectly by acting as a mitochondrial inhibitor, and it is possible that a direct activator could be more efficacious and/or have fewer side effects. AMPK is also activated physiologically by exercise, not only in skeletal and cardiac muscle but also in liver and adipose tissue, and it seem likely that it is responsible for many of the beneficial effects of regular exercise, particularly in protection against the development of obesity and type 2 diabetes.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA81

The TORC family of CREB coactivators: regulators of energy balance

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During fasting, mammals maintain glucose homeostasis by stimulating glucose production from the liver. Elevations in circulating glucagon trigger expression of the gluconeogenic program in part through induction of the cAMP responsive factor CREB. The effects of CREB on the gluconeogenic program are mediated by the cAMP responsive coactivator TORC2, which potentiates CREB activity in liver specifically under fasting but not feeding conditions. Sequestered in the cytoplasm under ad libitum feeding conditions, TORC2 is de-phosphorylated translocated to the nucleus in response to fasting where it stimulates gene expression through a direct interaction with CREB.
To monitor the effects of nutrient and energy sensing pathways on TORC2 activity in real time, we developed an in vivo hepatic imaging approach. We found that pancreatic and adipose-derived signals modulate glucose output through acute and long-term effects on TORC2 activity. The results illustrate a distinct mechanism through which nutrient and hormonal cues regulate the gluconeogenic program.

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Koo SH; Flechner L; Qi L; Zhang X; Screaton RA; Jeffries S; Hedrick S; Xu W; Boussouar F; Brindle P; Takemori H; Montminy M. The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature 2005, 437, 1109-1114.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA82

PGC-1 coativators and the control of energy homeostasis

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We have previously shown that the PGC-1 transcriptional coactivators are major regulators of several crucial aspects of energy metabolism. PGC-1a controls many aspects of oxidative metabolism, including mitochondrial biogenesis and respiration through the coactivation of many nuclear receptors, and factors outside the nuclear receptor family. ERRa NRF-1 and NRF2 are key targets of the PGC-1s in mitochodrial biogenesis. We have recently addressed the question of the role of PGC-1 coactivators in the metabolism of reactive oxygen species (ROS). Since these factors are very powerful inducers of mitochondrial biogenesis and respiration, it might have been expected that they could increase ROS formation. We now show that PGC-1a and b are induced when cells are given an oxidative stressor, H2O2. In fact, experiments with RNAi for the PGC-1s show that the ability of ROS to induce and ROS scavenging program depends entirely on the PGC-1s.This includes genes encoding mitochondrial proteins like SOD2, but also includes cytoplasmic proteins like catalase and GPX1. Cells lacking PGC1a are hypersensitive to death from oxidative stress caused by H2O2 or paraquat. Using mice deficient in PGC-1a, we have studied their resistance to oxidative challenges. The mice get excessive neurodegeneration when given kainic-acid induced seizures or MPTP, which causes Parkinsonism. These data show that the PGC-1s are important protective molecules against ROS generation and damage. The implications of this for diabetes and neurodegenerative diseases will be discussed.
PGC-1a controls the thermogenic gene program in brown fat, but we have now re-investigated factors that may control the determination of cell fate in the brown adipose lineage. We will discuss data showing that PRDM16 is a crucial factor controlling brown fat cell fate, including the ability to induce PGC-1a and UCP1. The implications of this for metabolic disease will be discussed.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA83

The IL-6 signalling in exercise and disease: Diabetes and inflammatory disease

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Growing evidence links type 2 diabetes to a state of low-grade chronic inflammation, and it has been suggested that interleukin (IL)-6 promotes insulin resistance due to the observation that plasma- IL-6 is often elevated in patients with metabolic disease. However, it is now well known that IL-6 is rapidly released into the circulation following exercise and from a simplistic physiological point of view, it seems paradoxical that working muscle would release a factor that inhibits insulin signalling when insulin action is enhanced in the immediate post-exercise period.
It is now well acknowledged that the IL-6 gene is rapidly activated during exercise and that the ac-tivation of this gene is further enhanced when muscle glycogen content is low. IL-6 is produced by contracting muscle fibers and released into the circulation, where it works in a hormone-like fash-ion. Acute IL-6 administration to humans increases insulin-stimulated glucose disposal and fatty acid oxidation in vivo, and IL-6 has strong anti-inflammatory effects.
Results from IL-6KO mice indicate that IL-6 can activate AMPK in muscle and adipose tissue, and that this contributes to, but

does not fully account for, the increase in AMPK activity in these tissues in response to exercise. They also suggest that a genetic lack of IL-6 is associated with a decrease in AMPK activity.
In vitro studies have demonstrated that IL-6 increases both basal and insulin stimulated glucose uptake, acetyl co-A carboxylase and fatty acid oxidation in mock, but not AMPK double negative cells, indicating that the metabolic effects of IL-6 are mediated by AMPK.
Our data challenge the commonly held view that IL-6 induces insulin resistance in all circum-stances and suggest that ligands that activate the heterodimeric IL-6R/gp130b receptor complex in muscle and fat cells are a viable drug target to treat peripheral insulin resistance.

Grants: The Centre of Inflammation and Metabolism is supported by a grant from the Danish Na-tional Research Foundation (# 02-512-55).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C36

Defining the essential mammalian endurance training transcriptome

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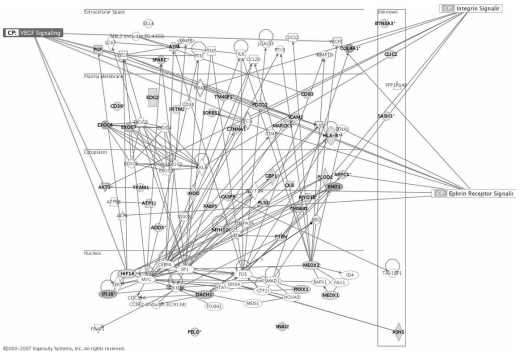
We have previously produced the first coding genome wide profile of the trancriptome responses to 6 weeks of endurance training in sedentary but otherwise healthy male subjects. This utilised a U95 Affymetrix gene chip platform and 8 subjects from 24 involved in the clinical study. Subsequent RT qPCR revealed that certain angiogenic and extracellular matrix (ECM) gene expression responses were modulated in proportion to physiological adaptation. This, for the first time, provide evidence that transcriptomic did not only catalogue changes with a stimulus but could reveal important gene networks likely to represent critical processes involved in physiological adpation. In the present study we have utilised the U133+2 Affymetrix genechip (n=48) in 24 subjects before and after 6 weeks of supervised endurance training (45min per day, at 70% VO2max). We discovered that 1100 transcripts are regulated 24hr post last training session, and these genes again reflected ECM processes, based on gene ontology classification. Further analysis revealed that 100 genes distinguish those that improved there aerobic function, from those that did not (20% of the cohort). Using the Ingenuity Database of hand-curated biological interactions we identified 5 prominent gene networks within this 100 gene list (Fig 1). Subsequent analysis of 30 of the 100 genes, in rodents bred for high and low training responsiveness, using real time qPCR identifies which of these marker genes are conserved from an evolutionary perspective. As these genes represent key factors relevant for adaptation to physical activity, it is also plausible that they represent "thirfty" genes, and further analysis, associating them with metabolic disease and diabetes is merited. In summary, we present the most detailed map of the molecular determinant of endurance training adaptability in mammals and identify the key signalling processes relevant for transducing physical activity into gains in aerobic capacity.



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All previous collaborators on this project, Affymetrix, Swedish Diabetes Society, Heriot Watt University and the Leverhulme Foundation

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C37

Effect of exercise and insulin on SREBP-1c expression in human skeletal muscle: potential roles for the ERK1/2 and Akt signalling pathways

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Sterol Regulatory Element Binding Protein-1c (SREBP-1c) is a transcription factor that regulates genes associated with glucose and fat metabolism (eg HKII, ACC and FAS). Animal studies have shown SREBP-1c to exhibit responsiveness to insulin and exercise. However, the signalling pathways regulating SREBP1c in human skeletal muscle are not clear. The Akt and the p38 mitogen-activated protein kinase (MAPK) pathways have been suggested as positive regulators whereas the STAT3 and extracellular signal-regulated kinase (ERK 1/2) pathways have been suggested as negative regulators of SREBP1c expres-

sion. The aim of this study was to examine the effects of exercise on basal and insulin-mediated changes in the activation (phosphorylation) of these signalling molecules and relate them to changes in SREBP-1c expression in human skeletal muscle. Eight healthy men (age 24 ± 2yrs, BMI 24 ± 1 kg/m2, mean ± SEM) underwent a hyperinsulinaemic (80 mU/l) euglycaemic (4.5 mmol/l) clamp for 4h, 24h after 90min of one-legged cycling at moderate intensity while keeping the other leg rested (control leg). During the subsequent 24h recovery, subjects consumed a mixed diet (55% CHO, 30% fat). Muscle biopsy samples were obtained from both legs after exercise (day 1), and before and after the insulin clamp (Day 2). Immediately after exercise, phosphorylation of pERK1 (0.41 ± 0.08 vs 0.32 ± 0.05, $P<0.05$, 1-way ANOVA), pERK2 (0.52 ± 0.10 vs 0.26 ± 0.05, $P<0.05$) and pAkt (0.78 ± 0.07 vs 0.63 ± 0.06, $P<0.05$) was higher in the exercise than the control leg, respectively. SREBP-1c mRNA content was not affected by exercise whereas its protein level was lower in the exercise than the control leg immediately after exercise (1.57 ± 0.51 vs 3.58 ± 0.99, $P<0.05$) and returned to normal levels 24h later. Similarly, SREBP-1c mRNA content was ~1.5 fold higher ($P<0.01$) in the exercise than the control leg 24h later. On day 2, insulin infusion upregulated SREBP1c mRNA levels by ~2-fold ($P<0.01$) in both legs but did not affect its protein levels. Although there were no insulin-induced changes in pERK1/2, insulin infusion increased pAkt levels in the control leg (0.97 ± 0.11 vs 0.66 ± 0.06, $P<0.05$) and tended to increase it in the exercise leg (0.90 ± 0.06 vs 0.63 ± 0.05, $P=0.065$). Neither exercise nor insulin affected STAT3 or p38 MAPK phosphorylation. These findings suggest that the exercise-induced reduction in skeletal muscle SREBP1c expression might be mediated by increases in the pERK1/2 pathway, whereas Akt might be an important positive regulator of SREBP1c mRNA in human skeletal muscle under insulin-stimulated conditions.

This work was supported by BBSRC and the Institute of Clinical Research, University of Nottingham.

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SA84

NADPH oxidase, nitric oxide and mitochondria in inflammatory neurodegeneration

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Inflammatory neurodegeneration is degeneration of neurons induced by inflammation. Inflammation occurs in most brain pathologies, and there is evidence that this inflammation plays both positive and negative roles in the pathology. For example, long-term use of anti-inflammatory drugs reduces the onset of Alzheimer's and Parkinson's diseases. In the brain, inflammation is mainly mediated by glial cells, in particular microglia, and these proliferate, migrate, release pro-inflammatory cytokines and become inflammatory-activated in many brain pathologies. In culture, inflammatory-activated glia kill co-cultured neurons, and this might contribute to neurodegeneration in vivo.

We have been investigating mechanisms by which glia become activated and subsequently kill neurons in culture. A variety of inflammatory mediators induce the expression of inducible nitric oxide synthase (iNOS) in microglia and astrocytes and/or activate the microglial NADPH oxidase (NOX), which produces superoxide and hydrogen peroxide. We find that a high level of glial iNOS expression induces neuronal death in synergy with hypoxia, basically by NO inhibition of neuronal respiration resulting in glutamate release and excitotoxicity. This suggests that the inflamed brain may be more sensitive to hypoxic damage. NO also induces glutamate release from astrocytes, but by a different, calcium-dependent mechanism. NO from nNOS can also synergise with hypoxia to induce neuronal death via inhibition of mitochondrial respiration (if glycolysis is blocked). Activation of the microglial NADPH oxidase (by PMA, ATP, arachidonate, IL-1β, Abeta or prion peptide) causes little or no death of co-cultured neurons. However, these agents stimulate microglial proliferation and TNF-alpha release, which is blocked by inhibiting NOX or by removing H2O2 with catalase, and is replicated by adding H2O2. Thus NOX appears to regulate microglial proliferation and activation through H2O2 production, but has little direct effect of neuronal death. However, if we activated NOX (with the above factors) in glia where iNOS had previously been induced there was considerable synergy in inducing death of co-cultured neurons. And this death was prevented either by inhibiting iNOS or NOX, or scavenging peroxynitrite (the neurotoxic product of NO reacting with superoxide). These mechanisms might contribute to neuronal death during neurodegenerative diseases.

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Mander, P. K. & Brown, G.C. (2005) Activation of microglial NADPH oxidase is synergistic with glial iNOS expression in inducing neuronal death: a dual-key mechanism of inflammatory neurodegeneration. Journal of Neuroinflammation 2005, 2:20.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA85

IL-1 and inflammatory neurodegeneration

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Inflammation occurs rapidly in response to acute brain insults such as stroke, haemorrhage or trauma, and can be sustained

for long periods of time for example in Alzheimer's, Parkinson's and multiple sclerosis. Experimental evidence indicates that inflammation plays a major role in neurodegeneration in these conditions and that the cytokine interleukin-1 (IL-1) is a pivotal mediator. IL-1 is expressed rapidly after neuronal injury, predominantly by microglia and elevated levels of endogenous or exogenous IL-1 markedly exacerbates injury. The naturally occurring IL-1 receptor antagonist (IL-1RA) markedly inhibits ischaemic, excitotoxic and traumatic brain injury in rodents, and has shown promise in a Phase II clinical trial in stroke patients.

The mechanisms of IL-1 expression, release and action in neurodegeneration are not fully elucidated and appear multiple. Systemic IL-1 markedly enhances ischaemic brain injury via release of neutrophils into circulation, neutrophil adhesion to injured cerebrovasculature and CNS invasion. Activation of matrix metalloproteinase 9 (MMP-9) occurs rapidly in neutrophils which have entered the CNS leading to cleavage of extracellular matrix and neuronal injury. IL-1 expressed within the CNS (primarily by microglia) acts on astrocytes to release neurotoxins including MMP-9, thus killing neurones indirectly via release of plasminogen. IL-1 can also influence neurones directly, for example to release IL-1β, via non-classical signalling pathways.

IL-1RA, delivered peripherally can enter the CNS in animals and patients and has no adverse effects in stroke or sub-arachnoid haemorrhage patients.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C38

Systemic inflammatory stimulus exacerbates brain damage after experimental stroke via a matrix metalloproteinase-9-dependent mechanism that targets the neurovascular unit

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Growing evidence suggests that systemic inflammation modulates the response to acute brain injury and the progression of neurodegenerative diseases (Emsley & Tyrrell, 2002; Perry, 2004). Peripheral inflammatory stimuli, such as infection, increase the risk of stroke and are associated with poorer outcome but the mechanisms are poorly defined (Smeeth et al. 2004; Palasik et al. 2005). We recently demonstrated that a systemic inflammatory challenge exacerbates brain damage after experimental stroke and that the cytokine interleukin-1 (IL-1) is a critical mediator in this paradigm (McColl et al. 2007).

In the current study, we further investigated the mechanisms involved. We tested the hypothesis that a systemic inflammatory challenge exacerbates brain damage after experimental stroke by potentiating neutrophil responses and investigated the role of matrix-metalloproteinase-9 (MMP9) in this paradigm.

Focal cerebral ischaemia was induced in C57Bl/6J mice by transient (30min) middle cerebral artery occlusion (under isoflurane anaesthesia (3.5% in 30% O_2-70% N_2O)). Systemic inflammation was induced by recombinant IL-1β challenge (i.p.) and the volume of ischaemic damage and neurological deficit determined. Cerebral neutrophil accumulation, MMP9 immunoreactivity and gelatinolytic activity were assessed 8h and 24h after MCAo. The role of MMP9 was tested by co-administration of an MMP9 inhibitor (SB-3CT) with IL-1β.

Systemic administration of recombinant IL-1β significantly exacerbated ischaemic brain damage and neurological deficit and potentiated circulating neutrophil counts and cortical neutrophil infiltration. Neutropenia attenuated the IL-1β-induced exacerbation of ischaemic damage. IL-1β increased cortical MMP9 immunoreactivity and gelatinolytic activity. MMP9 immunoreactivity was observed in neutrophils and blood vessels whereas gelatinolytic activity was observed primarily in the cerebral vasculature. Co-administration of the MMP9 inhibitor, SB-3CT, attenuated the IL-1β-mediated exacerbation of ischaemic damage.

These data show the detrimental effects of a systemic inflammatory challenge on experimental stroke outcome and indicate key roles for neutrophils and MMP9 in this paradigm. These mechanisms may underlie the poorer outcome in stroke patients presenting with infection and may have implications for stroke aetiology in general since most stroke patients will present with pre-existing systemic inflammation linked to co-morbidities such as atherosclerosis and heart disease.

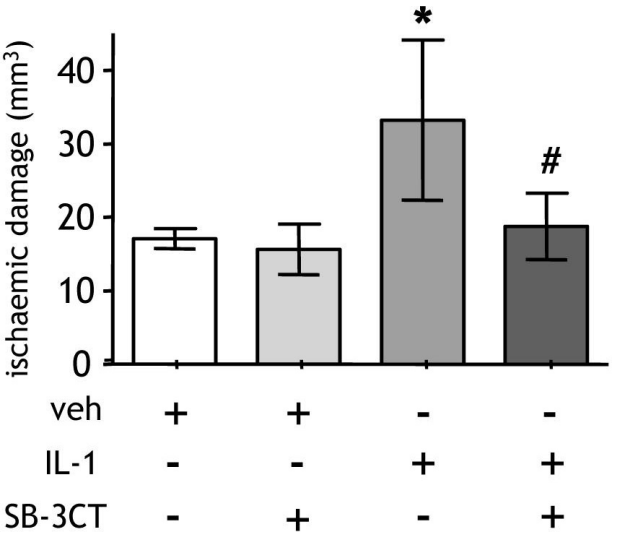

Figure1. Systemic challenge with recombinant IL-1β at the onset of MCAo significantly increased the extent of brain damage and this was attenuated by co-administration of SB-3CT.

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This work was supported by the Medical Research Council, UK.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C39

Lipoteichoic acid-induced loss of cerebellar granule cells is mediated by microglia and is dependent on direct neuronal-microglial interaction

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Bacterial meningitis still retains high rates of morbidity and mortality, and survivors often sustain debilitating neurological sequelae due to neuronal cell-death resulting from the infection. The pathophysiology of gram-positive bacterial meningitis can be simulated *in vitro* by application of the major cell-wall component lipoteichoic acid (LTA) – mimicking bacterial autolysis that occurs *in vivo*. This has been shown to result in loss of up to 50 % of cerebellar granule neurons in mixed neuronal-glial cultures. This effect presumably involved the phagocytosis of dying cells – a conclusion supported by the facts that dying cells were virtually absent in mixed cultures treated with LTA, and that cell-loss was not observed in pure neuronal cultures (lacking both microglia and astrocytes).[1]

To test the contribution of phagocytosis to neuronal loss, cytochalasin D, a compound that inhibits phagocytosis by blocking F-actin polymerization, was added here concomitantly with LTA. Cytochalasin D fully prevented neuronal loss for up to 7 days after LTA-treatment. Also, analysis of the phagocytic capacity of pure microglial cultures revealed that LTA-stimulation resulted in enhanced phagocytosis of polystyrene microspheres. This effect could be partially inhibited by cytochalasin D, confirming both a stimulation of phagocytic activity as a response to LTA-treatment as well as the inhibitory action of cytochalasin D on this process.

However, cytochalasin D also prevented microglial proliferation, which occurs in response to LTA. To exclude that the neuroprotective action of cytochalasin D was due to a side-effect, an alternative method to prove the importance of phagocytosis was used. First, microglia were selectively eliminated from mixed cultures by application of the lysosomotropic reagent, L-leucine-methyl-ester (LME) – a procedure that leaves the astrocyte population unaffected. This alone was sufficient to fully abolish neuronal loss in response to LTA. Alternatively, reconstitution of the culture system by addition of microglia to LME-treated cultures fully restored the original effect. However, when microglia were co-cultured in transwell-inserts, they were unable to induce neuronal death. Notably, microglial proliferation was observed in transwell-inserts – indicating that inflammatory microglial activation was still induced.

Our results show that LTA enhances the phagocytic capacity of microglia and that the loss of cerebellar granule neurons in response to LTA is fully dependent on the presence of microglia. More specifically, under these conditions neuronal death is not induced by soluble mediators (such as e.g. the pro-inflammatory cytokine TNF-α) but by a contact-dependent neuronal-microglial interaction.

Kinsner et al. (2005) J. Neurochemistry 95, 1132–1143

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA86

Extracellular μ calpain and microglia-mediated dopaminergic neurodegeneration: mechanisms of reactive microgliosis and progressive neurotoxicity

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Microglial activation and inflammation are linked to neuronal damage in numerous neurodegenerative diseases. Microglia are activated in Parkinson's disease, which is characterized by the selective and progressive loss of dopaminergic (DA) neurons. Recent evidence suggests that the microglial response to neuronal damage (i.e. reactive microgliosis) can actively induce neuronal death and fuel progressive neuronal loss. The mechanisms through which DA neuron injury activates microglia are poorly understood. Here, we address the novel possibility that μ calpain is a neuron-injury signal that activates microglia to further propagate DA neuron toxicity. Calpain is an intracellular protease that is upregulated in damaged DA neurons and is elevated in post-mortem PD patient brains. Western blot analysis revealed that μ calpain was present in conditioned media from MPP^+-treated cultures. μ Calpain was selectively neurotoxic to DA neurons when added to primary rat neuron-glia cultures. Addition of μ calpain to microglia-enriched cultures elicited both intracellular reactive oxygen species (ROS) and extracellular superoxide, which was abolished by the calpain inhibitor, calpeptin. Additionally, cultures from NADPH oxidase deficient mice, which are unable to produce extracellular superoxide, were insensitive to μ calpain-induced DA neurotoxicity, emphasizing the critical role of microglia-derived ROS. Further, inhibition of extracellular μ calpain with E64 attenuated MPP^+-induced DA neurotoxicity. Here, we are the first to identify μ calpain as a soluble neuron injury factor released by damaged DA neurons, which is then toxic to additional DA neurons through microglia-generated oxidative insult.

This work was supported in part by the Intramural Research Program of the National Institute of Health, the National Institute of Environmental Health Sciences. M.L.B. was supported by the NIH Pathway to Independence Award (NIEHS 1K99ES01549-01).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA87

Nitric oxide, ischemia and brain inflammation

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Stroke causes excitotoxicity, inflammation, cell death and compensatory neurogenesis. Resident and infiltrating cells generate

nitric oxide (NO) as a result of the activation of NO synthases (NOS)[1]. Experimental cerebral ischemia leads to the up-regulation of all three NOS isoforms, although the patterns of expression are temporally and spatially distinct. Classic gene knockout studies indicate that the activation of NOS-1 is detrimental, while the NO derived from NOS-3 is beneficial. Expression of NOS-2 is induced in both resident and infiltrating cells later than either NOS-1 or NOS-3, and this NO does not contribute to early pathology. Significant increases in expression of pro-inflammatory cytokines within a few hours of ischemic onset are assumed to trigger transcriptional activation of NOS-2. However, the promoter contains a hypoxia response element such that HIF-1α can activate the gene, and some neuroprotectants block cytokines without affecting NOS-2 expression[2]. Furthermore, transcriptional activation may not account for the very rapid appearance of NOS-2 positive cells infiltrating the infarct. While gene knockout studies suggest that NO produced by NOS-1 and NOS-2 is detrimental, and that derived from NOS-3 is beneficial, studies employing various NOS inhibitors provide conflicting results[3]. Collectively, NOS inhibitors significantly reduce infarct volume. Treatment before stroke onset reduces infarct volume in transient models, while early administration of NOS inhibitors after ischemic onset is effective in permanent stroke. Later treatment has a beneficial effect on infarct volume in both types of stroke model. Non-selective inhibitors do not alter infarct volume in permanent ischemia, whereas "selective" NOS-1 and NOS-2 inhibitors reduce lesion size regardless of experimental model. It is likely that the beneficial effects of non-selective inhibitors are limited because they inhibit NOS-3 to a similar degree. Consequently, they may aggravate ischemia by increasing platelet aggregation and white cell activity, raising blood pressure, and by restricting penumbral blood supply. Exogenously applied arginine (NOS substrate) has produced conflicting results: decreasing, having no effect, or even increasing infarct volume[4]. The same is true for the effect of arginine on cerebral blood flow which, if enhanced, could rescue salvageable tissue from the spreading ischemic core. This may be due to the ability of arginine to enhance NO from all three NOS isoforms. Conversely, experimental studies and human trials with NO donors show beneficial effects following experimental ischemia. However, unopposed high doses of NO donors might be detrimental due to profound vasodepressant effects. Following ischemic brain injury there is an increase in the rate of neurogenesis. These new cells arise in the dentate gyrus and subventricular zone, and populate subcortical and to a lesser extent cortical structures. While NO is reported to be cytostatic, or to promote terminal differentiation of neural stem cells in the uninjured brain, it appears to be anti-apoptotic in the ischemic brain. Administration of NO donors can enhance angiogenesis and neurogenesis, and significantly improve functional outcome. Mice deficient in NOS-2 do not display the predicted neurogenic response following ischemia. The mechanism is unknown but it could be that the resultant inflammatory reaction in the absence of NOS-2 derived NO proceeds unabated, resulting in an environment detrimental for neural progenitor cell differentiation and survival. While the generation of NO clearly contributes to pathology following ischemia it can also be beneficial[5]. Explanations for this apparent contradiction may be found in the cell type responsible, the amount of NO that is generated, and whether products of further NO oxidation are formed. A number of important questions have yet to be answered with respect to the roles for the NO generated by different cell types following acute injury. Seeking answers through the use of NOS inhibitors, or even knockout mice, will not be definitive. Conditional and/or cell-specific knockouts, in which NOS responses can be selectively manipulated, will be useful models for unpicking NO and neuropathology.

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Supported by USPHS-NIH grant NS29226.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA88

Coupling of exo- and endocytosis: insights from single-vesicle recordings

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During synaptic transmission small synaptic vesicles filled with neurotransmitter fuse with the plasma membrane to release their content. For maintaining synaptic transmission the exocytosed vesicle proteins have to be retrieved thereafter by compensatory endocytosis (1-3). What is the fate of synaptic vesicle proteins post fusion? Do they stay together in a raft-like structure (4), that can be retrieved efficiently in toto or do they disperse in the plasma membrane and have to be resorted and reclustered for retrieval? While it was recently shown that synaptic vesicles exocytosed and retrieved by compensatory endocytosis are non-identical with respect to their protein complement (5), this does not necessarily imply dispersion of vesicle proteins after fusion. By optically recording single fusion events with high-resolution scanning microscopy we show for four different transmembrane vesicle proteins, synaptobrevin 2, synaptotagmin 1, synaptophysin, and VGlut1, fast dispersion post fusion. Proteins diffused within the synaptic bouton membrane with diffusion constants around 0.2 μm2/s, but only 10 – 20 % were lost into the axonal membrane. This suggests a mechanism by which vesicle proteins are rapidly cleared from the release site to allow for the next docking and priming event, but are efficiently recaptured outside the active zone. Our findings at synapses tuned for high-fidelity signaling may hint at a general mechanism in organelle trafficking.

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This work was supported by grants of the Deutsche Forschungsgemeinschaft and the Human Frontier Science Project.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA89

Multiple vesicle recycling pathways in central synapses and their impact on neurotransmission

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Synaptic vesicle recycling is essential to sustain neurotransmission during activity in central synapses. However, it is yet uncertain if the only function of synaptic vesicle trafficking is to maintain fusion competence of synaptic vesicles and structural homeostasis of synapses in the long term. Or can it, in addition, modulate frequency dependence of synaptic responses during short-term synaptic plasticity?

Clathrin-mediated endocytosis comprises a ubiquitous means for vesicle recycling in most cell types. This pathway typically possesses well-defined morphological markers (coated pits, endosomal intermediates etc.) and adequate molecular tools are available to probe its properties in synapses. A rapid vesicle-recycling pathway, in contrast, may not employ the same molecular players and structural intermediates and is therefore harder to examine morphologically and molecularly. Therefore, most evidence in support of a fast retrieval and recycling mechanism for synaptic vesicles relies on electrophysiological and optical techniques with rapid time resolution. Our results from experiments using a combination of fluorescence imaging of synaptic vesicle trafficking and electrophysiological detection of short-term synaptic plasticity collectively suggest that a rapid form of synaptic vesicle recycling strongly contributes to neurotransmission in a frequency-dependent manner in hippocampal synapses. These findings can be outlined as follows:

1. 10-50% of styryl dye (FM1-43 or FM2-10) taken up during a bout of intense stimulation (e.g. 10 Hz for 10 s or 30 Hz for 3 s) can be rapidly re-available for exocytosis within 10 seconds after dye uptake. In addition, up to 50% of FM dye taken up during an exhaustive stimulation (30 Hz or 90 mM K+) can be re-released within 15 seconds.

2. Impairment of neurotransmitter refilling into endocytosed synaptic vesicles by inhibiting their re-acidification enables an electrophysiological means to test the role of vesicle reuse in synaptic depression. After inhibition of neurotransmitter refilling, synapses onto hippocampal CA1 pyramidal cells show faster depression with increasing stimulation frequencies. At 20 Hz, compromising neurotransmitter refilling increases depression within 300 ms reaching completion within 2 seconds. In contrast, at 1 Hz, inhibition of neurotransmitter refilling has a delayed effect on depression, which emerges gradually and becomes significant within 100 seconds. These observations suggest that rapid re-availability of endocytosed vesicles for fusion can slow the kinetics of short-term synaptic depression.

3. To image the effect of inhibiting vesicle re-acidification at the level of individual hippocampal synapses, we infected hippocampal cultures with lentivirus expressing synaptic vesicle protein synaptophysin tagged with superecliptic-pHluorin. Superecliptic-pHluorin is a GFP-based pH sensor that is normally quenched at pH 5.5 within the vesicle lumen but fluoresces once vesicles fuse with the plasma membrane and the fluorophore is exposed to extracellular pH (7.4). In response to stimulation at 20 Hz for 2 seconds, synaptophysin-pHluorin fluorescence shows a swift increase and a gradual slow return to baseline in agreement with earlier observations. After incubation with an inhibitor of vesicle re-acidification, however, in response to the same stimulation protocol, synaptophysin-pHluorin fluorescence shows a larger increase detectable within the first 500 ms consistent with rapid internalization of synaptophysin-pHluorin and re-acidification of synaptic vesicle interior in the absence of the inhibitor.

In addition, the same experiments outlined above also support the presence of a slow synaptic vesicle trafficking pathway that operates over minutes (e.g. slow decay of synaptophysin-pHluorin fluorescence after stimulation). Therefore, our current efforts are focused on examining the signaling mechanisms that potentially target vesicles to one of the two available pathways for synaptic vesicle recycling. Our initial results point to a strong influence of chronic activity in addition to acute frequency of presynaptic stimulation in determining the pathway through which synaptic vesicles can be recycled. Synaptic activity can influence signal transduction in nerve terminals in several ways, and therefore we are currently examining the roles of candidate mechanisms that may impact synaptic vesicle trafficking and alter resulting dynamics of neurotransmitter release.

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C40

Functional Characterisation of the N-terminal domain of Alsin, a protein involved in juvenile onset Motor Neurone Disease

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Mutations in the ALS2 gene cause juvenile onset amyotrophic lateral sclerosis, also known as motor neurone disease (MND) (1, 2). MND is a devastating neurodegenerative disorder causing a progressive paralysis. The ALS2 gene encodes the 180kD protein alsin, which has guanine nucleotide exchange (GEF) activity for Rab5 and Rac1 (3), thereby implicating a role in endosome fusion and intracellular trafficking. ALS2-deficient mice do not show an obvious MND-like phenotype, although they do show irregularities in endosome trafficking (4). We are investigating the role of the N-domain, which has sequence homology to RCC1 (regulator of chromatin condensation-1), a known beta-propeller structure.

We have predicted the N-terminal domain of alsin using comparative modelling, and propose it is likely to form a seven-bladed beta propeller structure containing a disordered loop located within one of the blades. The propeller, like RCC1, is formed of anti-parallel beta-sheet blades radially arranged around a hydrophobic core. We aim to use circular dichroism to confirm the secondary structure, thereby providing practical evidence for the model.

Beta-propeller structures are often sites of protein-protein interaction. We investigated whether the N-terminal of alsin contains protein interaction surfaces by using complementary and parallel techniques of co-immunoprecipitation, a yeast-2-hybrid screen and GST-pulldown. Preliminary findings have identified microtubule-associated proteins, neurofilaments, as well as proteins involved in endo- and exocytosis and associated with synaptic vesicles. These results are consistent with a role for alsin in endosome trafficking and axon maintenance, and strengthen well-established findings that neurofilament proteins are of high importance in MND pathology. We are now in the process of confirming these interactors, using human and mouse alsin constructs in parallel to investigate possible interspecies differences.

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This work was funded by SHERT and the Wellcome Trust

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C41

Anterograde tracing study and co-localization of NK-1 and Muc3 receptors in the palate of Rana pipiens

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Mucociliary activity is an important clearance mechanism in the respiratory system of air breathing vertebrates. Substance P (SP) and acetylcholine play an important role in the stimulation of the mucociliary transport in the frog palate. In this study, anterograde neuronal tracing was combined with immunocytochemistry for NK-1R (SP receptor) and Muc3R (acetylcholine receptor) in the palate. The nerve fibers in the palate were labeled with the anterograde tracer Biotinylated Dextran Amine (BDA). The optimal labeling of BDA fibers in the palate was obtained at 8 days of exposure. Immunoflorecence shows that NK-1R and Muc3R are co-localized in the membranes of epithelial and goblet cells in the palate. Molecular characterization of SP receptor was performed by isolation of RNA from brain and palate followed by cDNA synthesis. Ultrastructural study of the palate showed axonal-like endings with vesicles in connection with epithelial and goblet cells. These results further support the concerted action of both neurotransmitters in the regulation of mucociliary activity in the frog palate.

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NIH- RISE

NIH- PRAABRE

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SA90

Clathrin-mediated endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses

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Neurons transmit information at chemical synapses by the release of neurotransmitter contained within small vesicles. The maintenance of synaptic transmission requires that these vesicles be recycled after fusion with the cell surface, but these endocytic mechanisms have been. It is established that synaptic vesicles can collapse on fusion and the machinery for retrieving this collapsed membrane by clathrin-mediated endocytosis (CME) is enriched at hippocampal boutons (Takei et al., 1996). What is less clear is the speed at which CME operates at small central synapses and its importance compared to other mechanisms of vesicle retrieval. In fact, it has been suggested that the large majority of vesicles released by physiological stimulation are recycled by a second, faster mechanism called "kiss-and-run," which operates in 1 s or less (Aravanis et al. 2003). A key feature of the kiss-and-run model is that the vesicle is retrieved at the site of fusion before it has collapsed into the surface membrane (Gandhi & Stevens, 2003).

What is the relative importance of these different endocytic mechanisms during normal synaptic activity? To address this question, we designed sypHy, a new and improved optical reporter of exocytosis and endocytosis made by fusing pH-sensitive GFP (Miesenbock et al., 1998) to the synaptic vesicle protein synaptophysin. The fluorescence of sypHy increases when vesicles fuse, and then declines when they are re-acidified after endocytosis. Vesicle reacidification occurred with a time-constant of 4 s, allowing us to estimate the rate of endocytosis from the decline in the sypHy signal. The average release probability

reported by sypHy was slightly larger than the value of 0.35 measured electrophysiologically, indicating that pHluorin reliably records all fusion events and can therefore provide an unbiased view of the kinetics of endocytosis.
We measured synaptic vesicle retrieval after single action potentials (APs), when fast kiss-and-run has been reported to be the predominant mode of fusion, but found only one mode of endocytosis, which occurred with a time constant of 15 s at room temperature. The speed of endocytosis was the same at synapses of high and low release probability and constant for a range of stimulus strengths up to 40 APs at 20 Hz. Monitoring the connection of a fused vesicle with the surface of the cell by altering the external pH provided an alternative method of estimating the rate of endocytosis which yielded a time-constant of 12 s. Two different methods of inhibiting CME blocked vesicle retrieval after weak stimuli: overexpression of a dominant-negative construct (AP180-C) and knockdown of clathrin heavy chain using RNAi. These results provide clear evidence against the idea that fast, clathrin-independent mechanisms of vesicle retrieval play a significant role at hippocampal synapses. We therefore conclude that CME is the physiological mechanism of vesicle retrieval (Granseth et al. 2006).

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA91

Control of slow endocytosis by protein dephosphorylation

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Synaptic vesicle retrieval in central nerve terminals can occur via at least two different pathways, fast and slow endocytosis. Slow endocytosis is only activated by strong physiological stimulation and the molecules that trigger and mediate this process are still being identified. We have recently shown that the phosphorylation cycle of the group of proteins called the dephosphins selectively control the slow endocytic pathway in central nerve terminals. One of the dephosphins is the large GTPase dynamin I, whose dephosphorylation is essential for at least one form of synaptic vesicle endocytosis. The dephosphorylation of dynamin I allows an interaction with the endocytosis protein syndapin I, placing syndapin I as the possible effector in slow endocytosis. Thus the dephosphins may be key sensors for activation of slow endocytosis during strong stimulation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA92

Intestinal glucose sensing and regulation of glucose absorption

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Dietary sugars are transported from the intestinal lumen into enterocytes by the sodium-dependent glucose transporter (SGLT1). Although expression of SGLT1 is regulated by luminal monosaccharides, the luminal glucose sensor mediating this process was unknown. Here, we show that the sweet taste receptor subunit T1R3 and G-protein α-gustducin, expressed in enteroendocrine cells, underlie intestinal sugar sensing and SGLT1 upregulation. Dietary sugars and artificial sweeteners increase SGLT1 expression and glucose absorptive capacity in wild-type mice, but not in T1R3 or α-gustducin knock out mice. We demonstrate that sweeteners acting upon sweet taste receptors stimulate secretion of gut hormones implicated in SGLT1 upregulation.

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SA93

Physiological significance of L-amino acid sensing by extracellular Ca^{2+}-sensing receptors

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Throughout the body, amino acid sensing mechanisms couple protein ingestion to physiological responses including the control of growth, tissue maintenance, and digestion and absorption. The molecular identities of the amino acid sensors in endocrine glands, the gut, kidney and other tissues, however, have been unclear. Recent work demonstrates that several class 3 G-protein coupled receptors (GPCRs) are broad-spectrum amino acid sensors that couple increases in amino acid levels to the activation of phospholipase C and intracellular Ca2+ mobilization. The calcium-sensing receptor (CaR) is one broad-spectrum amino acid sensor of the class 3 sub-group with apparent selectivity for aromatic, aliphatic and polar amino acids. Its most potent amino acid activators include L-Phe and L-Trp, which are recognized activators of gastrin and gastric acid secretion as well as cholecystokinin and attendant pancreatic enzyme secretion. Based on considerations of fasting and fed amino acid concentrations, however, it is apparent that the CaR exhibits equivalent responses to as many as 6-8 amino acids and is highly sensitive to changes in the integrated plasma amino acid concentration. With respect to intracellular Ca2+ mobilization, the CaR responds to both extracellular Ca2+ (Ca2+o) and amino acids and exhibits strong positive interactions between the two classes of activators. In the human parathyroid, which expresses the CaR at high levels endogenously, CaR-active amino acids

markedly promote intracellular Ca2+ mobilization and reversibly suppress the secretion of the key calcium-regulating peptide hormone, PTH. Human adenomatous parathyroid cells, which exhibit markedly reduced CaR expression when compared to normal cells, however, are much less sensitive to amino acids. Recent work suggests that thyroid parafollicular C-cells, which secrete another calcium-regulating peptide hormone calcitonin under CaR control, are also sensitive to acute changes in amino acid concentrations. In the gut, CaR expression is widespread, potentially explaining diverse effects of calcium ions and aromatic amino acids on digestion and epithelial transport. For example, gastric parietal cells exhibit marked positive interactions between Ca2+ and the aromatic amino acid L-Phe with respect to gastric acid secretion. The CaR is also widely expressed in the kidney, and recent work demonstrates that L-Phe stimulates renal calcium excretion, consistent with CaR-dependent inhibitory regulation of calcium reabsorption in the cortical thick ascending limb. Taken together, the results indicate that the CaR, as well as several other closely related class 3 GPCRs, represent physiologically and, potentially medically, significant targets for L-amino acids.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C42

In HepG2 the LXR-responsive elements (LXREs) in the promoters of fatty acid synthase (FAS) and its transcriptional activator, SREBP-1c, respond differently to RXR ligands

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Treatment of the hepatoma cell line HepG2 with all-trans retinoc acid (RA) induced expression of both mRNA and protein of fatty acid synthase (FAS) and SREBP-1c, an important transcriptional activator for FAS. Transient transfection of HepG2 showed that the rat FAS and murine SREBP-1c promoters responded positively to 9 cis-RA and all-trans RA. Cotransfection experiments using a human expression vector for retinoid-X receptor α identified two cis-elements essential for the RA response of the FAS promoter: the NF-Y-binding CAAT-box at -100 and the SREBP/USF-binding element at –65, known to be involved in glucose/insulin and sterol regulation. A LXR/RXR heterodimer binding site (LXRE at -660) reported to be essential for FAS regulation during lipogenesis contributed marginally to the RA-dependent upregulation of the FAS promoter. However, the LXR/RXR heterodimer binding site at -234 (Yoshikawa T. et al., 2001) conferred the full RA response on the SREBP-1c promoter in HepG2. Our data clearly demonstrate the existence of functional differences in LXREs which may well imply different roles in vivo.

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The authors are indebted to Dr. Hitoshi Shimano and Takashi Yamamoto (Institute of Clinical Medicine, University of Tsukuba, Tsukuba City, Japan) for the SREBP-1c promoter constructs.

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C43

Amino acid transporters and cell signalling in a *Drosophila* cell line (Schneider (S2) cells)

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We have recently shown a novel role for an amino transporter, Path, in signalling in the fruit fly Drosophila melanogaster (Goberdhan et al 2005). This is a member of the proton-coupled amino acid transporter PAT family, with highly unusual functional properties. We describe here preliminary experiments using a cell line designed to complement our functional understanding of both this group as well as of the CD98 related group of amino acid transporters in this model organism.

Figure 1 shows that S2 cells, known to express at least two (CG13384 and Path) of the 11 PAT genes found in the *Drosophila* genome, express readily-detectable transport properties expected for proton-coupled transporters (stimulation by acidification under sodium-free conditions; inhibition by the characteristic substrates Ala, Pro, Gly); however additionally the differing potency for inhibition by Leucine of the transport of the substrates Ala and Gly shows that more than a single PAT transporter must be involved.

We have also shown that the system-L specific-substrate BCH is a potent inhibitor (under sodium-free conditions) of radiolabelled leucine influx into S2 cells. This means that these cells must express both the heavy chain (CD98 homologue) as well as the appropriate light chain (since system L is a heterodimeric transporter). S2 cells express a putative CD98 heavy chain homologue (CG2791) as well as five possible light chains. Knock-down of CG2791 using a standard RNAi protocol reduces system L-mediated leucine influx specifically.

We conclude that S2 cells express endogenous transporters that will allow transport/signalling roles of both these families (PATs and CD98-related transporters) to be studied systematically *in vitro* while also exploiting the unrivalled genetic tractability of the fruitfly to understand their functional significance *in vivo.*

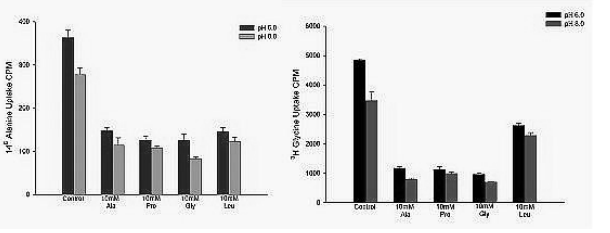

Figure 1: Influx of radiolabelled L-alanine (left) and of glycine (right) into S2 cells under sodium-free conditions: the external pH was respectively 6.0 or 8.0. Inhibition by 10mM unlabelled alanine, proline, glycine and leucine is shown. Note the pH dependence of uptake; and the different pattern of inhibition by leucine for the two substrates.

Goberdhan D et al (2005) Development 132 2365-75

Acknowledgements: we are grateful to the CR UK for funding

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA94

How does your gut taste? Nutrient detection in the wall of the gastrointestinal tract

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The presence of nutrients in the intestinal lumen initiates release of regulatory peptides from endocrine cells in the gut wall resulting in a number of signaling events in the epithelium, including activation of extrinsic and intrinsic neurons, and subsequent changes in gastrointestinal function and regulation of food intake. However, the mechanisms that underlie these events are not fully understood. This presentation will review three aspects of this process; 1. the process by which endocrine cells respond to nutrients and the role of different proteins either expressed in the apical membrane of the cells, including G protein coupled receptors and nutrient transporter-related proteins, or intracellular proteins, such as apolipoprotein A-IV; 2. the regulatory peptides released from gut endocrine cells that activate vagal afferent activity via expression of specific receptors for several gut regulatory peptides, including cholecystokinin (CCK) type 1, peptide YY type 2, 5-HT type 3, leptin, ghrelin, cannabinoid type 1, and glucagon-like peptides (GLP)-1 and -2 receptors; 3. the changes in sensitivity of this pathway that lead to changes in gastrointestinal function and dysregulation of food intake, and how these mechanisms and pathways might be altered in diseases, such as inflammation and obesity.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA95

Long chain fatty acid sensing in the gastrointestinal tract

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The gastrointestinal tract is highly adapted for the digestion and absorption of long chain fat: a rich energy source. Indeed much of the machinery (delayed gastric emptying, increased surface area, bile secretion) is only truly necessary for efficient lipid handling. This process is not passive: the gut epithelium is able to sense the presence of fat, responding rapidly and appropriately. It is now clear that the key sensory cell type responsible is the enteroendocrine cell (EEC). The best exemplar for fat sensing is the CCK-secreting I-cell, principally situated in the duodenum. CCK acts as a classical hormone, but also via vagal afferent fibres. The effects of fat on the gut via CCK are highly chain length specific. We established that there is a key requirement for a chain length of C12 and longer in humans (1). Consequent differential effects on gall bladder emptying and gastric emptying can be observed, and are not seen with shorter fatty acids. This also translates into a differential effect on gastric capacity, which may have salience for satiety (2).

We have begun to explore the cellular basis for the fat sensory system. The murine cell line STC-1 is the best available model, and responds in a highly similar manner to that observed in vivo (3). There is a key dependency on intracellular calcium, with evidence both for mobilisation of intracellular and extracellular pools. The signalling pathway remains elusive, although there is some evidence fatty acids may at least in part transduce their own signal (4). There is a clear need for free fatty acid to induce signalling: intact triglycerides are inert. The physicochemical properties of fat may also be important: hydrophobicity increases with increasing chain length. C12 is the first saturated fatty acid to be solid at body temperature, and appreciably less soluble in water than chains up to C10.

The recent deorphanisation of GPCRs (GPR 40, 41,43,120) and reassignment as fatty acid receptors has potentially transformed this area. GPR41 and 43 are more likely to be short chain fatty acid receptors. Although the best evidence exists for GPR40 as the pancreatic beta cell fatty acid sensor, there is evidence for its expression in the gut and in EEC cell line models. Heterologous expression of GPR40 subcloned from STC-1 cells in Xenopus laevis oocytes confers fatty acid responsiveness similar to that observed in STC1 cells (5). However, the current lack of a specific antibody and of techniques to isolate EEC has hampered confirmation of this as the primary EEC lipid sensor.

Other cell types have been postulated to play a role in fatty aid sensing including the enterocyte via apolipoprotein A-IV, and the brush cell population. It is unclear whether submucosal sensory nerves have an independent fat sensing capability: this seems more likely to be the case for short chain fatty acids.

Given the central role of EEC in fat sensing it is now essential to develop models to study primary cell physiology in health and disease. There is now evidence that EEC hyperfunctionality is implicated in the reduction in food intake observed in intestinal inflammation. Mimickry of these mechanisms in the absence of fat would therefore have potential applications in the control of food intake, and perhaps in the gastrointestinal handling of oral drugs.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA96

Pathophysiology of Ischaemic stroke: insights from imaging and implications for therapy

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Ischaemic stroke remains a major killer and the first cause of acquired handicap in the UK, costing billions of pounds yearly. Preventing death and limiting handicap are therefore major goals. This can be achieved only if the pathophysiology of infarct expansion is properly understood. Work in the animal, especially the non human primate, has shown that following occlusion of the middle cerebral artery (MCA) - the most frequent and prototypical stroke - there is a gradient of reduction in tissue blood flow from the periphery of the cortical MCA territory towards its centre, from perfusion being normal or only mildly reduced in the former to very low (but never abolished) in the latter. Tissue fate has been shown to locally depend on the severity of hypoperfusion and duration of occlusion, with a fraction of the MCA territory being initially in a "penumbral" state, i.e, electrically silent but still salvageable by early reperfusion. Translating this knowledge in the human has proven a long and difficult journey, but was eventually achieved using physiological quantitative imaging, specifically PET. However, PET in MCA stroke has also revealed the presence of considerable pathophysiological heterogeneity from patient to patient, largely unpredictable from either elapsed time since onset or clinical deficit. At time of scanning, some patients show spontaneous recanalization of the MCA, with efficient and actually excessive reperfusion, and these patients do well and end up with very small infarcts and excellent functional recovery. The remaining patients still have MCA occlusion, but within this group, two main patterns are encountered: i) small "core" of already irreversible damage but extensive "penumbral" hypoperfusion; and ii) extensive "core" with little or no remaining penumbra. The latter patients have poor prognosis, and often develop "malignant" MCA stroke, while the former have unpredictable outcome, with the amount of clinical recovery proportional to the volume of penumbra that escapes infarction. Importantly, all three pathophysiological patterns can be seen within 3 hrs of clinical onset, while persistent penumbra can be encountered as late as 16-18 hrs after onset, suggesting a prolonged window for therapeutic intervention. While these observations underpinned key trials of thrombolysis, which have shown clinical benefit up to 3 hrs (now part of clinical routine), they also indicate that not all patients may be rational candidates for thrombolysis, and that only those who are likely to benefit should be exposed to the risks of this intervention. It is increasingly recognized that this underlying pathophysiological heterogeneity needs to be accounted for if thrombolysis is contemplated, and accordingly pathophysiological diagnosis with imaging is rapidly becoming an essential component of stroke assessment and customized management, replacing the clock by individual pathophysiology. To implement this, diffusion- and perfusion-weighted MR (DWI-PWI) and CT-based perfusion imaging are increasingly used, although still undergoing formal validated against gold-standard PET. Beyond thrombolysis per se, knowledge of the individual pathophysiology also guides management of physiological variables like blood pressure, blood glucose, oxygen saturation and temperature, which can otherwise precipitate the penumbra into the core, and the mildly hypoperfused tissue into the penumbra. We propose that future trials of neuroprotection use physiological imaging to select the patient category that best matches the drug's presumed mode of action, rather than lumping together patients with entirely different pathophysiological patterns in so-called "large trials", which have all failed so far.

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SA97

Neurovascular pathophysiology in stroke

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Stroke research continues to face many challenges as we struggle to overcome translational hurdles between basic science and clinical applications. To date, the only established therapy is thrombolytic reperfusion, whereas numerous trials of pure neuroprotection have not yielded clear efficacy. In this presentation, we will assess the hypothesis that effective translational research must be based on mechanisms, and that linked platforms of cell-to-tissue-to-whole-animal models are required. Protease mechanisms that underlie cell-cell and cell-matrix signaling at the neurovascular interface will be examined as a case study of how acute injury is inextricably linked to delayed remodeling. Some complications of tPA reperfusion may be associated with dysregulation of matrix metalloproteinase (MMP) networks. Degradation of neurovascular matrix may underlie BBB injury, edema, hemorrhage and cell death. In contrast, brain tissue remodeling and neurogenesis may in turn be dependent on regulated MMP processes during stroke recovery. MMP and inflammatory responses at both tissue and biomarker levels will be assessed as an example of how analysis at multiple cell levels may provide information useful for interpretation of animal model systems. Attention to neurovascular mechanisms at molecular, cellular and organ levels of analysis may yet provide new opportunities for translating our basic science into meaningful clinical therapies.

Supported in part by grants from teh NIH and AHA.

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C44

Examination of the cytoprotective action of NXY-059 in vitro using a neuroblastoma-derived cell-line

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Background. The nitrone compound NXY-059 (disodium 2,4-sulphophenyl-N-tert-butylnitrone) is a free radical trapping agent that is neuroprotective in both rodent and primate models of acute ischaemic stroke (see Green and Ashwood 2005). However, evidence for cytoproprotective activity of nitrones in vitro is sparse. We have therefore investigated whether NXY-059 is cytoprotective in an in vitro preparation, using a neuron-like cell culture model to examine free radical-induced cell damage. Damage was produced by addition of the free radical generating agent sodium nitroprusside (SNP) and we examined the effect of both a cocktail of known antioxidant compounds and NXY-059 on SNP-induced cell death.
Methods. Cells of a murine neuroblastoma-derived cell-line (N1E-115) were exposed to sodium nitroprusside (SNP). Cell death was assessed at 24 h by use of the MTT assay of mitochondrial complex II activity and also by microscopic examination of nuclear morphology using Hoechst 33342 chromatin stain to identify apoptotic cells.
Results. SNP (10 -1000 μM) produced concentration-dependent cell death as determined by the MTT assay (EC50 ~ 100 μM at 24 h). A cocktail of known anti-oxidant agents (ascorbate, reduced glutathione and dithiothreitol, all at 100 μM) produced significant protection ($P<0.05$) against cell death induced by SNP (100 μM), assessed by both the MTT assay (65% reduction in damage) and nuclear morphology counting (100% reduction). In contrast, NXY-059 at a concentration (300 μM) approximately two-fold higher than the plasma concentration required to produce neuroprotection in vivo (Sydserff et al, 2002) was without effect on SNP-induced cytotoxicity. NXY-059 (300 μM) was also without effect on cell death mediated by a related free radical stimulus (100 μM H2O2; 24 h).
Conclusion. These results suggest that NXY-059 may not act to protect neurones in the brain following an ischaemic insult and complements recent data suggesting that rather the drug may act to protect the brain endothelium (Culot et al 2006). This proposal supports the concept of neuroprotection by an action at the neurovascular unit (del Zoppo 2006).

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We are grateful to The Wellcome Trust and AstraZeneca Global CNS R&D for financial support.

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C45

Acute oestrogenic effects on vascular contractility of middle cerebral artery

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Activation of oestrogen receptor (ER) is neuroprotective in transient cerebral ischaemia. (Carswell et al, 2004). Vasodilatory effects of oestrogen are well documented (Pelligrino et al, 1998). Endothelial and smooth muscle cells express membrane and classic nuclear oestrogen receptors, ER-∝ and ER-β. However, it is unclear whether vasodilatory effects in cerebral arteries are mediated via membrane and/or nuclear ER or independent of both. MCA of male 3 month old Sprague Dawley rats (152 ± 2μm, n=84) were isolated and mounted on a wire myograph, the arteries were allowed to equilibrate (37○C, 20mins). Following constriction with U46619 (EC50) the relaxant potential of the following drugs was assessed: Bradykinin (Bk;1μM), 17β-estradiol (E2;0.1-100μM), selective oestrogen receptor modulators (SERMs: LY2120310 and LY362321; 0.01nM-10μM), ER-β agonist, DPN (0.1-100μM) and ER-∝ agonist, PPT (0.1-100μM). The NOS inhibitor L-NAME (100μM) was used to evaluate the role of NO in relaxation. The non-selective ER antagonist ICI 182780 (1μM) was used to assess whether the relaxation is receptor dependent. Results are expressed as % relaxation of U46619-induced tone. Data are expressed as mean ± s.e.m., $n \leq 5$ and analysed using unpaired t-test.
Relaxation induced by Bk (61 ± 4%) was virtually abolished by L-NAME (6 ± 4%, $p<0.05$). In contrast relaxation induced by E2 (87 ± 3%) was unaffected by L-NAME (93 ± 1%) or ICI 182780 (80 ± 4%). SERM LY2120310 and SERM LY362321 had no significant effect on vessel tone (-72 ± 26% v/s -72 ± 15% vehicle and -20 ± 8% v/s -25 ± 10% vehicle, respectively). Relaxation induced by DPN (60 ± 7%) and PPT (97 ± 7%) was similarly not inhibited by ICI 182780 (74 ± 2%) and (95 ± 3%), respectively.
The present study shows, for the first time, the vascular actions of SERMs and agonists on the MCA. E2 does not cause relaxation via the endothelial NOS pathway. There was no relaxation induced by SERMs. However, the PPT and DPN results indicate that the acute vasodilatory effects of oestrogen in the MCA may be via activation of ER-∝ and β, possibly at the level of the plasma membrane. This is in agreement with Montgomery et al (2003) who used mesenteric arteries from male rats and who speculate that such direct membrane effects may involve inhibition of calcium entry mechanisms.

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The authors would like to acknowledge Dr. M. O'Neill and Dr. J. Dogde at Eli Lilly for supply of SERMs.

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SA98

Pharmacological approaches to stroke: reperfusion certainly, neuroprotection possibly

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There are limited therapeutic options for treating acute ischaemic stroke. Two approaches have received considerable attention, thrombolysis and neuroprotection. Thrombolysis has provided the only clinically approved drug, recombinant tissue plasminogen activator (rtPA). However patients must undergo a scan before its administration, it must be given within 3 h of symptom onset, and it increases the risk of haemorrhagic complications. Consequently no more than 5% of patients receive rtPA. New thrombolytics and anti-aggregation compounds are in development, including desmoteplase, a genetically engineered version of a thrombolytic protein in Vampire Bat saliva. Preliminary evidence suggested that desmoteplase-treated patients had a higher rate of early reperfusion, better clinical outcome and no increase in haemorrhage rate compared to the placebo group. Another approach to increase cerebral blood flow is the defibrinogenating compound ancrod. Success with these compounds in current Phase III trials has to encompass not only improved functional outcomes compared to placebo but also a longer therapeutic window than rtPA. These compounds would also have to possess a satisfactory adverse event profile, particularly a low incidence of hemorrhagic transformation, a problem that has limited clinical acceptance of rtPA.

Neuroprotection is an entirely different approach. The role of a neuroprotectant is to interfere with the biochemical chain of events that results in neurodegeneration, thereby limiting tissue damage. The basis of neuroprotection is that the area of markedly reduced blood flow (the core) is surrounded by the penumbra which, while compromised, can be protected either by reflow or administration of a neuroprotectant. Without intervention cells in the penumbra die. Over the last 20 years huge efforts have been expended in developing neuroprotectants. Around 1000 compounds have been developed preclinically with many showing efficacy in preclinical stroke models. Over 100 reached clinical evaluation but none has clearly demonstrated efficacy. Because of these many failures a group of experts formulated a set of guidelines to be used for preclinical drug development before a compound progressed to the clinic (Stroke Therapy Academic Industry Roundtable, 1999). At the time the guidelines were published the novel free radical trapping neuroprotectant NXY-059 was being developed and its preclinical programme was focussed on following the guidelines which included full dose-response information, validation in permanent focal ischemia models, short and long term functional outcome measures and confirmation of results in non-company academic centres. NXY-059 fulfilled all criteria (Green & Ashwood, 2005) and was therefore examined in acute stroke patients. A first Phase III trial (1700 patients) was positive on the primary end point (Lees et al. 2006) but a second trial (3200 patients) failed to confirm efficacy. This result both emphasises the importance of conducting more than one large trial in order to prove clinical efficacy and, crucially, questions the validity of the STAIR criteria. There remain 2 compounds in late clinical development, DP-b99 and citicoline, but the outcome of their clinical efficacy remains uncertain, given the apparent lack of predictive value of current preclinical evaluation techniques.

Why has success for the neuroprotection approach been elusive? Possibly because we are not modelling the disease closely enough. Many stroke patients are old, with co-morbid conditions such as hypertension and diabetes and these are not being modelled when young healthy animals are used. Such co-morbid conditions may alter cerebrovascular functions such as the structure of the blood brain barrier or the neuroimmune system. The future of neuroprotection research may require better animal models. Alternatively, the failure of NXY-059, a compound developed with close regard to the best established guidelines of preclinical and clinical methodology, may result in pharmaceutical companies switching strategies. Few companies will feel the huge costs of a neuroprotectant clinical trial programme are justified in the light of so many failures. Consequently we are likely to see increasing research and development on approaches which involve neuro-restoration and repair in the region of the cerebral infarct. There is already much information on the mechanisms associated with cell death and cell survival in the CNS. Manipulation of such mechanisms may prove valuable following a stroke. What remains uncertain is how one might "switch on" or "switch off" such mechanisms in the damaged brain without disrupting homeostasis in the many other regions of the organism where such intervention would almost certainly prove problematic.

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SA99

Clinical pharmacological issues in the development of acute stroke therapies

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The development of effective acute stroke therapies is a high priority because of the substantial death and long term disability that result from acute stroke. Reducing the initial extent of brain damage is key if outcomes from acute stroke are to be improved. In 85-90% of cases stroke is due to infarction usually secondary to atherothrombotic or cardioembolic disease causing vessel occlusion in the anterior circulation. The two main drug targets in ischaemic stroke are lysis of the acute thrombus in the target vessel and protection of the ischaemic penumbra. Drug development of thrombolytic therapy has successfully occurred although dose selection to optimise the risk/benefit ratio (haemorrhage/ recanalisation) has proved a difficult challenge. The efficacy of thrombolysis has been clearly demonstrated within 3 hours and translation of animal studies was achieved in proof of

concept studies in man examining recanalisation in acute stroke, then efficacy in phase III studies. Phase IV studies have an important role in defining safety in routine clinical practice and in patient subgroups such as the very elderly, poorly represented in phase III clinical trials.

Translating neuroprotective drug effects seen in animal models into positive phase II/III studies has been unsuccessful despite considerable industry efforts. Early studies had many problems in study design with failure to achieve plasma concentrations consistent with animal studies, patient selection with lack of initial imaging, and long time windows. The 1999 and 2001 STAIR consensus criteria listed preclinical and phase II/III design requirements intended to improve chances of demonstrating efficacy. Despite improvements in clinical study design the lack of a 'proof of concept' model to demonstrate salvage of the ischaemic penumbra in phase II studies remains a major barrier. The recent failure to demonstrate efficacy of the free radical scavenger NXY-059 following an initial positive phase III trial and achievement of most of the STAIR criteria place a question mark over the future of neuroprotective drug development. However demonstration of the benefits of hypothermia following cardiac arrest indicate neuroprotection is achievable in man. From a clinical pharmacological perspective a number of potentially correctable problems in the development of neuroprotection remain including: increasing quality, reporting and analysis of pre-clinical studies; efficacy in animal models more reflective of the older stroke patient with physiological derangement; efficacy in in-vitro human studies (cell culture, tissue slices); demonstration of drug access through the blood brain barrier to the putative site of action (PET, intra-operative studies); selection of patients with salvageable tissue (MR, CT perfusion selection); very early treatment (pre-hospital paramedic based trials); adaptive study design to identify most promising doses for phase III studies; standardisation and refinement of clinical measures of neurological impairment and disability; and physiological optimisation in proof of concept human studies. There remain concerns that rodent acute stroke studies are a poor model of the human ischaemic penumbra. Further refinement and validation of rodent models, and consideration of the role of primate models is required. Despite many difficulties and disappointments neuroprotection in stroke remains a tantalising, achievable goal and one of the great challenges of translation for the next decade.

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SA100

Ryanodine receptor-calcium release channel (RyR2) dysfunction in cardiac pathology

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The ryanodine receptor-calcium release channel (RyR) is an integral membrane protein that mediates rapid calcium efflux from the endoplasmic reticulum. RyRs play a direct role in the calcium signalling that is responsible for triggering numerous calcium-activated physiological processes, including contraction, neurotransmitter release and hormone secretion. The most characterised RyR-mediated process is excitation-contraction coupling in striated muscle, where electrical excitation of the plasma membrane is transmitted to the cell interior and results in a massive calcium efflux that triggers myocyte contraction (1,2,3).

The RyR is a homotetramer of 560,000 Dalton subunits, forming a ~2.3 megaDalton complex that associates with a multitude of accessory proteins including calmodulin, FK506 binding protein (FKBP12), calsequestrin, snapin, triadin and sorcin (1). The predicted transmembrane domain is located in the C-terminal one fifth of the molecule with the remaining ~80% thought to project into the cytoplasm. Several phosphorylation sites are located on the cytoplasmic portion that can regulate accessory protein interactions and channel function. The RyR complex comprises a ion permeation pathway of high unitary conductance that is weakly selective for divalent versus monovalent cations.

Recently, approximately 70 single residue mutations in the cardiac RyR2 have been identified in families that exhibit catecholaminergic polymorphic ventricular tachycardia (CPVT), a condition in which physical or emotional stress can trigger severe tachyarrhythmias that can lead to sudden cardiac death (1,2,3). The RyR2 mutations in CPVT are clustered in the N- and C-terminal domains, as well as in a central domain. Further, a critical signalling role for dysfunctional RyR2 has also been implicated in the generation of arrhythmias in the common condition of heart failure.

We have cloned the full-length cDNA encoding human cardiac RyR (RyR2) and generated RyR2 plasmids with various CPVT mutations to enable the heterologous expression and analysis of calcium release mediated by the wild type and mutated RyR2. These studies suggest that the mutational locus may be a significant factor in the mechanism underlying RyR2-mediated calcium channel dysfunction (1,2,3,4). Further understanding the causes of aberrant calcium release via RyR2 should assist in the development of effective treatments for the ventricular arrhythmias that often leads to sudden death in both heart failure and in CPVT.

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Our work is supported by grants from the British Heart Foundation, the European Union and the Medical Research Council.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA101

Sarcoplasmic reticulum calcium leak and cardiac arrhythmias

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Cardiac arrhythmia is an important cause of death in patients with heart failure (HF) and inherited arrhythmia syndromes, such as catecholaminergic polymorphic ventricular tachycardia (CPVT) [1]. Recent studies indicate that alterations in intracellular calcium (Ca2+) release channels known as ryanodine receptors (RyR2) play an important role in triggering ventricular arrhythmias [2]. For example, inherited mutations in the RyR2 gene have been associated with exercise-induced ventricular arrhythmias and sudden cardiac death in patients with CPVT. Electrophysiological studies have revealed that CPVT-mutant RyR2 channels are more likely to open aberrantly during diastole, which may provide the trigger for delayed afterdepolarizations and premature ventricular contractions. Studies in cardiomyocytes isolated from knockin mice carrying a CPVT-mutation in RyR2 demonstrated an increased propensity towards spontaneous Ca2+ release events during diastole [3]. Some CPVT mutations are also thought to decrease the binding affinity of the channel-stabilizing subunit calstabin2 (FKBP12.6), which may further enhance diastolic Ca2+ release from the sarcoplasmic reticulum (SR) [2]. Interestingly, decreased binding of calstabin2 to RyR2 has also been reported in patients and animals with heart failure. Indeed, enhanced diastolic SR Ca2+ leak has been demonstrated in cardiomyocytes isolated from calstabin2-deficient mice [2,4]. Moreover, an increased open probability of RyR2 isolated has been reported in several animal models of heart failure [5]. Taken together, these findings show that reopening of RyR2 channels during diastole, when the channel normally remains closed, cause SR Ca2+ leak which may initiate delayed afterdepolarizations and triggered activity leading to ventricular arrhythmias.

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American Heart Association, March of Dimes Foundation

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SA102

Mitofusin-2, mutated in Charcot-Marie-Tooth IIa, links endoplasmic reticulum to mitochondria

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Juxtaposition between endoplasmic reticulum (ER) and mitochondria provides physical basis for their intercommunication during Ca^{2+} signalling
and apoptosis. Molecular mechanisms controlling this interaction are unknown. Mitofusin-2 (MFN2), a mitochondrial dynamin-related protein mutated in Charcot-Marie-Tooth type IIa (CMTIIa), localized also at the ER
and was required for ER shape and its interaction with mitochondria. Localization of MFN2 at the ER proved essential to complement morphology of the organelle and interaction with mitochondria. Conversely, CMTIIa mutants of MFN2 restored morphology of mitochondria, but not of ER and juxtaposition of these organelles. ER-MFN2 bridged the
two organelles via homo and heterotypic interactions with mitochondrial MFN 1 and 2. Thus, MFN2 physically links ER and mitochondria, suggesting a role for ER and ER-mitochondria tethering in pathogenesis of CMTIIa.

L.S. is an Assistant Telethon Scientist of the Dulbecco-

Telethon Institute and an EMBO Young Investigator. Supported by a "Bolsa de Doutoramento" of the "Fundação para a Ciência e Tecnologia", Portugal. Telethon Italy, Compagnia di San Paolo Italy, Human Frontier Science Program Organization, United Mitochondrial Disease Fund USA, AIRC Italy.

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SA103

Inositol 1,4,5-trisphosphate-induced Ca^{2+} release; a master regulator of cardiac physiology

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Changes in intracellular Ca^{2+} are required for contraction of cardiac myocytes. To meet the increased hameodynamic requirements associated with exercise or stress, cardiac output is enhanced through an increase in the frequency and force of contraction of cardiomyocytes. As a result of a prolonged increased in workload the heart undergoes hypertrophy. This adaptive response is manifest as an enlargement of the heart ventricles

that is due to an increase in cell size without an increase in cell number. Interestingly, as well as controlling cardiomyocyte contraction, Ca^{2+} also plays a key role in regulating the transcription of genes involved in the hypertrophic response. Several models have been proposed to explain how Ca^{2+} can simultaneously, and with great fidelity, specifically control transcription and contraction in cardiac myocytes. Contraction and transcription are regulated in a linear fashion by the frequency and/or amplitude of Ca^{2+} transients. Alternatively, contraction and transcription are controlled by Ca^{2+} elevations that are either temporally or spatially distinct.

Recently, inositol 1,4,5-trisphosphate receptor (InsP3Rs) intracellular Ca^{2+} release channels have been shown to be expressed and functional in adult cardiac myocytes. InsP3Rs are expressed at approximately 100-fold lower level than ryanodine receptors (RyRs), which are responsible for mediating the Ca^{2+} release required to induce contraction. InsP3Rs also exhibit a restricted distribution in cardiac myocytes and unlike RyRs, which are located throughout the sarcoplasmic reticulum, they exhibit a sub-plasmalemmal and perinuclear distribution (1). This raises the possibility that they could give rise to Ca^{2+} signals that are distinct from those arising through RyRs. Recent evidence indicates that Ca^{2+} release through InsP3Rs occurs in response to cellular stimulation with endothelin-1 (ET-1). ET-1 is a well-known vasoactive hormone and a potent pro-hypertrophic agonist. The dual roles of ET-1 and InsP3-induced Ca^{2+} release in myocyte contraction and hypertrophy will be discussed.

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HLR is a recipient of a Royal Society University Research Fellowship. DH is supported by a PhD studentship from the MRC. We would also like to thank the BBSRC and the Babraham Institute for funding this work.

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C46

Study of the functional role of Bcl-2 family proteins in regulating Ca^{2+} signaling in apoptotic cells

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In mitochondria-dependent apoptosis in mammalian cells, the downstream signals (such as the release of cytochrome c) and the activation of caspases family were well studied. However, the upstream signal of mitochondria is still not clear. It is known that the Bcl-2 family proteins play important roles in controlling the permeabilization of the mitochondrial outer membrane. Alternatively, there is evidence suggesting that Ca^{2+} signal is also involved in regulating the apoptotic process. Recently, it was found that Bcl-2 family proteins can affect the apoptotic process by modifying Ca^{2+} released from the ER (1). The objective of this work is to understand the cross talk between the Bcl-2 signaling pathway and the Ca^{2+} signaling pathway. First, we examined whether mitochondrial Ca^{2+} plays a role in the apoptotic process. Previously, we found that cytosolic Ca^{2+} signal was upstream of cytochrome c release during apoptosis in HeLa cells (2). Using Rhod-2-AM as a mitochondrial Ca^{2+} indicator, we observed a bi-phase mitochondrial Ca^{2+} elevation during the apoptotic process. In HeLa cells undergoing the early stage of UV-induced apoptosis, we observed mitochondrial Ca^{2+} oscillation that was synchronized with cytosolic Ca^{2+} elevation. At a later apoptotic stage, a broader mitochondrial Ca^{2+} elevation was observed before mitochondrial membrane potential decrease. Second, we examined how the bcl-2 family proteins affect the Ca^{2+} mobilization from ER to mitochondria. We fused various bcl-2 proteins with different GFP and dsRed mutants and examined their overexpression effects on Ca^{2+} signal. We found that Bcl-2 and Bcl-xL can affect the ER Ca^{2+} homeostasis, while another mitochondria-localized Bcl-2 family protein, Mcl-1, can reduce mitochondrial Ca^{2+} uptake. Third, we investigated the functional role of IP3 receptors (IP3Rs) in apoptosis. We found that IR3Rs inhibitors (2-APB, Heparin) in general were anti-apoptotic, while an IP3R binding protein, RACK1, which can sensitize IP3Rs, promoted UV-induced apoptosis. These findings support the hypothesis that suppressing the Ca^{2+} mobilization between ER and mitochondria has an anti-apoptotic effect. We are currently conducting further experiments to study the interaction between IP3Rs and Bcl-2 family proteins.

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This work is supported by grants from the Research Grants Council of Hong Kong, HKUST6466/05M and N_HKUST616/05.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C47

Proteasomal alterations in the ratios of two novel PKC isoforms regulates myocardial injury following ischemia/reperfusion

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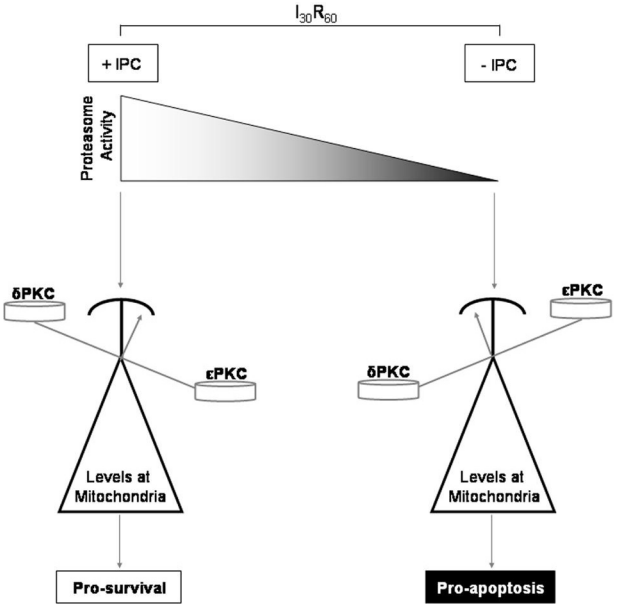
Reperfusion of blood flow to ischemic myocardium is associated with increased necrosis. Short bouts of ischemia before prolonged ischemia (ischemic preconditioning, IPC) can protect from reperfusion injury. Protein kinase-c (PKC) is a family of serine/threonine kinases that play important roles in many cellular processes including cardiomyocyte viability. Mitochondrial translocation of δPKC during cardiac reperfusion impairs mitochondrial function and initiates apoptosis. Conversely, activation of εPKC prior to ischemia maintains mitochondrial func-

tion resulting in reduced injury. Little is known about what regulates the opposing roles of these two isozymes in the pathogenesis of damage during ischemia/reperfusion (I/R). Therefore we wanted to determine the spatial and temporal relationships between δPKC and εPKC in the context of I/R and IPC to better understand their roles in cardioprotection. Using an ex vivo rat model of I/R, we determined by Western blot that IPC prior to ischemia diminished δPKC and enhanced εPKC translocation to mitochondria during reperfusion. Inhibition of εPKC with a peptide inhibitor reversed this and blocked IPC-mediated protection resulting in increased necrosis. Total levels of δPKC were decreased in response to IPC, whereas the content of εPKC did not change. Interestingly, ischemia induced a decline in proteasome activity that was protected by IPC. To better understand how cellular levels of δPKC were regulated, we blocked proteasome function using the inhibitor lactacystin. Inhibition of the proteasome prevented IPC-induced reduction of total δPKC and resulted in aberrant translocation to mitochondria with a concurrent loss in εPKC translocation. Importantly, inhibition of the proteasome prevented IPC mediated protection by restoring δPKC translocation, increasing cytochrome c release, decreasing pro-survival signaling, and increasing cellular oncosis, all of which resulted in increased tissue necrosis. Therefore, as shown in the figure, protection from I/R injury is in part dependent upon preservation of proteasome activity. Proteasome activity controls the ratio of δPKC and εPKC at mitochondria and thus anti- and pro-survival processes. Inhibition of the proteasome during ischemia tips the scale, favoring accumulation of δPKC at mitochondria resulting in the induction of apoptosis through cytochrome c release (black). In contrast, IPC prior to ischemia protects proteasome function increasing δPKC degradation allowing for εPKC accumulation at mitochondria and protection (white). Regulation of proteasome activity to control the ratios of two opposing PKCs represents a novel way to control cellular viability.



Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA104

Inhibition of vasopressin neuron activity by dendritic dynorphin release

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Hypothalamic vasopressin neurons fire action potentials (spikes) in alternating periods of activity (phasic bursts) and silence to optimize the release of vasopressin (the anti-diuretic hormone) from the posterior pituitary gland. During phasic bursts, spikes are superimposed on plateau potentials, generated by summation of post-spike depolarizing after-potentials (DAPs).

Using extracellular single unit recording in vivo, we have shown that kappa-opioid receptor antagonist administration increases the activity of vasopressin neurons by increasing the firing rate during, and the duration of, the active periods (1). However, kappa-opioid receptor antagonists do not alter the firing rate of vasopressin neurons at the beginning of bursts, but rather the increases in activity emerge as bursts progress, indicating that there must be a progressive endogenous activation of kappa-opioid receptor mechanisms during bursts (2). By contrast to the effects of kappa-opioid receptor blockade, V1 vasopressin receptor antagonist administration increases firing rate throughout bursts in anaesthetised rats, indicating that the actions of endogenous vasopressin are generally inhibitory and tonically present (2). The kappa-opioid peptide, dynorphin, is co-packaged in vasopressin neurosecretory vesicles and exocytosed from vasopressin cell dendrites during periods of activity; our data indicate that this dendritically released dynorphin feeds back to inhibit vasopressin neurons, terminating activity.

Using intracellular sharp electrode recording in vitro, we have demonstrated that DAPs are subject to activity dependent inhibition and that this inhibition is prevented by neurosecretory vesicle depletion or kappa-opioid receptor antagonist administration, but not by oxytocin/vasopressin receptor antagonist administration or mu-opioid receptor antagonist administration, indicating that endogenous activation of kappa-opioid receptors is responsible for activity dependent inhibition of DAPs (3). We have also demonstrated that kappa-opioid receptor antagonist administration enhances plateau potential amplitude during spontaneous firing to increase post-spike excitability and firing rate (4). Overall, these data are consistent with hypothesis that dendritic dynorphin terminates firing in vasopressin neurons through activity dependent plateau potential inhibition (5).

During dehydration, vasopressin cells display a higher frequency of activity than normal, which should decrease the duration of active periods due to the feedback inhibition defined above. However, the active periods are actually prolonged during dehydration, indicating that dehydration might induce adaptations in vasopressin neurons that reduce, and/or overcome, the effects of activity dependent feedback; we are currently investigating whether kappa-opioid receptor antagonism is less effective at increasing vasopressin neuron activity in dehydrated rats.

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Supported by The New Zealand Lotteries Health Board (CHB), The Wellcome Trust (CHB), The Medical Research Council (GL) and The Canadian Institutes of Health Research (CWB).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA105

Retrograde and autocrine synaptic modulation in the supraoptic nucleus

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The supraoptic nucleus (SON) is well known for its two major roles: 1) the control of blood pressure and water balance through it is synthesis of the neuropeptide, arginine vasopressin (AVP), and 2) the control of milk letdown and facilitation of parturition mediated by the related peptide, oxytocin (OXT). Magnocellular neurons (MCNs) of the SON send projections to the posterior pituitary where the peptide contents are released. In addition, there is now a large body of evidence indicating that these peptides are also released from dendrites of magnocellular neurons. We have used SON slices to investigate the role of AVP and OXT in synaptic transmission in the SON. Stimulation of afferent fibers to MCNs elicits excitatory of inhibitory post synaptic potentials (EPSPs, IPSPs), or, when cells are held under voltage clamp EPSCs or IPSCs. AVP appears to inhibit EPSCs impinging onto AVP neurons through an action at a post synaptic site, in keeping with the known location of AVP receptors on AVP neurons. In putative OXT neurons that bear OXT receptors, OXT enhances post synaptic AMPA receptors, again at a likely postsynaptic site. However, in putative AVP neurons, through analysis of paired pulse facilitation and miniature analysis, we also observed a reduction in EPSCs by OXT through an action at the presynaptic terminal; release of endogenous OXT from MCNs mimicked the effect of exogenous OXT.

MCNs, like many peptidergic neurons, also contain a variety of other transmitters and modulators. Thus we have investigated the possible action of these substances. Endogenous cannabinoids (CBs) are now known to be ubiquitously located through many brain structures and CB1 receptors can be localized to presynaptic terminals in the SON. When we applied CB receptor agonists to the SON, we observed a depression in EPSCs and IPSCs similar to what is seen with OXT. Interestingly, the depression of excitatory transmission than can be observed after depolarization of the postsynaptic cell body can be blocked by both CB1 receptor antagonists as well as OXT/AVP receptor antagonists. We believe that excitation of the MCNs causes release of OXT or AVP that, through autocrine actions of the peptides on MCNs, causes synthesis and release of endogenous CBs that act on presynaptic receptors to inhibit afferent transmission.

We have also studied other peptides and modulators found in SON MCNs- galanin, pituitary adenylate cyclase activating polypeptide (PACAP), nitric oxide and endothelin. The majority of these substances have potent presynaptic effects, but unlike those elicited by OXT, they do not appear to be mediated via secondary release of endogenous cannabinoids. With respect to galanin and endothelin, we have been unsuccessful in demonstrating that they are released from MCNs, and it appears that the likely source of peptide to influence EPSCs in the SON is from fibers extrinsic to the nucleus.

In summary, we are beginning to unravel the receptor location and local circuitry underlying the actions of a number of the neuromodulators and transmitters of the SON; however, little is known at this time of the overall role of these substances in the functioning of the SON.

Supported by CIHR.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA106

The role of intrahypothalamic release of oxytocin in the regulation of appetite

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Magnocellular neurons in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) secrete oxytocin not only from nerve terminals in the posterior pituitary, but also from their dendrites in the hypothalamus. These neurones are the major source of oxytocin in the brain as very large amounts of oxytocin can be released within the SON, and this can occur independently of release from nerve terminals. Because of the long half-life of peptides in the brain, it is likely that oxytocin released from neuronal dendrites within the hypothalamus can diffuse through the brain over considerable distances to reach some of the many different target sites that express high-affinity receptors for oxytocin(1). In the brain, oxytocin is involved in many behaviours, including sexual and feeding behaviours.

Research on appetite has progressed rapidly in the last ten years, but there is still little understanding of how appetite-related signals are processed in the brain in vivo. During feeding, peripheral signals that inform our brain about metabolic status are integrated by peptidergic neurones in the hypothalamus and brainstem. This information is relayed to "satiety centres", especially the ventromedial nucleus of the hypothalamus (VMH), and to the arcuate nucleus, where alpha-melanocyte-stimulating hormone (α-MSH), a potent inhibitor of food intake, is produced. The SON oxytocin cells are a target for α-MSH. We showed recently that α-MSH triggers Fos expression in SON oxytocin cells. However, surprisingly, α-MSH inhibits the electrical activity of oxytocin cells; this is mediated by endocannabinoids as CB1 cannabinoid receptor antagonist blocks the inhibitory effect

of α-MSH. Remarkably, although α-MSH inhibits oxytocin cells, it stimulates oxytocin release from dendrites, this release occurs because α-MSH triggers a rise in intracellular [Ca2+] by mobilising intracellular Ca2+ stores(2,3).
Centrally, both α-MSH and oxytocin inhibit food intake. It is possible therefore that the appetite-inhibiting effects of α-MSH are, at least in part, mediated by dendritically-released oxytocin. If so, a likely target for oxytocin is the VMH, which contains a high density of oxytocin receptors, and is a well-established "satiety centre". To investigate whether oxytocin affects the electrical activity of VMH neurones in vivo, we need to identify the various types of VMH neurones by their electrical characteristics, as there is very little known about the behaviour of these neurones in vivo. In current in vivo electrophysiological experiments, we expose the ventral surface of the brain by transpharyngeal surgery and make extracellular recordings of single VMH neurons. A cannula is inserted ventrally into the third ventricle to allow central injection of oxytocin. So far, of 150 spontaneously active VMH neurones recorded, most (about 80%) are within 6 clearly distinct subpopulations of VMH neurones according to their distinctive, spontaneous firing patterns, such as the mean firing rate, interspike-interval distribution. Each of these subpopulations is then further characterised by its responses to oxytocin injected centrally via the third ventricle, and also to appetite-related peptides such as leptin, cholecystokinin (CCK), and GHRP6 (Growth Hormone Releasing Peptide 6) injected intravenously. Surprisingly, CCK although it potently inhibits feeding, has an inhibitory effect on most VMH neurones while oxytocin seems to either activate, or inhibit, or has no effect, depending on the neuronal subtype recorded.

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My work is supported by the Royal Society of Edinburgh/Scottish Executive.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA107

Dendritic release of oxytocin within the brain: when, where and why?

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The brain oxytocin system has served as a distinguished model system in neuroendocrinology to study detailed mechanisms of dendritic release and its behavioural and neuroendocrine consequences. It has been shown that oxytocin is released within various brain regions. However, evidence for dendritic release has only been provided for the main sites of oxytocin synthesis, i.e. the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei.
In pregnancy and lactation, the brain oxytocin system is highly activated as indicated by an elevated level of neuronal oxytocin synthesis, neuropeptide release and receptor binding within selected brain regions. Thus, during parturition and in response to the suckling stimulus during lactation, oxytocin secretion into blood is accompanied by local oxytocin release within the hypothalamus. Moreover, exposure to various psychological or physical stressors triggers dendritic oxytocin release as monitored by intracerebral microdialysis within the SON and PVN during ongoing behavioural testing. The intracerebral release of the neuropeptide is not restricted to hypothalamic regions, as oxytocin release has also been found within the central amygdala and the septum in response to stressor exposure.
Such locally released oxytocin has been shown to be an important modulator of emotionality and hormonal stress responses, as it regulates active versus passive stress-coping strategies, and reduces the level of anxiety and the activity of the hypothalamo-pituitary-adrenal (HPA) axis probably by modulating the local release of excitatory amino acids. Oxytocin effects could be localized within the PVN and the amygdala. Moreover, in lactating dams, oxytocin specifically promotes maternal aggressive behaviour towards an intruder while it also reduces the level of anxiety-related behaviour peripartum. The high activity of the brain oxytocin system found in the peripartum period may therefore be an important factor attenuating the generell stress responsiveness found at this time. The effects of oxytocin are likely to be mediated via G-protein coupled receptors and the activation of MAP kinase pathways. Brain oxytocin exerts important anxiolytic, stress-protective and prosocial actions. Therefore, in order to develop novel therapeutic strategies, detailed knowledge on mechanisms of release and intracerebral actions of the endogenous neuropeptide is needed.

Supported by VolkswagenStiftung and Deutsche Forschungsgemeinschaft

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA108

Age-related loss of skeletal muscle mass and function: mechanisms of prevention by heat shock proteins

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Ageing is accompanied by a reduction in skeletal muscle mass and strength which leads to instability, increased risk of falls and an inability to perform everyday tasks. In addition, skeletal muscle of old mammals is more susceptible to exercise-induced damage and has a markedly impaired ability to fully regenerate following damage. The mechanisms underlying these age-related functional defects are unclear although several reports suggest that deficits may be associated with accumulation of oxidative damage. Muscles of young individuals can adapt to prevent damage following exercise. One of the major components of this adaptation is the increased production of heat shock proteins (HSPs). This induction of HSPs is impaired in muscles of old

rodents (Vasilaki et al, 2006). Studies from our laboratory have demonstrated that this inability to produce HSPs plays a role in the development of age-related functional deficits. Lifelong overexpression of HSP70 in skeletal muscles of mice resulted in protection against some aspects of exercise- induced muscle damage, but most strikingly, resulted in efficient and successful recovery of muscles of old mice following a severe damaging exercise (McArdle et al, 2004). Muscles of old wild type mice demonstrated biochemical changes consistent with increased oxidative damage and an inability to activate other redox-sensitive transcription factors such as NFkB and AP-1 (Broome et al, 2006). In contrast, these changes were not evident in muscles of old HSP70 overexpressors (Broome et al, 2006). Studies examining the effect of overexpression of other HSPs demonstrate cytoprotection against other aspects of contraction-induced damage, demonstrating the importance of different components of the stress response in cytoprotection. The mechanisms by which increased HSP content confers protection appears to be due to a reduced accumulation of oxidative damage and maintenance of the ability of muscles to activate other redox-responsive transcription factors. Further evidence for this comes from examination of muscles from SOD1 null mice which demonstrate aberrant activation of NFkB and an accelerated loss of muscle mass and function with age (Muller et al, 2006).

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The authors would like to thank Research into Ageing, the Wellcome Trust and the National Institute on Aging (PO1,AG20591) for funding this work.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C48

Ageing to arrhythmias: Changes in cardiac ionic regulation with age

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Age associates with increasing problems in cardiac function. Even healthy progressive ageing limits the heart's ability to respond to stress and is associated with increasing susceptibility to arrhythmias and myocardial dysfunction. This may be caused by altered regulation of intracellular calcium ions ($[Ca^{2+}]_i$). Exercise has been suggested as a way to reverse or limit the effects of ageing on the heart (Bronikowski *et al.* 2003).

This investigation sought to examine the effects of healthy progressive ageing on the mouse ventricle, focusing on changes in proteins regulating $[Ca^{2+}]_i$. Further investigations assessed whether acute or life-long exercise influenced changes in the response of the heart to ageing, beneficially or otherwise.

Mice (C57/Bl6) at 3 months & 24 months of age were randomly assigned to sedentary or exercise training groups. Exercise training was performed by treadmill running at 14 $m.min^{-1}$ for 15 minutes three times per week for a total of ten weeks. A further group of mice aged 12 months undertook this training protocol for 12 months. Immunohistochemical analysis was performed using fluorescently-labelled monoclonal antibodies to the L-type, D-type and T-type calcium channels and sodium-calcium exchanger (NCX), in conjunction with laser-scanning confocal microscopy.

Results are displayed below (Table 1). Ageing and exercise both induced hypertrophy. Ageing had no effect on the L-type and D-type calcium channel but significantly increased T-type calcium channel and NCX expression. Exercise in young mice significantly increased L-type channel and NCX expression, however, no change in D-type and T-type calcium channel isoforms were observed. Exercise in the elderly induced significant increases of all calcium channels and NCX compared with young and old sedentary controls. No differences were observed between acute and long-term responses to exercise in the elderly heart.

Exercise produces differential effects in the old and young heart but does not reverse the effects of ageing.

Table 1

	Young Sedentary	Young Trained	Old Sedentary	Old Trained	Old 12 Months Training
Cell Cross-sectional Area (μm^2)	1.00 ± 0.24	1.43 ± 0.41 #	1.31 ± 0.34 #	1.93 ± 0.49 # *	1.61 ± 0.41 #
L-Type Calcium Channel (per cell)	1.00 ± 0.28	1.48 ± 0.58 # *	0.85 ± 0.12	1.84 ± 0.22 # *	1.30 ± 0.35 # *
D-Type Calcium Channel (per cell)	1.00 ± 0.16	1.39 ± 0.05	1.17 ± 0.14	2.25 ± 0.48 # *	1.68 ± 0.35 # *
T-Type Calcium Channel (per cell)	1.00 ± 0.34	1.21 ± 0.25	1.31 ± 0.35 #	2.54 ± 0.50 # *	2.26 ± 0.45 # *
Sodium-Calcium Exchange (per cell)	1.00 ± 0.14	1.60 ± 0.21 #	1.55 ± 0.12 #	_	1.88 ± 0.31 # *

Mean +/- standard deviation of single-cell cross-sectional area and labelling for calcium regulatory proteins as expressed relative to the young sedentary control group. # indicates significantly different compared to the young sedentary control ($p<0.05$); * significantly different to the old sedentary group ($p<0.05$) by ANOVA.

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Funded by the BBSRC - SPARC initiative and Research into Ageing

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C49

Bone strength of the tibia and the radius in master runners, master walkers and sedentary people: a peripheral quantitative computer tomography study

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Introduction. Few studies have addressed whether above average bone mass and strength, as often observed in young physically-active people, persist with age in athletes [1]. Exercise interventions in elderly people yielded heterogeneous results [2, 3]. To investigate life-long running and walking against age-related loss in bone mass and strength [4], this cross-sectional study focuses on two questions: 1) Do bone mass and strength parameters differ between Master sprinters, middle and long distance runners, walkers and sedentary people? 2) Are bone mass and strength maintained in Master runners and walkers?
Methods. 158 male and 149 female Master athletes and 75 sedentary control people aged between 33 and 87 years were included into this study. Athletes participated at World or European Veteran Championships and achieved on average 82% of the predicted age and gender world record performance. Tomographic scans (XCT 2000, STRATEC, Germany) were taken at 4% and 60% of the ulna length and 4% and 38% of the tibia length. Data was analysed in terms of trabecular volumetric bone mineral density (tBMD; epiphysis), bone mineral content (BMC; epiphysis, diaphysis), bone area (A; epiphysis, diaphysis) and polar moment of resistance (RPol; diaphysis).
Results. Both genders showed evident group differences in diaphyseal BMC, A, and RPol within the weight-bearing tibia of runners, whilst tibia epiphysis and radius parameters were mostly similar. In the tibia diaphysis, data values followed a pattern of: Sprinters > middle distance runners > long distance runners > walkers > control participants. Group differences between sprinters and control participants amounted to 23%-26% in females and to 14%-17% in males ($P < 0.001$). 2) Age-related declines were mainly found within the females' tibia and radius shafts. Declines of approx. 0.5% per year applied to diaphyseal tibia BMC and A of all female athlete groups. Age-related changes of radius BMC and A were found within all female groups, except from long distance runners. However, enhanced tibia bone mass and strength in the athletes in younger age led to a higher or similar bone mass and strength in older age compared to control people. Conclusion. Loading of bones by running leads to higher bone mass and strength of Master athletes, especially in the weight-bearing tibia diaphysis compared to sedentary people. In men, this difference persists over the entire lifespan whilst it tends to diminish with increasing age in women. It is unclear at this stage whether the latter is directly related to the ageing process or to hormonal changes occurring after menopause.

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SA109

Exercise-induced cardioprotection against ischemia-reperfusion injury

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Myocardial ischemia-reperfusion (I-R) injury is the major contributor to the morbidity and mortality associated with coronary artery disease. Although several factors contribute to I-R-mediated myocardial injury, strong evidence indicates that production of reactive oxygen species and calcium overload are important mediators of this type of cardiac damage. This tutorial lecture will discuss recent experiments examining the effects of endurance exercise training in providing cardiac protection against I-R injury. In this regard, evidence indicates that both short term (days) and long-term (weeks) endurance exercise training significantly reduces I-R-induced myocardial injury. At present, the mechanism(s) responsible for exercise-induced cardioprotection remain debatable. Experiments investigating the role of exercise-induced increases in cardiac antioxidants and ATP-sensitive potassium channels in exercise-mediated cardioprotection will be discussed.

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Much of the research discussed during this session was supported by grants from the National Institutes of Health (HL072789 and HL067855) awarded to Scott K. Powers.

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SA110

An ageing problem of cardiac excitation-contraction coupling: But is this a male-only concern?

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Mammalian hearts show important age-related deficits in contractile function, in particular when heart rates are rapid (Lakatta, 2003). Previous studies have shown that these defects in contractile function also are present in ventricular myocytes isolated from ageing hearts (Xiao et al., 1994; Lim et al., 2000; Isenberg et al., 2003; Dibb et al., 2004). However, previous studies of excitation-contraction (EC)-coupling in ageing myocytes have been conducted in cells isolated from male animals only, female animals only or from animals where the sex has not been

specified. Thus, whether ageing affects cardiac EC-coupling differently in myocytes from male and female animals has not yet been investigated. The goals of this study were to characterize age-related alterations in EC-coupling in ventricular myocytes and to investigate whether these alterations were affected by the sex of the animal. Experiments utilized myocytes isolated from young adult (7 months) and aged (24 months) male and female B6SJLF1/J mice or young adult and aged male Fischer 344 rats. All studies were performed at 37 degrees Celsius. Voltage clamp experiments were conducted with high resistance microelectrodes in isolated ventricular myocytes. Cells were paced with a series of conditioning pulses delivered at a frequency of 2 Hz prior to test steps from -40 to 0 mV. Myocytes were loaded with fura-2 AM to measure intracellular calcium concentrations. Sarcoplasmic reticulum (SR) calcium content was assessed by rapid application of 10 mM caffeine. Unloaded cell shortening was measured with a video edge detector. Results showed that the amplitudes of fractional cell shortening and calcium current densities were significantly smaller in aged male myocytes when compared to cells from younger males (mean calcium current densities were -6.0 ± 0.4 vs. -3.2 ± 0.9 pA/pF for young adult and aged male mice; $p<0.05$; n=13/group; values were -8.2 ± 0.7 vs. -6.2 ± 0.4 pA/pF for young adult and aged male rats; $p<0.05$; n=16-23/group). In addition, calcium transients were significantly smaller and had slower rise times in aged male myocytes than in younger adult cells (mean calcium transient amplitudes were 167.4 ± 22.4 vs. 83.0 ± 23.2 nM for young adult and aged male mice; $p<0.05$; n=7-10/group). Similar results were obtained in myocytes from young adult and aged rats. Systolic and diastolic calcium concentrations also were significantly lower in aged male myocytes than in cells from younger animals. Interestingly, SR calcium content declined with age in male myocytes. However, the amount of calcium released per unit calcium current (an estimate of EC-coupling gain) was similar in young adult and aged male cells (values were 828.0 ± 180.9 vs. 715.2 ± 237.3 nM $sec^{-1}/pA/pF^{-1}$ for young adult and aged male mice; $p<0.05$; n=7-9/group). EC-coupling gain also was decreased significantly in aged male rat cells when compared to younger myocytes (values were 314.4 ± 62.9 vs. 178.8 ± 25.3 nM $sec^{-1}/pA/pF^{-1}$ for young adult and aged male rats; $p<0.05$; n=16-23/group). Furthermore, the fractional release of SR calcium was similar in young adult and aged male cells. These results show that there is an age-related decrease in cardiac contractile function in ventricular myocytes from male mice and rats. Results in myocytes obtained from female animals were markedly different. In contrast to results in male animals, fractional shortening and calcium current densities were similar in young adult and aged myocytes isolated from female hearts (mean calcium current densities were -4.6 ± 0.4 vs. -3.5 ± 0.5 pA/pF for young adult and aged female mice; n=11-20/group). Furthermore, calcium transient amplitudes and rates of rise were unaffected by age in female cells (mean calcium transient amplitudes were 140.7 ± 23.5 vs. 112.0 ± 16.2 nM for young adult and aged female mice; n=9-21/group). In addition, systolic and diastolic calcium concentrations were similar in young adult and aged female myocytes. Interestingly, SR calcium content actually was elevated in aged female myocytes compared to cells from younger animals and fractional SR calcium release declined with age in females. However, the gain of EC coupling was similar in myocytes from young adult and aged female mice (values were 859.6 ± 272.3 vs. 909.5 ± 145.4 nM $sec^{-1}/pA/pF^{-1}$ for young adult and aged females; n=9-21/group). These data demonstrate that age-related deficits in cardiac EC-coupling are prominent in ventricular myocytes from male hearts but not in cells from female hearts. Thus, age-related changes in cardiac EC-coupling in murine ventricular myocytes are influenced markedly by the sex of the animal. These observations suggest that it is important to consider sex as a variable in studies of the effects of ageing on cardiac EC-coupling.

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Canadian Institutes of Health Research

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA111

MEK partner 1 function in cell spreading and migration

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In the budding yeast, scaffolding proteins direct their assembled MAP kinase signalling modules to appropriate upstream activators and downstream targets to effect specific biological endpoints. Thus, the yeast MAP kinase module entrained by STE5 prepares cells for mating by promoting cell cycle arrest and by re-organizing actin structures necessary for schmooing. We recently have found that a putative mammalian MAP kinase scaffold, MEK partner 1 (MP1), controls the extracellular-signal regulated kinase (ERK) pathway and actin and focal adhesion organization in the context of cell adhesion and spreading. Independent lines of published evidence implicate ERK signalling and endocytosis in focal adhesion disassembly. By virtue of its localization to late endosomes and lysosomes, and its regulation of ERK cascade components, MP1 is ideally placed to integrate these requirements for cytoskeletal re-organization during cell morphogenesis. We will discuss possible mechanistic connections between MP1 and cytoskeletal regulators.

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SA112

Mapping and functional elucidation of partnerships involving signal scaffold proteins that tether cAMP phosphodiesterase-4

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It's becoming increasingly apparent that precisely 'where' processes occur within the 3-D matrix of the cell and 'who' the partners are, is crucial to allow for effective intracellular signalling. Delineating these fundamental processes will give insight into not only normal signalling function but also pathological changes and thus is likely to provide ways to generate novel therapeutics and diagnostics. In this arena, studies of cAMP signalling have provided important paradigms. Isoforms of the cAMP specific phosphodiesterase-4 (PDE4) family have provided the paradigm for compartmentalised cAMP signalling, with their N-terminal portions shown to contain motifs acting as 'zip codes' for precise intracellular targeting of specific isoforms so as to assemble complexes that then orchestrate the formation and shaping of intracellular 'clouds' and gradients of cAMP. Distinct PDE4 isoforms control output through major cAMP effector proteins by binding directly to the cAMP-stimulated GTP exchange factor, EPAC and by binding indirectly with protein kinase A (PKA) through interaction with various members of the PKA anchor protein family (AKAPs). Distinct PDE4 isoforms also exert spatially constrained actions by interacting with signal scaffold proteins such as beta-arrestin, RACK1 and DISC1. PDE4s can be regulated through direct interaction with proteins such as AIP/Ara9/XAP2 and indirectly, through interaction with kinases such as ERK, which cause their phosphorylation. PDE4s are regulated by post-translational modification, including multi-site phosphorylation by ERK, PKA and as a consequence of PI3 kinase activation. Dominant negative constructs and peptide displacers allow insight into spatially constrained functions os specific PDE4 isoforms and point to the generation of novel, isoform-specific therapeutics. In this regard PDE4 selective inhibitors have been shown to have potential for treating COPD, RA, asthma, septic shock, spinal cord injury, depression and schizophrenia.

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This work was supported by grants from the Medical Research Council (U.K.) (G8604010; 0600765), the Leducq Foundation (Paris) and by the European Union (037189).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA113

Ubiquitin signaling pathways

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Originally described as a destruction tag for misfolded or disused proteins ubiquitin (Ub) has recently entered centre stage in many fundamental processes like cell cycle, apoptosis, DNA repair or endocytosis. In these processes Ub acts as a signalling component able to trigger molecular events in cells. Ub does so by operating as a reversible and highly versatile regulatory signal for an expanding number of Ub-binding domains (UBD) present in cellular proteins that convey Ub signals into appropriate cellular phenotypes. In addition, it is becoming apparent that deregulation of Ub pathways results in the development of human diseases including many types of tumours. The common principles and specific features of Ub and other Ub-like modifiers and their binding domains in the regulation of signaling pathways will be discussed.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C50

A subset of GAF domains are evolutionarily conserved sodium sensors

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The ability of an organism or cell to maintain intracellular inorganic ion concentrations when challenged by extracellular fluctuations is among the most ancient and fundamental cellular processes. Despite the importance of inorganic ions in physiology, little is known of the molecular mechanisms by which the predominant inorganic ions of the cellular environment are detected. Here we describe work on the identification and characterization of an intramolecular signalling complex responsive to sodium ion.

The ubiquitous GAF domain is an important site of signal perception in eukaryotes and prokaryotes. GAF domains from diverse species have equally diverse ligands including NO in the NorR sensor of *Escherichia coli*, 2-oxoglutarate in NifA of *Azotobacter vinelandii*, and the cyclic nucleotides cAMP and cGMP in cyanobacteria, unicellular parasitic eukaryotes, and mammals. The mammalian cyclic nucleotide phosphodiesterases (PDE) are integral to the regulation of cellular levels of cAMP and cGMP by controlling the rate of degradation. Eleven families of PDE whose activity can be regulated by their N-termini are known. Of these regulatory domains PDEs types 2, 5, 6, 10, and 11 possess GAF domains regulated by cyclic nucleotides. The CyaB1

and CyaB2 adenylyl cyclases (AC) of the model cyanobacterium *Anabaena* PCC 7120 also bind cAMP through one (CyaB1) or two (CyaB2) N-terminal GAF domains to mediate positive feedback regulation of the AC domain.

Na^+ but not other monovalent cations regulates the function of CyaB1 by blocking cAMP mediated autoregulation. Mutation of the cAMP binding GAF domain of CyaB1 (GAF-B) blocks Na^+ regulation while mutation of GAF-A has no effect. A chimera of the GAF domain motif of CyaB2 with the AC domain of CyaB1 shows a similar response to Na^+. Na^+ bound recombinant GAF domains with substantially greater affinity than K^+. Exogenous cAMP blocked Na^+ binding to GAF domains but Na^+ had no effect on cAMP binding. Circular dichroism spectroscopy revealed that Na^+ maintains the domain in a conformation unable to signal and cAMP removes this constraint. Genetic ablation of the *cyaB1* and *cyaB2* genes gives strains defective in homeostasis at limiting Na^+ due to compromised Na^+/H^+ antiporter activity. To investigate whether Na+ regulation of GAF domain function is of more global relevance we investigated a chimera of the cGMP regulated GAF domain motif of mammalian PDE2 contiguous with the CyaB1 AC catalytic domain. This experiment revealed that Na^+ inhibition of AC specific activity was now dependent upon cGMP and that Na^+ regulation of GAF domain function has been conserved since the eukaryotic/bacterial divergence. The GAF domain is the first identified protein domain able to functionally sense and signal changes in environmental Na^+.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C51

Functional domains of the mouse β3-adrenoceptor associated with differential G-protein coupling

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Alternative splicing of the mouse β3-adrenoceptor (AR) produces two isoforms: the β3a-AR couples to Gs and the β3b-AR couples to both Gs and Gi (Hutchinson et al, 2002). To define the residues involved in this differential G protein coupling, truncated receptors were expressed in CHO-K1 cells and their properties examined. In these cells, maximal cAMP accumulation following stimulation with CL316243 was increased relative to control (control, 1.00±0.07; PTX, 2.20±0.22, n=5, P<0.0001) after pretreatment with pertussis toxin (PTX, 100ng ml-1). These results suggested that the β3a-AR is restrained from coupling to Gi by residues in the C-terminus. This view was supported by studies using a cell penetrating peptide transportan-10 (Tp10) to introduce peptides comprising the β3-AR C-terminal tail fragments into cells. Treatment with β3a-Tp10 caused the β3a-AR to become PTX sensitive, whilst the other peptides did not affect cAMP responses to stimulation of any receptor (Sato et al., 2005). Site directed mutagenesis was used to identify the residues in the C-terminus of the β3a-AR responsible for inhibition of coupling to Gi. Mutation of a putative caveolin binding site caused β3a-AR mediated cAMP accumulation to become PTX sensitive (control, 1.00±0.16; PTX, 1.75±0.14, n=4, P<0.0001). Furthermore, filipin III, an agent that disrupts lipid rafts such as caveolae, also caused the wild type β3a-AR to become PTX sensitive (control, 1.00±0.19; PTX, 1.56±0.04, n=4, P<0.0001), but in addition concentration-response curves to CL316243 were markedly shifted to the right (pEC50 control: 9.76±0.03, +filipin III: 8.73±0.21). To determine if this signalling mechanism had physiological significance we examined the effects of filipin III on mouse brown adipocytes that predominantly express β3a-AR (Chernogubova et al., 2005). CL316243 caused a concentration-dependent increase in cyclic AMP levels in primary brown adipocytes from FVB mice, that was unaffected by pre-treatment of PTX. Filipin III (1μg ml-1) significantly reduced the cAMP response to CL316243 (P<0.0001), indicating that disruption of caveolae limited the Gs/AC/cAMP pathway. Addition of PTX after filipin III treatment caused a significant increase of in the maximal cAMP response to CL316243 compared to control (P<0.05). The study suggests that β3a-ARs are restricted to caveolae and that localization of the receptor may play a specific role in G-protein mediated signalling.

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This work was supported by the National Health & Medical Research Council (NH&MRC) of Australia Project Grant #236884 (RJS). DSH is an NH&MRC CJ Martin Fellow, MS is a Monash University Research Scholar.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA114

Acetylcholine binding proteins: structural models of the extracellular domain of the nicotinic receptors

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Nicotinic acetylcholine receptors (nAChRs) are members of the ligand-gated ion channel (LGIC) family that mediate and/or modulate synaptic signaling. They are members of the pharmaceutically important subfamily of pentameric ligand gated ion channels that includes GABAA, GABAC, 5HT3 serotonin, and glycine receptors. nAChRs play important roles in memory and learning processes and receptor alteration is thought to contribute to diseases including schizophrenia, Alzheimers disease, drug addiction. Moreover they play a determinant role in the autoimmune disease myasthenia gravis and have been associated to nocturnal frontal lobe epilepsy. nAChRs are the prime mediators of nicotine addiction in tobacco smokers. Because nAChRs have prominent roles in disease of the nervous system, they have become major targets in drug discovery programs.

nAChRs exist in subtypes with distinct physiological and pharmacology properties. The lack of detailed structural information about these receptors has hampered rational drug design. Structural information about the ligand-binding domains and the subunit interfaces has expanded upon discovery and crystallization of the water-soluble homologue of the ligand-binding domain of nicotinic receptors, the acetylcholine binding protein (AChBP)(1,2). The crystal structure of AChBP has become an established model for the extracellular domain of the pentameric LGICs and homology models have been generated to analyze receptor-ligand interactions. AChBP has pharmacological properties resembling those of the homomeric alpha-7 subtype, with weak affinity for acetylcholine and a 10-fold higher affinity for nicotine. The ligand binding site of AChBP is characterized by the presence of aromatic and hydrophobic residues that are contributed by two adjacent subunits. The crystal structures of AChBP in complex with nicotine and carbamylcholine (3) have elucidated the molecular contacts between ligand and protein and are in excellent agreement with biochemical data obtained from nAChR binding studies. AChBP in complex with the nAChR agonists carbamylcholine and nicotine has revealed that both ligands bind at the same position and cause similar local conformational changes within the protein. These structures are useful tools for the development of new drugs for the nicotinic acetylcholine receptor and its family members.
In addition to this, we determined the 2.4 Å structure of α-Conotoxin PnIA (A10L D14K), a potent blocker of the α7 nAChR, which binds with high affinity to Aplysia californica AChBP (Ac-AChBP)(4). Alpha-Ctx is buried deep within the ligand-binding site and interacts with residues on both faces of adjacent subunits. The toxin itself does not change conformation, but displaces the C-loop of AChBP and induces a rigid-body subunit movement. Moreover we revealed the 2.2- Å crystal structure of Ac-AChBP in complex with alpha-conotoxin ImI (5). This toxin also forms interactions in the ligand-binding site that were not seen in the complex of Ac-AChBP with PnIA(A10L D14K). In contrast to ImI, conotoxin PnIA(A10L D14K) lacks binding selectivity to AChBP homologs. The knowledge of these toxin-AChBP contacts will advance rationalized design of ligands using the Ctx framework and may lead to compounds with increased receptor subtype selectivity.

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SA115

Molecular pharmacological characterisation of neuronal nicotinic acetylcholine receptors: targets for drug discovery and commercially important insecticides

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Nicotinic acetylcholine receptors (nAChRs) are excitatory neurotransmitter receptors with important roles in both vertebrate and invertebrate species. Mammalian and insect neuronal nAChRs have attracted considerable attention as target sites for drug and insecticide development. Human neuronal nAChRs have been implicated in several neurological disorders and, in addition, insect nAChRs are target sites for commercially important insecticides which are used extensively in animal health and crop protection applications. Nicotinic receptors display considerable heterogeneity in their subunit composition (17 different nAChRs subunits have been identified in vertebrates and about 10 subunits in insect species). Heterologous expression of nAChRs subunits cloned from mammalian and insect species has helped to establish the influence of nAChR subunit composition upon pharmacological and functional properties. A major goal for both drug discovery and insecticide development is the identification of subtype-selective compounds. We have employed radioligand binding and electrophysiological approaches to examine the pharmacological properties of recombinant nAChRs. The importance of subunit composition and the influence of subunit domains have been examined by the expression of hybrid receptors, artificial subunit chimeras and by site-directed mutagensis. These approaches are currently being used to characterise a variety of nicotinic agonists, antagonists and potentiators. For example, recent studies of mammalian nAChRs have identified amino-acids important in determining subtype-selective and species-selective potency of novel nAChR agonists and revealed their importance in the coupling of binding to channel gating. Neonicotinoid insecticides, which act on insect nAChRs, have been used extensively for crop protection and animal health applications since their introduction in the early 1990s. Compared to some other insecticide groups, resistance to neonicotinoids has been slow to develop but is now established in field populations of certain insect pests. We have reported recently the identification of a resistance-associated target-site mutation (Y151S) within two nicotinic acetylcholine receptor (nAChR) subunits (Nlα1 and Nlα3) from the brown planthopper Nilaparvata lugans, a major rice pest in many parts of Asia. The influence of this mutation upon the functional properties of recombinant nAChRs has been examined by heterologous expression in Xenopus oocytes and a cultured Drosophila cell line. The Y151S mutation was found to have no significant effect on the maximal current observed with the endogenous nAChR agonist acetylcholine. In contrast, a significant reduction in maximal current and a rightward shift in agonist dose-response curves was observed for all neonicotinoid compounds.

Interestingly, substantial differences were observed in the magnitude of these effects with different neonicotinoid compounds examined.

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Funding provided by the BBSRC, MRC and Wellcome Trust

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA116

Nicotinic receptors control dopamine-mediated locomotor behaviors

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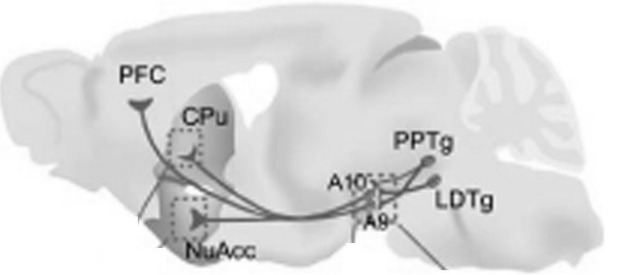
The neurotransmitter dopamine is involved in a broad range of brain functions, including motor activity, cognition and reinforcement. Midbrain dopaminergic (DA) ascending pathways have been classically divided into two major tracts: i) The nigrostriatal pathway projects from the Substantia Nigra pars compacta (SNpc; A9 cell group) to the dorsal striatum and is primarily involved in the regulation of motor activity; its degeneration in humans leads to Parkinson's Disease (PD). ii) The meso-corticolimbic tract projects from the Ventral Tegmental Area (VTA; A10) to the Nucleus Accumbens (NuAcc) of the ventral striatum, limbic areas and prefrontal cortex (PFC) and is mainly implicated in cognition, reward-based learning and addiction. Over the past 40 years the DA system has been the focus of intense research, particularly due to its relationship with PD and drug addiction. Today, the basic aspects of DA control over the basal ganglia are well understood, however, little information is available on the regulation of DA neurons by various other neurotransmitters acting on the midbrain DA nuclei and their striatal projections.

Recent evidence suggests an important role of acetylcholine (ACh) in the regulation of DA activity. The cholinergic afferences from the Pedunculopontine Tegmental Nucleus (PPTg) and the Laterodorsal Pontine Tegmental Nucleus (LDTg) innervate the SNpc and VTA nuclei, respectively. Additionally, cholinergic interneurons in the ventral and dorsal striatum provide a further anatomical and functional basis for the interaction of ACh with DA pathways (see Fig. 1). Further evidence suggests that nicotinic ACh receptors containing the β2 subunit (β2*-nAChRs) are implicated in the cholinergic action over DA activity: β2*-nAChRs are present in the VTA and SNpc of all mammals, both in the soma of DA nuclei as well as in GABAergic interneurons, and also at the terminal of DA striatal projections. We have recently described the regulation of the firing pattern of DA neurons in vivo through somato-dendritic β2*-nAChRs in anaesthetized mice (1). In addition, striatal cholinergic interneurons are known to exert a local control over DA release, mainly through the activation of presynaptic β2*-nAChRs in DA terminals.

Previous studies have shown the involvement of β2*-nAChRs in various types of DA mediated behaviors including those associated with the reinforcing properties of nicotine originating from their activity within the VTA (2). However, a main question that remains to be addressed is the direct participation of β2*-nAChRs in the control of DA-mediated spontaneous locomotor activity.

Mice lacking functional β2*-nAChRs (β2KO) show a striking hyperactive phenotype, displaying enhanced navigation and decreased exploration in the open field(3), evidence of a deficit in behavioral flexibility. Our hypothesis is that this feature reflects an imbalance of DA neurotransmission at meso-corticolimbic and/or nigrostriatal pathways. Given the classical view of the anatomical and functional dissociation of the two major ascending DA pathways, we tested the specific contribution of endogenous ACh via β2*-nAChRs to the locomotor behaviors regulated by each of these systems in vivo.

To this end, we performed a targeted lentiviral genetic rescue of functional β2*-nAChRs in the SNpc or the VTA of β2KO mice and quantified the behavioral outcome of this selective restoration.



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M.E.A. acknowledges financial support from Fondation pour la Recherche Médicale and Région Ile-de-France. This work was supported by Institut Pasteur, CNRS URA 2182, Collège de France, ARC and the French National Science Foundation grant ANR 'Neuroscience, Neurologie et Psychiatrie 2005' to U.M.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA117

Presynaptic nicotinic receptors and striatal dopamine neurotransmission: a dynamic and endogenous cholinergic filter

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Mesostriatal dopamine neurons and striatal cholinergic interneurons (tonically active neurons, TANs) participate in signalling the behavioural or reward-related significance of stimuli in the environment. Both populations of neurons signal reward-related

events by briefly modifying their firing frequencies in a synchronous but opposite manner. Furthermore, some form of antagonistic balance between dopamine and acetylcholine (ACh) is well known to regulate postsynaptic signal integration in striatum. Recent findings have revealed direct presynaptic ACh/dopamine interactions via nicotinic receptors that can play a key role in governing striatal dopamine signalling and that suggest that these synchronous changes in neuron activities may have key physiological implications.

Striatal ACh appears to operate a powerful but complex neuromodulatory control over dopamine release probability via striatal nicotinic acetylcholine receptors (nAChRs) on dopamine axons. Tonic levels of striatal ACh at β2-subunit-containing (β2*)-nAChRs promote dopamine release by individual action potentials but, owing to accompanying short-term depression, minimize the subsequent release of dopamine by multiple action potentials in a high-frequency (or 'reward-related') burst (Rice and Cragg, 2004). Nicotine, at concentrations seen in smokers, desensitizes tonically active β2*-nAChRs and thus can reduce initial dopamine release probability but also consequently facilitate release by bursts thus enhancing the contrast in dopamine signals by high frequency, burst activity. In conjunction with the excitation of midbrain dopamine neurons, this filtering action offers a mechanism through which nicotine may promote how burst activity in dopamine neurons facilitates goal-directed behaviour and reinforcement processing.

Here, using data obtained by monitoring action potential-dependent dopamine release in real-time using fast-scan cyclic voltammetry at carbon-fibre microelectrodes in rodent striatal slices, we consider the role of pauses in ACh interneuron activity on the presynaptic filtering of dopamine release by nicotinic receptors. We have explored the role of the different nAChR subtypes formed by the molecular subunit diversity expressed by dopamine neurons.

Reductions in striatal ACh tone at nAChRs that might accompany pauses in TAN activity powerfully polarize how opposing dopamine neuron activities are transduced into dopamine release. Like nicotine, blockade of ACh tone at nAChRs powerfully enhances dopamine signals offered by reward-related bursts and diminishes dopamine signals released by tonic activity or pauses in dopamine neurons. These data suggest that reward-related dopamine signals could therefore be enhanced by the concomitant pause activity in TANs. Like nicotine, TAN pauses could powerfully enhance the contrast, or salience, of dopamine signals offered by reward-related bursts, and even by reward omission-related pauses, in dopamine neurons. Exploration of the nAChR subtypes responsible for this presynaptic filtering of dopamine release probability suggest key roles for α6β2 and for non-α6, β2-subunit containing nAChRs in this presynaptic filtering of dopamine release probability by endogenous ACh.

Together these data reveal that endogenous ACh, acting at α6β2 and/or non-α6, β2-subunit containing nAChRs exerts a powerful presynaptic filter on dopamine signalling and that the concomitant changes in dopamine and ACh neuron activity that accompany reward-related information could act in concert to promote dopamine signalling (Cragg 2006). These functionally cooperative TAN-dopamine interactions within striatum may shed light on pharmacotherapies for addiction and parkinsonian disorders.

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Michael J. Fox Foundation for Parkinson's Research, Parkinson's Disease Society, Paton Fellowship

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C52

α7- and non-α7-containing nicotinic acetylcholine receptors modulate dopamine release in rat medial prefrontal cortex

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The mesocortical dopamine (DA) system within the prefrontal cortex (PFC) is implicated in the regulation of cognitive processing like attention and working memory (Jones, 2002). Hypo-DA activity within the medial PFC (mPFC) contributes to the major symptoms of schizophrenia, including deficits in sensory gating, attention and working memory (Castner *et al.* 2004). Nicotine improves the attentional processes associated with working memory in laboratory animals (Levin *et al.* 1998) and in schizophrenic patients (Harris *et al.* 2004), possibly by facilitating DA release. Accordingly, the modulation of DA release by nicotinic acetylcholine receptors (nAChR) has been demonstrated in various brain regions, including the accumbens and striatum (Marshall *et al.* 1997). The present study characterized nAChR-mediated DA release in the mPFC using *in vivo* microdialysis in freely moving rats.

Adult male Sprague-Dawley rats (300-320 g) were anaesthetized with ketamine (75 mg/kg, i.p.) and medetomidine (0.5 mg/kg, i.p.) and implanted with unilateral microdialysis probes (AN69, 4 mm active membrane) into the mPFC using standard stereotaxic surgery procedures. Buprenorphine (0.05 mg/kg, s.c.) was administered as analgesic and anaesthesia reversed with atipamezole (1 mg/kg, s.c.). After 24 h, the probes were perfused with artificial cerebrospinal fluid (aCSF, 2 μl/min) and 15 min fractions were collected and analyzed for DA by HPLC–amperometry.

Local perfusion of nicotine produced a concentration-dependent increase in extracellular DA levels which was blocked by the nonspecific nAChR antagonist chlorisondamine (CHL) given by continuous local infusion (100 μM), or systemic administration (10mg/kg, i.p.). Nicotine-evoked DA release was partially attenuated by continuous local infusion of the β2*-selective nAChR antagonist dihydro-beta-erythroidine (DHβE, 10 μM). The involvement of α4β2* nAChR was supported by the ability of the more selective agonist 5-IA-85380 to increase extracellular DA levels in mPFC. This response was completely abolished by continuous local infusion of DHβE (10 μM). Local infusion of the specific α7 nAChR agonist Compound A also facilitated DA release within the mPFC concentration dependently. This effect was attenuated by the α7-specific antagonist methylcaconitine (MLA) administered locally (100 μM) or systemically (3mg/kg, i.p.). Further the α7 nAChR allosteric modulator PNU-120596 (10 μM) on co-infusion with Compound A, potentiated its DA facilitating effect. PNU-120596 alone on systemic (1mg/kg, s.c.) but not local administration (10 μM) produced DA release within mPFC. This suggests tonic cholinergic control of DA release in mPFC by extra cortical α7 receptors.

These data demonstrate that local α7 and β2* nAChR within the mPFC can facilitate DA release.

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Supported by BBSCRC grant BBS/B/15600. Compound A and PNU-120596 were provided by Dr J. Kew, GSK, Harlow, UK.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA118

Tuberculosis: evolution in millennia and minutes

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Tuberculosis is one of the most important infectious threats to human health today with more than a third of the population infected. The organism has co-evolved with humans for more than 10 millennia with periodic epidemics. Throughout this time the organism has undergone deletions of significant parts of its genome allowing us to plot the divergence of important subspecies such as *M. africanum* and *M. bovis*. It may be that, as in the case of other pathogens, this has increased the pathogenicity of the organism. Moreover, the impact of other pathogens, notably the human immunodeficiency virus, has resulted in an upsurge in the number and seriousness of cases as it increases the likelihood of reactivation of latent disease.

Recent advances in molecular techniques such as insertion sequence, mycobacterial intergenic repeat unit (MIRU) and spoligo-typing have enabled us to plot the evolution of *M. tuberculosis* over shorter periods. Using these techniques lineages of related organisms can be identified and followed in human populations. Thus we are able to identify lineages with enhanced pathogenicity and the ability to develop antibiotic resistance.

The recent description of extensively resistant *M. tuberculosis* (XDRTB) has once again thrown the spotlight on the importance of antibiotic resistance. *M. tuberculosis* becomes resistant to antibiotics due to point mutations in the chromosome. For each of the different antibiotics mutations have been described. Rifampicin resistance provides a model system which demonstrates that each mutation is associated with a different reduction in the Darwinian fitness of the organism. The situation becomes more complex when multiple resistance determinants are considered. There is evidence, that, as strains are transmitted between individuals that the fitness deficit is ameliorated as the organism adapts to the new host. This provides an intriguing insight on micro-evolution over very short time frames.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA119

Prospects for new tuberculosis drugs

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Tuberculosis (TB) is one of the oldest infectious diseases known to man and has infected one third of the world's population. As a result, someone dies from the disease every 15 seconds and 30 million more people will lose their lives to TB in the next decade. Although directly observed short course chemotherapy (DOTS) is available to treat the disease, this treatment is old, slow and inefficient by the current standards of the pharmaceutical industry. In recent years, with increased public and private funding, some of the most innovative approaches have been used to identify and validate targets for new drugs, and to implement the screening and medicinal chemistry processes required to identify lead compounds for the generation of candidate drugs.

New TB drugs should have the following desired properties:

1) High potency to reduce treatment duration,
2) Activity against persistent bacilli,
3) Inhibition of new target classes,
4) Activity against multidrug resistant TB,
5) Specificity for Mycobacterium tuberculosis.

Among the validated targets that we are pursuing in the NM4TB project, as part of the European Commission's 6th Framework programme, are several enzymes involved in highly druggable areas such as cell wall biogenesis, nucleic acid synthesis and central metabolic pathways for which assays amenable to high-throughput screening are available. Intensive efforts are being focused on rapidly emerging targets that impact upon two as-yet untouched areas of the physiology of M. tuberculosis namely signal transduction pathways and persistence. Details of the former will be presented.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA120

Probing host-pathogen interactions, persistence and drug tolerance in *Mycobacterium tuberculosis* using microarrays

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Mycobacterium tuberculosis is the causative agent of tuberculosis, a disease with a global impact of >8 million new cases and 2 million deaths each year. *M.tuberculosis* also latently infects a third of the world's population and can reactivate to cause disease at 5% life-time risk or 10% per year for HIV co-infections. Treatment requires prolonged antibiotic therapy for 6 months and treatment failures are leading to the emergence of multiple and extreme drug resistance, threatening the world with untreatable TB. Genome sequence information reveals much about the biology of this pathogen and its metabolic potential, including a complex regulatory potential for gene expression control which implies pathogenicity is driven by differential gene expression in response to multiple and changing environments within the host.

Post-genomic technologies such as whole genome DNA microarrays have been used to measure changes in transcriptional responses of *M.tuberculosis* to a range of different environments, such as low oxygen, low iron or exposure to drugs. Expression profiles of mRNA at a whole genome level are called the Transcriptome and can be used to reveal the nature of the environment of the bacterium as well as reveal the physiological state of the bacteria. Studying the *M.tuberculosis* transcriptome during association with the host and in models of intracellular infection such as macrophage cultures [1], reveals how *M.tuberculosis* interacts with the host and responds to in vivo signals. Applying these powerful technologies to models of infection and naturally infected tissue is the next challenge and microarrays are now being used to reveal the metabolic process that underpins the clinically and biologically important state of latency. Advances in molecular biology that allow amplification of mRNA populations have been linked with microarray analysis to derive the transcriptome from low numbers of bacilli likely to be found using in vivo tissue or samples. Considerations of mRNA stability and differential harvesting are also important in order to allow in vivo transcriptome profiling. Slow growing or persistent organisms in natural infections are drug tolerant and subpopulations of bacilli in pulmonary tuberculosis account for the prolonged 6 month antibiotic therapy regimes. Identifying metabolic processes associated with drug tolerant persister populations in human sputum samples may therefore allow drug target identification for new, more rapidly sterilising antibiotics. Microarrays therefore provide an important experimental approach to dissect out the host-pathogen interactions in tuberculosis that hopefully will facilitate the quest for better drugs and vaccines for the treatment and prevention of tuberculosis.

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Some work presented was supported by EU Sixth Framework Programme Priority (LHP-CT-2004-012187). I acknowledge the Wellcome Trust and its Functional Genomics Resources Initiative for funding the multi-collaborative microbial pathogen microarray facility at St George's (BμG@S)which provided the whole genome *M.tuberculosis* microarrays.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA121

Structure, function and biosynthesis of the Mycobacterium tuberculosis cell wall: The identification of new drug targets

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In spite of effective antibiotics to treat tuberculosis in the late 1950s and early 1960s we enter the new millennium with tuberculosis (TB) currently the leading cause of death from a single infectious agent, killing more than three million people worldwide each year. Thus, an understanding of drug-resistance mechanisms, the immunobiology of cell wall components to elucidate host-pathogen interactions and the discovery of new drug targets are now required for the treatment of TB. Above the plasma membrane is a classical chemotype IV peptidoglycan to which is attached the macromolecular structure, mycolyl-arabinogalactan via a unique diglycosylphosphoryl bridge (often referred to as the mAGP complex). Clearly, cell wall assembly is a profitable choice for the design of novel anti-TB agents. In addition, new targets within the complex may be identified as well as establishing the mode of action of existing agents, mechanisms of drug resistance and the immunomodulatory role of cell wall constituents. In summary, the presentation will discuss the assembly of the mAGP, it's associated lipids and the site of action of several major anti-TB drugs, along with the completion of the TB genome bringing forward a new era in TB research and focus for new drugs to combat multi-drug resistant TB.

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GSB acknowledges support in the form of a Personal Research Chair from Mr. James Bardrick, as a former Lister Institute-Jenner Research Fellow, the Medical Research Council (UK), and the Wellcome Trust.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA122

Overview of small animal PET imaging

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Small animal Positron Emission Tomography (PET) is being utilized in animal models of a wide variety of diseases including cancer, cardiac disease, pulmonary disease and neurological diseases. High sensitivity/high resolution small animal PET scanners are currently commercially available allowing imaging utilizing multiple radiopharmaceuticals and consecutive imaging

over relatively long time frames. Examples from the types of disease models listed above will be given. One of the major challenges in small animal PET imaging is the conversion of qualitative image data into quantitative parametric images. In order to accomplish this arterial time activity curves need to be measured and in the majority of cases tracer metabolism quantified. Accomplishing this as a major challenge particularly when utilizing mouse models of diseases. Approaches to quantify cardiac blood flow and metabolism will be presented to show how quantitative data can be obtained.

There is currently great interest in the application of nanoparticles as delivery vehicles both for diagnostic agents and therapeutic drugs. MicroPET imaging has played a role in the evaluation of the pharmacokinetics of these novel agents. Examples of this application will be presented. Radiopharmaceuticals utilized with small animal PET scanners range from small molecules to labeled peptides to labeled antibodies and to the nanoparticles discussed above. Examples of imaging of all of these types of agents will be presented.

The author acknowledges the assistance of the Washington University Small Animal Imaging Resource and the Small Animal Imaging Core of the Siteman Cancer Center. This work is supported by the Washington University Small Animal Imaging Resource Grant CA83060, Siteman Cancer Center Support Grant CA9184203 and Cyclotron Produced Isotopes in Biology and Medicine Grant HL13851.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA123

Imaging of peptide receptors using microPET

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Peptides and their interaction with receptors play a major role in normal physiology as well as in pathophysiology. One important group of these receptor systems is the seven-transmembrane G-protein coupled receptor (GPCR), targeted by about 50% of the currently used drugs. In addition, a number of novel peptide receptor systems predicted from the human genome and their cognate ligands have recently been identified. The aim of our work is to investigate if these peptides and their receptor subtypes play a role in the human cardiovascular system (Maguire & Davenport, 2005), and if so to quantify any changes associated with disease and to identify new targets for novel drugs.

Positron emission tomography (PET) is the only technique available to image and quantify receptors *in vivo* with high sensitivity. With the recent development of dedicated PET scanners for small animals such as the microPET, it is now possible to perform functional imaging in rodents with a spatial resolution sufficient to delineate discrete organs and their larger substructures.

In this presentation we will illustrate our approach to explore novel peptide receptors using PET with our recent work on the endothelin receptor system (Johnström *et al.* 2003, 2004, 2005, 2006). Endothelin-1 (ET-1) is a multifunctional peptide in humans and alteration in the ET receptor system has been linked to a number of vascular diseases. ET-1 is produced from its precursor peptide bigET-1 by the action of endothelin converting enzymes (ECE). Two different strategies are currently being pursued to prevent the vasoconstrictor actions of endothelin: selective receptor antagonists or blocking synthesis of the peptide by inhibition of ECE. For this purpose we have synthesised novel subtype selective PET radioligands as well as labelled the endogenous peptide ET-1 and its precursor peptide big ET-1 with ^{18}F and investigated these radioligands pharmacodynamic and pharmacokinetic properties *in vivo* in anaesthetised animals with microPET.

[^{18}F]-ET-1 is rapidly cleared from the circulation with a subsequent fast accumulation in ET receptor rich tissue such as lung, kidney and liver, consistent with receptor binding. The *in vivo* distribution correlated with the anatomical localisation of receptors detected *in vitro* using [^{125}I]-ET-1. However, the receptor density visualised in the heart was unexpectedly low compared with that predicted from the *in vitro* measurements. Uptake in lung and kidney was significantly blocked when the animal was pre-treated with the ET_B selective antagonist BQ788, confirming that the fast clearance from the circulation was ET_B receptor mediated. In contrast, radioactivity increased in the liver suggesting binding of increased levels of circulating ET-1 to ET_A receptors. Hence the ET_B receptor plays an important role as a clearing receptor removing ET-1 from the circulation and this mechanism may be beneficial in protecting the cardiovascular system from the detrimental effects caused by upregulated ET-1 in disease.

Infused [^{18}F]-big ET-1 showed a localised distribution in lung suggesting tissue specific conversion of big ET-1 to ET-1 and binding to ET_A receptors in the vasculature. This was confirmed by dynamic PET where pre-treatment with the ECE inhibitor phosphoramidon or the ET_A selective antagonist FR139317 reduced binding of ET-1 to ET_A receptors due to inhibition of ECE activity or ET_A receptor blockade. This demonstrate that tissue-specific conversion of big ET-1 in the vasculature may increase levels of circulating ET-1 and subsequent binding to ET_A receptors suggesting that inhibition of ECE activity will be beneficial in limiting the harmful effects of ET-1 in disease.

Our results clearly demonstrate that we can study ET receptor pharmacology *in vivo* in small animals using microPET. Thus we have the potential to obtain pharmacodynamic information of novel drugs, image animal models to monitor disease progression or effect of treatment to further clarify the significance of the ET receptor system in disease. Furthermore, they show the potential for studying other emerging orphan receptor systems facilitating the rapid translation of information from the human genome into function and into the clinic.

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This work was supported by grants from the British Heart Foundation, the Medical Research Council and for the microPET a JREI grant from HEFCE and Merck Sharp & Dohme, Ltd.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA124

Imaging animal models of addiction

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Much research suggests that certain personality traits predispose humans to drug abuse and addiction, including sensation- (or novelty-) seeking, risk-taking, impulsivity and antisocial conduct disorder. However, from studies of human drug addicts alone, it is difficult to determine whether co-morbid impulsivity and cognitive dysfunction pre-date the onset of drug use or emerge as a consequence of chronic drug use. Accumulating evidence from studies in animals suggests that distinct behavioural and physiological traits predict individual differences in drug-taking behaviour. Of these the best characterized is the HR (high responder) rat described by Piazza PV *et al.* (1989). *Science* 245, 1511-1513, which show high levels of locomotor activity in a novel environment and increased propensity to self-administer low doses of psychostimulant drugs such as d-amphetamine and cocaine. A key neural substrate underlying individual differences in drug vulnerability is thought to involve the brain dopamine (DA) systems, in particular the nigrostriatal, mesolimbic and mesocortical DA pathways innervating the dorsal striatum (caudate and putamen), nucleus accumbens and prefrontal cortex. A recent positron emission tomography (PET) study in non-human primates has indicated that low DA D2 receptor availability in the striatum inversely predicts subsequent levels of intravenous cocaine self-administration (Nader MA *et al.* (2006). *Nat Neurosci* **9**, 1050-1056). However, it is not clear how D2 receptor availability in the striatum relates to a specific behavioural endophenotype that confers vulnerability to drug addiction. This symposium considers the relevance of a spontaneously occurring form of impulsivity in outbred rats to intravenous cocaine self-administration and to underlying changes in striatal D2 receptor function, as measured by PET (see Dalley JW *et al. Science* Mar 2nd 2007). Rats were screened for trait high impulsivity (HI) using a 5-choice serial reaction time task (5-CSRTT) of sustained visual attention (Robbins TW 2002. *Psychopharmacology* 15 617-634). Impulsivity was defined as high levels of anticipatory responses made before the presentation of a food-predictive, brief light stimulus, a trait which is present in roughly 7% of subjects screened. We found that HI rats show a clear increase in their rate of intravenous cocaine self-administration as well as a vertical shift in the cocaine dose-response function compared with non-impulsive (NI) rats. We also found using PET and the selective high-affinity DA D2/3 receptor antagonist [18F]fallypride that D2/3 receptor availability is significantly reduced in the nucleus accumbens, but not dorsal striatum, of cocaine-naïve HI rats compared with cocaine-naïve NI rats. As there were no accompanying changes in DA release in the nucleus accumbens the most likely explanation for the significant reduction in D2/3 receptor availability in HI rats was a reduction in Bmax. In a final experiment, we investigated the consequences of intravenous cocaine self-administration on pre-trained 5-CSRTT performance. Intriguingly, prolonged exposure of HI rats to cocaine dramatically, and selectively, reduced impulsive responding on the 5-CSRTT. Overall, therefore, these findings expand on previous findings in abstinent human cocaine addicts (Volkow *et al.* (1993. *Synapse* 14, 169-177) by demonstrating that decreased D2 receptor availability in the striatum may be a pre-disposing neurobiological trait and not only a consequence of chronic cocaine exposure. They also highlight an important causal relationship between trait impulsivity and susceptibility to drug taking. Thus, these findings may be consistent with the hypothesis that stimulant drug addiction represents a transition from initial impulsivity mediated by DA dysfunction in the nucleus accumbens to the emergence of compulsive habitual drug responding mediated by the dorsal striatum.

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This research was supported by a joint award from the UK Medical Research Council (MRC) and the Wellcome Trust and by an MRC Pathfinder grant to JWD, BJE and TWR (G0401068). DEH was supported by the Wellcome Trust. ESJR holds an RCUK Academic Fellowship supported by the British Pharmacological Society Integrative Pharmacology Fund. We thank Merck, Sharp, & Dohme for originally donating the micro-PET scanner to Cambridge University.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA125

The quaternary structure of G protein-coupled receptors: A day in the life

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It is now generally accepted that rhodopsin-like, family A G protein-coupled receptors (GPCRs) possess quaternary structure and can form dimers or higher-order oligomers. It is less clear if these are constitutive interactions or whether their organisational structure can vary during their life history. By developing an endoplasmic reticulum (ER) trapping strategy, based on addition of the C-terminal 14 amino acids of the alpha2C-adrenoceptor to the C-terminal tail of other GPCRs that normally traffic successfully to the cell surface, we were able to demonstrate that such a modified form of the chemokine CXCR1 receptor became retained in the ER. This modified receptor acted as a 'dominant negative' and prevented cell surface delivery of wild type forms of both the CXCR1 and CXCR2 receptors. However, it was without effect on trafficking of alpha1-adrenoceptors, which do not interact with the CXCR1, (Wilson et al., 2005). Such observations indicate that GPCR-GPCR interactions already occur during protein synthesis/maturation and prior to cell surface delivery. alpha1-adrenoceptors also possess quaternary structure and mutations, selected based on likely protein-protein contact sites, determined by receptor fragment interaction mapping studies (Carrillo et al., 2004), that modulate the effectiveness of protein-protein interactions can result in incomplete receptor maturation and trapping of the complex in the ER/Golgi, even though bi-molecular fluorescence complementation (BiFC) studies indicate that protein-protein interactions are not ablated (Lopez-Gimenez et al., 2007). BiFC approaches can also be employed to show that wild type and ligand-rescued mutant alpha1-adrenoceptors traffic to the cell surface as dimers/oligomers. A wide range of studies have indicate that GPCR hetero-dimerisation can modulate the function and pharmacology of GPCRs. In part, this involves allosteric effects across the dimer interface, such that ligand that have no inherent affinity for a GPCR can regulate the function of the GPCR if it forms a hetero-dimer with a GPCR that binds the ligand in question (Ellis et al., 2006). Combinations of BiFC employing GPCRs that constitutive internalise, such as the alpha1-adrenoceptor, and fluorescent ligands that bind and co-internalise, have also demonstrated that at least certain GPCRs internalise from the cell surface into the cell as dimers/oligomers (Lopez-Gimenez et al., 2007). Such studies also indicate that GPCR dimers interact with beta-arrestins but it remained to be established if the recently reported dimerisation of beta-arrestins is integral to this process.

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These studies were supported by the Medical Research Council, the Biotechnology and Biosciences Research Council and the Wellcome Trust.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA126

Kinetic analysis of GPCR-mediated signalling events

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Signalling via G-protein-coupled receptors occurs in a temporally and spatially organized manner. However, conventional techniques offer very little resolution of this spatial and temporal patterning. In order to permit a better analysis of these processes, we have developed a series of fluorescent methods to record and image various steps in the sequence of signalling via G-protein-coupled receptors: ligand binding, receptor activation via their conformational change, receptor/G-protein interaction, G-protein activation (via subunit rearrangement), ion channel activation (by patch clamp analysis), and the generation of the second messenger cAMP.

Our data show that ligand binding is rate limiting at low ligand concentrations, and that it may occur as a biphasic process. At higher ligand concentrations, most receptors activate within about 50 ms, and receptor/G-protein- interaction occurs with the same time course, suggesting virtually instantaneous interaction of activated receptors with G-proteins. In contrast, activation of various types of G-proteins is about ten-fold slower, with time constants in the range of 500 ms. G-protein activation appears to by tightly linked to effector activation such as monitored by opening of the GIRK K-channel.

Increases in the concentrations of the second messenger cAMP occur over seconds to minutes. In most cells, cAMP appears to diffuse freely throughout the cytosol, enabling ubiquitous responses. However, in cardiomyocytes cAMP does not seem to diffuse freely, and cAMP signals caused by stimulation of beta2-adrenergic recepors appear to stay localized, whereas those initiated by beta1-receptor stimulation are more generalized. Simultaneous recording of different second messengers indicate complex interactions between the levels of cAMP, cGMP and calcium that can be mediated both by effects on the generation and on the degradation of these messengers.

Taken together, our studies show a differentiated pattern of signal propagation along signalling chains, and and a great complexity of the spatial and temporal characteristics of the signals transduced via these pathways. The physiological relevance of

such distinct patterns remains to be elucidated, but it is clear that they permit a much better encoding of biological messages than previously anticipated.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C53

Coupling of seven-transmembrane receptors with G15 protein challenges the paradigm of desensitization

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Desensitization of agonist-induced stimulation is considered a universal paradigm of seven-transmembrane (7TM) receptors. This concept is now challenged by our present findings showing that coupling with G_{15}, renders 7TM receptors resistant to β-arrestin-mediated desensitization.

G_{15} is a member of the $G_{q/11}$ subfamily of heterotrimeric G proteins that is known to couple with most 7TM receptors independently from their G protein coupling specificities. Accordingly, we show here that agonist stimulation of V2 vasopressin (V2R), δ-opioid (DOR), and β2-adrenergic (β2AR) receptors, individually co-expressed with the α-subunit of G_{15} ($G_{15}\alpha$ in transfected COS-7 cells, increased the intracellular accumulation of inositol phosphates (IPs). Moreover, the R137H mutant of V2R, that being constitutively desensitized causes nephrogenic diabetes insipidus in humans, re-gained functional coupling to phospholipase C-β when co-expressed with $G_{15}\alpha$ in transfected COS-7 cells while remaining unresponsive to agonist-induced accumulation of IPs or cAMP when co-expressed with $G_q\alpha$ or $G_s\alpha$, respectively. Furthermore, in COS-7 cells, $G_{15}\alpha$-mediated IP accumulation induced by agonist stimulation of wild type V2R, DOR, and β2AR was totally refractory to desensitization induced by over-expressed β-arrestin-2, whereas endogenous $G_q\alpha$-mediated stimulation of IPs by V2R was desensitized.

Whether refractoriness to desensitization would also be proven for endogenous 7TM receptors coupled with G_{15} a mechanism to generate sustained intracellular signals in certain physiological and pathological conditions would then be demonstrated.

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The work was supported by a FIRB grant (nr. RBIN04CKYN_002) from the Italian Ministry for University and Research to MP.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C54

Arrestin-3 interacts with specific Gα proteins and COPI, and regulates export of G protein-coupled receptors

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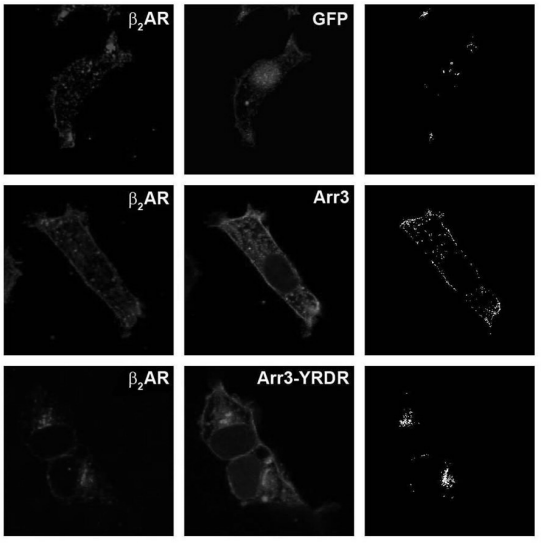
The role of arrestins in the endocytosis and signalling of G protein-coupled receptors (GPCRs) is well established. Here, we report a new role for arrestin-3 in the anterograde transport of GPCRs. Overexpression of arrestin-3 promoted, while arrestin-3 knock-down by siRNA inhibited, the cell surface expression of the β2-adrenergic, the thromboxane A2 and the angiotensin II type 1 receptors in HEK293 cells. Surprisingly, we show that arrestin-3 binds directly to Gαs and Gαq, but not to Gαi proteins. An arrestin-3 mutant (arrestin-3 YRDR) deficient in Gαs and Gαq binding strongly inhibited the cell surface expression of the GPCRs that we studied. Confocal microscopy revealed that arrestin-3 can localize at the Golgi where the arrestin-3 YRDR mutant retained the receptors. We also report that arrestin-3 interact with the COPI complex of proteins involved in Golgi vesicular transport. Interestingly, we observed that Gα proteins were co-immunoprecipitated with COPI, which was promoted by overexpression of arrestin-3. We propose a new model where arrestin-3 regulates the anterograde transport of GPCRs by forming a molecular complex with Gα proteins and COPI.



Arrestin-3 promotes cell surface targeting of the β2AR while the arrestin-3 YRDR mutant retains the receptor intracellularly. HEK293 cells were co-transfected with pcDNA3-HA-β2AR and either pEGFP-N1, pEGFP-N1-arrestin-3, or pEGFP-N1-arrestin-3 YRDR. The cells were processed for confocal microscopy. Co-localizing pixels were extracted by scatter plots and shown in the right panel.

This work was supported by a grant to J.L.P. from the Canadian Institutes for Health Research (CIHR). G.L received a studentship from the Heart & Stroke Foundation of Canada. A.P. holds a doctoral fellowship from NSERC. During this work, J.L.P. received salary support from a New Investigator Award from CIHR and from a "Chercheur-boursier Junior 2" scholarship from the Fonds de la Recherche en Santé du Québec.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA127

At the end of the day, receptors get tired too

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Following prolonged agonist activation, G protein-coupled receptors (GPCRs) become desensitised to prevent excessive stimulation. Desensitisation is usually triggered by receptor phosphorylation, either by G protein-coupled receptor kinases (GRKs) or by second messenger-dependent protein kinases (e.g. PKC). Following phosphorylation by GRKs, arrestins bind to the receptor, which uncouples the GPCR from its G protein[1]. Arrestins also target the receptor for internalisation via clathrin-coated pits, following which the receptor is either dephosphorylated and recycled back to the cell surface, or it is targeted to lysosomes for degradation.

We have used a combination of heterologous expression of receptor and analysis of endogenous receptors in mature neurones to identify the mechanisms underlying morphine-induced desensitisation of μ-opioid receptors (MOR). There is still much controversy about whether morphine actually induces significant MOR desensitisation in neurones, and by what mechanism, and also whether or not these molecular events bear any relationship to the important problem of morphine tolerance.

For our heterologous expression system, we have stably transfected HEK293 cells with the G protein-coupled inwardly rectifying K^+ (GIRK) channel subunits Kir3.1 and Kir3.2A and then transiently transfected them with MOR. The evoked current thus provides a real-time readout of receptor activation. Following addition of saturating concentrations of agonist, the evoked GIRK current reaches a peak and then declines (desensitises) in the continued presence of drug. The $t_{0.5}$ for both morphine and DAMGO (a peptide MOR agonist)-induced desensitisation was rapid at 1-2 min, and the extent of desensitisation was the same for the two agonists (~70%). However, the mechanism of desensitisation was different; DAMGO-induced desensitisation was blocked by a Dominant Negative Mutant construct of GRK2 (DNM GRK2), whilst morphine-induced desensitisation was unaffected by DNM GRK2, but was instead reduced by inhibitors of PKC[2]. Thus in a heterologous system, agonists at the same GPCR that induce the same cellular response can undergo desensitisation by different mechanisms.

Crucially, we have now shown that agonist-selective mechanisms of MOR desensitisation also occurs with endogenous MORs in mature neurones. Using rat brain slices containing locus coeruleus (LC) neurones, DAMGO induces rapid MOR desensitisation, whilst morphine can only do so ($t_{0.5}$ ~ 3 min) when PKC activity in the neurones is elevated, such as by co-activation of neuronal M2 muscarinic receptors with oxotremorine[3]. By injecting an adenoviral construct expressing DNM GRK2 into LC, we have shown that DAMGO-induced desensitisation in LC neurones is GRK-mediated, whilst the construct did not affect morphine plus PKC-mediated desensitisation. Importantly we have used selective membrane permeable RACK (Receptors for Activated C-Kinase) inhibitors of PKC to demonstrate that morphine-induced MOR desensitisation in LC neurones is mediated specifically by the PKCα isoform. We have also found that morphine plus PKC - induced desensitisation is absent in LC neurons from PKCα knockout mice. Thus in LC neurones PKCα mediates morphine desensitisation, whereas GRKs mediate DAMGO-induced desensitisation. It is likely that the agonists induce different conformations of MOR that, although able to couple to K^+ current activation, nevertheless trigger different mechanisms of desensitisation.

We have also developed a model for cellular tolerance to morphine, where prolonged (>6 h) exposure to morphine (30 μM) alone in LC neurons induces extensive MOR desensitisation (>80%), as quantified by operational analysis of functional receptor loss[4]. Again, PKC activity was essential to maintain morphine-induced MOR desensitisation because exposure to PKC inhibitors for only the last 30-50 min of a 6–9 h exposure to morphine reversed the MOR desensitisation. In contrast to morphine-induced desensitisation, both acute and prolonged MOR desensitisation induced by DAMGO was reduced by DNM GRK2 expression.

Since PKC inhibitors can reverse morphine tolerance *in vivo*[5], then it seems likely that PKC-mediated desensitisation of MORs underlies morphine tolerance. We are trying to identify the molecular mechanism(s) of PKC-mediated desensitisation of the morphine-activated MOR; initial studies indicate that PKC can phosphorylate GST fusion proteins expressing the 3rd i.c. loop and C-terminal tail of the receptor. Once the phosphorylated residues are identified, we can introduce point mutations into the intact MOR to assess their importance in morphine-induced MOR desensitisation and morphine tolerance.

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This work was supported by project grants from the Wellcome Trust.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA128

Mechanisms and consequences of GPCR sorting in the endocytic pathway

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Endocytic membrane trafficking represents a fundamental means for controlling receptor-mediated signal transduction in diverse

cell types. Our laboratory is focused on understanding mechanisms by which seven-transmembrane G protein-coupled receptors (GPCRs) are sorted between distinct, and functionally important, membrane pathways following ligand-induced endocytosis. The talk with summarize current understanding of (a) sorting of internalized GPCRs between lysosomal and recycling pathways, (b) receptor-mediated regulation of the endocytic machinery itself, and (c) functional consequences of GPCR sorting. Recent progress toward visualizing and analyzing specific receptor trafficking events in real time will be discussed, focusing on membrane traffic controlling the number and location of specific receptors on dendrites of neurons cultured from the central nervous system.

Many colleagues, both within the lab and elsewhere, have made important contributions relevant to our work. Research discussed was supported by the National Institutes of Health and the American Heart Association.

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SA129

Biochemistry and functional significance of collagen cross-linking

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The biophysical characteristics of vascular tissues are dependent to a large extent on the properties of their main structural components, fibrillar collagens. Stabilization of fibrillar collagens occurs initially through formation of difunctional, lysyl oxidase mediated cross-links whereby lysine or hydroxylysine residues in the non-helical portions of the molecule (telopeptides) are converted to aldehydes which then condense with hydroxylysine and other residues in neighbouring molecules to form intermolecular bonds. These borohydride reducible bonds are intermediates that are converted during maturation to trifunctional cross-links which include pyridinium, pyrrole and histidine-containing compounds [1]. Whether lysine or hydroxylysine residues are present in the telopeptides is crucial in directing the pathway of cross-link formation in a tissue-specific manner, so that complete hydroxylation of telopeptide lysine leads to pyridinium cross-link formation whereas collagen lacking telopeptide hydroxylysine forms histidine adducts of the difunctional bonds. The hydroxylation of telopeptide lysine is accomplished intracellularly by a separate lysyl hydroxylase enzyme to that which hydroxylates lysine residues destined to be in the helix. This enzyme, the long form of lysyl hydroxylase-2, is, therefore, the primary control for the pattern of lysyl oxidase-mediated cross-linking in collagen [2]. Functionally, the importance of these lysine-derived cross-links is well demonstrated by the effects of the lathyrogen, β-aminoproprionitrile, which, as an irreversible inhibitor of lysyl oxidase, results in the assembly of collagen fibrils lacking tensile strength because the pathways to cross-link formation are blocked. The differential effects of the various lysine-derived cross-links are not well established, although it is clear that the transformation from intermediate to mature forms is accompanied by increased inter-microfibrillar bonding, a feature that results in transverse arrays of cross-links across the fibril giving resistance to shear stresses.

A second type of cross-linking mechanism that may also involve collagen lysine residues, but which should not be confused with lysyl oxidase-mediated processes, is that involving glycation or lipid oxidation products. Glycation has been the focus of much research and the importance in vascular biology of Advanced Glycation Endproducts (AGEs) as well as their receptor is increasingly recognised. In contrast to the maturation of lysyl oxidase-mediated cross-links, formation of AGEs in collagen is a true ageing process that is generally deleterious to the function of the tissue. Another important difference for age-related cross-linking is that these bonds are formed between collagen helices and do not involve the telopeptides. This leads to major functional consequences in terms of tissue stiffness and increased resistance to enzymatic degradation. The major effect of inter-helical cross-links on enzyme degradation of collagen is exemplified in skin collagen where increasing concentrations of an inter-helical, histidine adduct cross-link is matched by increased resistance to solubilisation by pepsin [3]. The structure and biochemistry of the many AGE compounds described to date will not be discussed in detail as this is the topic of another presentation in the present symposium. In many cases, however, the amounts of specific forms of these compounds in collagen are too low to have any functional significance, and compounds such as pentosidine should be viewed as markers of an ageing process rather than themselves being significant cross-links. Concentrations of the lysine-arginine cross-link, glucosepane, do, however, approach those of the lysyl oxidase-mediated cross-links in skin collagen of the elderly [4].

The properties of cardiovascular tissue are dependent on many factors and, in addition to collagen cross-linking, it is important to be able to assess the contribution of different collagen types as well as measure the other principal constituents of the extracellular matrix, elastic tissue and proteoglycans. New techniques being developed to accomplish these aims will be outlined, including cross-link analyses by mass spectrometry using multiple reaction monitoring, a procedure that overcomes problems in measuring collagen cross-links using conventional methods caused by the presence of reduced elastin-derived components. The overall aim of these studies is to provide information on how changes in the extracellular matrix affect the properties of both normal and diseased cardiovascular tissues.

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Support of the Scottish Executive Environment and Rural Affairs Department is gratefully acknowledged.

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SA130

Cross-link breakers as a new therapeutic approach to cardiovascular disease

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The unique organization of collagen and elastin fibers in extracellular matrix of cardiovascular tissue provides the basis for its structural integrity and optimal function. Physiologically, collagen and elastin fibers are enzymatically cross-linked to form matrix. In addition, collagen fibers may be linked non-enzymatically, most notably by formation of advanced glycation end-products (AGEs). AGEs are formed by a reaction between reducing sugars and body proteins; they slowly accumulate during the lifespan, and their formation is accelerated in diabetes mellitus and hypertension. Importantly, AGEs adversely affect cardiovascular structure and function. Increased collagen cross-linking increases vascular and myocardial stiffness; this leads to elevated systolic and pulse pressures, impaired ventricular relaxation, and diastolic dysfunction. Furthermore, AGEs interact with receptors on endothelial and smooth muscle cells with consequent inflammatory reactions, release of growth factors and cytokines, and increased oxidative stress.

Recently, breakers of AGEs related cross-links have been discovered and the cardiovascular effects of one of them, Alagebrium (ALT-711) have been studied. In aged dogs, ALT-711 enhanced diastolic compliance, which was associated with improved diastolic filling and cardiac output (1). Studies in older monkeys demonstrated that ALT-711 improved both arterial and ventricular function, as evidenced by decreased pulse wave velocity and improved stroke volume index and fractional shortening (2). In another study, diabetes was induced with aloxan in 9-12 year old mongrel dogs and five months later one-half of the diabetic dogs received ALT-711, the other half received placebo (3). The results showed that induction of diabetes in aging dogs increased heart mass by 14%, decreased ejection fraction, increased myocardial collagen content, and increased aortic stiffness. Treatment with ALT-711 prevented the increase in heart mass, restored ejection fraction, and reduced aortic stiffness. Furthermore, the treatment also increased left ventricular collagen solubility and reduced collagen content. These results demonstrated that AGEs-related collagen cross-linking have a causative role in the development of cardiovascular complications of diabetes and aging. Two studies in diabetic rats also demonstrated that, in addition to reducing cardiovascular stiffness, beneficial effects of ALT-711 may be mediated by reduction of profibrotic growth factors and cytokines, as well as by diminishing oxidative stress. The beneficial effects of ALT-711 have also been shown clinically (4). In older human subjects with elevation of arterial pulse pressure and reduced arterial compliance at the initiation of the study, the 8-week treatment with ALT-711 lowered pulse pressure and improved arterial compliance.

We examined (5, unpublished results) the cardiovascular and renal effects of prolonged treatment (over 6 months) with ALT-711 in 40 weeks old spontaneously hypertensive rats. They were divided into two groups (60 rats in each) that were closely matched as to baseline urinary protein excretion and arterial pressure. One group received ALT-711 (1 mg/kg/day) whereas the other received vehicle by gastric gavage. When the SHR rats were 70 weeks old, 15 rats from each group were randomly selected for hemodynamic study. The remaining rats continued to receive their respective treatment until death. Urinary protein excretion increased with time (34.8±1.7; 58.5±2.3; and 95.5±2.6 mg/24h, at baseline and 3 and 6 months later, respectively) and this increase was attenuated (<0.05) in ALT-treated rats (36.1±1.6; 48.6±2.0; 68.2±2.4 mg/24h). Hemodynamic studies revealed no difference in arterial pressure, cardiac index, total peripheral resistance, and heart rate between the groups. Aortic distensibility was greater in ALT treated rats than in controls (1.12±0.08 microm/mmHg vs. 0.73±0.07, $p<0.05$) whereas pulse wave velocity was reduced in treated rats (435±13 cm/sec vs. 499±12, $p<0.05$); both findings suggesting reduced aortic stiffness with ALT-711. No differences in the left ventricular end-diastolic pressure and maximal rate of pressure rise (+dP/dT) between the groups was found. Two indices of left ventricular diastolic function, maximal rate of pressure fall (-dP/dT) (-7435±205 mmHg/sec in ALT-treated vs. -6254±254 in control, $p<0.05$) and diastolic time constant (15.5±0.4 msec in ALT-treated vs 17.5±0.4, $p<0.05$), improved in treated rats. Life-span was also extended in ALT-treated rats: control rats lived 84±2 weeks, treated 92±2 ($p<0.05$).

In conclusion, numerous experimental and few clinical studies demonstrate that agents that break AGEs-related collagen cross-links may be useful in the treatment of disorders associated with aging, diabetes and hypertension.

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C55

Collagen cross-link measurement as an indicator of human carotid artery atherosclerotic plaque vulnerability

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Atherosclerosis is a progressive vascular disease that is a major risk factor for acute myocardial infarction and stroke (Libby, 2002). An important determinant of atherosclerotic plaque vulnerability is the stability of the fibrous cap, afforded in part by the presence of collagen. The formation of intermolecular cross-links within newly formed collagen fibrils gives collagen its stability, and is essential for tissue function. Intermediate (difunctional) cross-links undergo spontaneous maturation, characterised by the production of trifunctional pyridinium compounds, pyridinoline (Pyd) (Robins & Duncan, 1983) and deoxypyridinoline (Dpd) (Ogawa et al., 1982). Therefore, the

level of mature collagen cross-links within the region of the fibrous cap may dictate the vulnerability of the plaque to rupture. Plaques were obtained from patients undergoing carotid endarterectomy (CEA). CEA samples were dissected out into healthy marginal tissue, plaque tissue and underlying media for biochemical analysis of collagen cross-links by HPLC. Portions of healthy marginal tissue and whole plaque were also used for histological determination of total collagen to cellular ratio by staining with Masson's trichrome. Detection of CD68 by IHC was used as a macrophage marker in plaque sections, and an arbitrary scoring system assigned. Total collagen and cross-link data is expressed as mean ± s.e.m and assessed for statistical significance using one-way ANOVA with Tukey's post-hoc test. The level of Pyd cross-links (n=12) contained within the plaque was significantly higher than that found in the underlying media ($p \leq 0.01$) and healthy tissue ($p \leq 0.01$). However, levels of Dpd (n=8) were considerably lower in the plaque than in the underlying media ($p \leq 0.01$). The ratio of total collagen to cellular material (n=16) was high in plaque and underlying media (1.3±0.04), and equal in healthy tissue (1.0±0.05). The highest incidence of macrophage frequency was found to be predominant in the subcap region of the underlying media (n=9). These results show for the first time that it is possible to measure mature collagen cross-links in human atherosclerotic artery samples, and that Dpd in the plaque is considerably lower than that measured in the underlying media. Therefore, levels of Dpd may be crucial to the stability of the plaque, serving as a marker of vulnerability. Future work will assess the levels of bifunctional cross-links prior to maturation to pyridinium compounds, which may account for reduced Dpd.

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C56

Hypoxic inhibition of human cardiac myofibroblast invasion and MMP-2 activation may impair adaptive myocardial remodelling

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Cardiac fibroblasts account for up to two-thirds of the total number of cells in the normal heart and are responsible for extracellular matrix homeostasis. In vitro type I collagen, the predominant myocardial collagen stimulates proteolytic activation of constitutively secreted proMMP-2 at the cell membrane and requires formation of a ternary complex with membrane-type metalloproteinase (MT1-MMP) and TIMP-2. Following myocardial infarction (MI) normally quiescent fibroblasts initiate a wound healing response by transforming into a proliferative and invasive myofibroblast phenotype. Myocardial oxygen deprivation is an inevitable consequence of MI, therefore this reparative event occurs under chronically hypoxic conditions. The aim of this study was to investigate the effect of hypoxia on fibroblast migration and invasion, and to investigate whether modulation of MMP-2 activation was involved.

Human cardiac myofibroblasts were cultured from biopsies of right atrial appendage from patients undergoing coronary artery bypass surgery. All experiments were performed on cells from at least 5 different patients in medium supplemented with 50 mg/ml Type I rat tail collagen under conditions of normoxia (21% O2) and hypoxia (1% O2) for 48 h. Invasion assays were performed using a modified Boyden chamber technique with Matrigel coated membranes and 2.5% FCS as chemoattractant. Invaded cells were quantified by counting H & E stained cell nuclei on the underside of each membrane under light microscopy. Migration was quantified using a scratch wound assay by making a uniform linear wound in a confluent monolayer of myofibroblasts. Quantification was performed from digital images of the wound before and after incubation. Cell supernatants were collected at the end of all experiments, and analysed by gelatin zymography to quantify pro and active MMP-2 secretion.

2.5% FCS significantly stimulated myofibroblast invasion, which did not occur in the absence of chemoattractant. Hypoxia (1% O2) significantly attenuated invasion by ~50% ($P<0.05$, paired t-test, n=7). The inhibition was accompanied by reduced MMP-2 activation in invasion assay supernatants of ~50% ($P<0.05$, paired ratio t-test, n=7). In contrast, in the wound scratch assays, despite observing a significant inhibition of MMP-2 activation ($P<0.01$, n=5), hypoxia had no effect on cell migration (P=NS, n=5).

In conclusion, the lack of effect of hypoxia on migration suggests that inhibition of invasion is mediated by inhibition of MMP-2 activation but not by modulation of fibroblast motility. Decreased activation of MMP-2 following hypoxia potentially impairs early, post-MI remodelling. Determining the exact mechanism by which this occurs may provide a target for therapeutic modulation.

Supported by the British Heart Foundation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA131

Matrix metalloproteinases and plaque stability

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The rupture of an atherosclerotic plaque with subsequent thrombotic lumenal occlusion is a primary cause of acute coronary events, accounting for 60% of sudden deaths. The present dogma proposes that inflammatory cells play a major role in the pathophysiology of both atherosclerotic plaque development and rupture. Entrapment of low-density lipoprotein (LDL) within the extracellular matrix (ECM) of the vessel wall results in its modification and oxidation, which instigates an inflammatory response. Leukocytes interact with numerous adhesion molecules, and various cytokines and growth factors secreted from endothelial cells and underlying vascular smooth muscle cells (VSMC), prompting their adhesion, transmigration and maturation. This inflammatory infiltrate triggers a series of events resulting in the formation of a complex atherosclerotic plaque. Such lesions contain a lipid/necrotic core that is highly thrombogenic but is protected from the circulating blood by the VSMC-rich, fibrillar collagen-rich fibrous cap which confers mechanical strength. Thus rupture of an atherosclerotic plaque exposes

the thrombogenic lipid core to the circulating blood, initiating thrombus formation that can rapidly occlude the artery, leading to clinical symptoms such as unstable angina, myocardial infarction and stroke.

Many of the multifactorial processes that participate to plaque development and destabilisation involve extracellular matrix turnover. Within the normal vessel wall extracellular matrix degradation is tightly regulated through a balance of proteinases and their endogenous inhibitors. However, within the atherosclerotic plaque the balance may become shifted towards matrix degradation, particularly at the rupture-prone shoulder region of the fibrous cap where accumulating inflammatory cells and phenotypically altered smooth muscle cells secrete a plethora of proteinases including matrix metalloproteinases (MMPs). Indeed, studies in human atherosclerotic plaques have suggested a decisive role for MMPs in plaque destabilisation. Similarly, in the atherosclerotic lesions that form in cholesterol-fed rabbits and mice, an increase in MMP secretion and activity paralleled the severity of atheroma formation observed in these models. Subsequently, numerous studies have used genetic manipulation, gene therapy and synthetic MMP inhibitors to dissect the roles MMPs may play during atherosclerotic plaque development and stability in hypercholesteroleamic animals. These studies have demonstrated that MMPs play both beneficial and detrimental roles during atherosclerosis, not just through there capacity to degrade extracellular proteins, but also non-matrix proteins that influence the behaviour of plaque cells such as smooth muscle cells and macrophages. For instance some MMPs can facilitate VSMC migration and subsequent fibrous cap formation whilst others MMPs aid macrophage plaque-infiltration and lipid-core formation, factors known to trigger plaque rupture.

The more we learn about the multifactorial events that underlie plaque instability, particularly with regards to the interactions MMPs have with surrounding cellular and extracellular components, the more complicated the whole process appears. For example, smooth muscle cell growth may be beneficial in unstable atherosclerotic plaques, promoting fibrous cap formation, but detrimental in stable lesions by increasing lesion size and stenosis. With this in mind, and considering that utilisation of broad-spectrum MMP inhibitors has additionally highlighted the dual role MMPs play, the generation of more selective MMP inhibitors is warranted, particularly if MMP inhibition is to be considered as a therapeutic target for atherosclerotic plaque stabilisation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA132

Mast cells, matrix degradation, and loss of atherosclerotic plaque stability

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Mast cells have been assigned a role in the development of atherosclerotic lesions and their clinical complications. The mast cells are multipotent effector cells which originate in the bone marrow, circulate as progenitor cells, and ultimately find their ways into various tissues. Like in other tissues, also in the inner layer of the arterial wall, the intima, the progenitor cells remain in the close vicinity of endothelial cells, and slowly differentiate into mature mast cells which are filled with cytoplasmic secretory granules. The granules contain histamine, heparin, and neutral serine proteases tryptase, chymase, and cathepsin G. In addition, the granules contain several growth factors such as bFGF, VEGF, and TGF-beta, and proinflammatory cytokines such as TNF-alpha. Once stimulated, the mast cells degranulate, and so exocytose the effector substances into the immediate surrounding of the activated mast cells.

Atherosclerotic lesions develop in the intima. Originally, blood-derived lipids accumulate within intimal macrophages, then the macrophages become converted into foam cells, and fatty streaks develop. Later, the fatty streaks develop into mature atherosclerotic plaques along two principal histopathological pathways: they develop either into inward-growing fibrotic lipid-poor lesions, or into outward-growing non-fibrotic lipid-rich lesions. The former ones cause significant stenosis and local turbulent flow conditions, while the latter ones do not cause such intraluminal changes. Irrespective of the mode of growth, the plaques show signs of inflammation, and may cause atherothrombotic complications, when the subendothelial thrombogenic surface becomes exposed to blood. As a result, an arterial platelet-rich thrombus develops, and may cause myocardial infarction or stroke.

The mechanisms of plaque disruption leading to atherothrombotic complications depend on the type of the plaque: a stenosis-causing fibrotic plaque usually erodes, while a non-stenotic lipid-rich plaque typically ruptures. What are the suggested mechanism by which intimal mast cells might contribute to the development of the above-described atherothrombotic events? Regarding erosion, activated subendothelial mast cells secrete proteases which degrade various components of the endothelial basement membrane, and so render the endothelial cells susceptible to physical detachment by the forces exerted by the local turbulent flow of blood. Proteolytic degradation of the pericellular matrix of the endothelial cells may also cause loss of the outside-in survival signaling, which, together with mast cell-derived TNF-alpha induces apoptotic death of the endothelial cells. Support for the mast cell-dependent erosion of coronary artery plaques comes from the observation that coronary microthrombi are often located above subendothelial mast cells.

Regarding plaque rupture, following mechanisms have been suggested to be operative in the lipid-rich lesions. In the lipid-rich lesions, a collagen-containing fibrous cap is synthesized and maintained by viable smooth muscle cells (SMCs). The fibrous cap separates the large lipid core from the circulating blood. If activated to degranulate, the mast cells present in the cap do secrete heparin and the neutral protease chymase, which together exert multiple inhibitory actions on their neighboring SMCs. Heparin inhibits growth of SMCs and chymase inhibits collagen synthesis by these cells. By degrading fibronectin of the pericellular matrix of the SMCs, chymase triggers their apoptotic death. Together, these actions by mast cells tend to cease collagen synthesis in the cap. Moreover, the mast cell-derived proteases chymase, tryptase, and cathepsin G, activate matrix metalloproteinases (MMPs), which again, degrade collagen and other components of the extracellular matrix in the fibrous cap. Finally, by secreting TNF-alpha, mast cells stimulate neighboring macrophages to synthesize and secrete pro-MMP-9, which may then be activated in the plaque. The ability of mast cells to simultaneously attenuate matrix

synthesis and to accelerate matrix degradation contributes to the local catabolic state within inflamed areas of the fibrous plaque. As a consequence, in these areas the cap becomes thin and fragile, and the lipid-rich plaque becomes vulnerable to rupture.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA133

Plasmid encoded drug resistance

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Bacteria have been around on planet Earth for three billion years or so. In that time they have become adept at protecting themselves against toxic chemicals. Nowhere is this better illustrated than in the case of the development of resistance to antibiotics. Indeed, clinical medicine, as practiced over the last six to seven decades can be viewed as one vast test of the Darwinian thesis "survival of the fittest" as applied to bacteria. That antibiotic resistance is now a major clinical problem all over the world, virtually irrespective of the specific antibiotic, attests to the success and speed of bacterial adaptation.

Mechanisms of antibiotic resistance in bacteria are varied and include target protection, target substitution, antibiotic detoxification and block of intracellular antibiotic accumulation. The acquisition of the genes needed to elaborate the various mechanisms is greatly aided by the existence of a variety of promiscuous gene transfer systems, such as bacterial conjugative plasmids, transposable elements and integron systems, that permit movement of genes from one DNA system to another and from one bacterial cell to another, not necessarily one related to the gene donor. Bacterial plasmids serve as scaffolds on which are assembled arrays of antibiotic resistnce genes, acquired in the main by transposition (transposable elements) and site-specific recombination mechanisms (integron gene cassettes), mechanisms that when working in concert are able to construct complex resistance gene arrangements (1).

The evidence suggests that ABR-genes in human bacterial pathogens originate from a multitude of bacterial sources, indicating that the genomes of all bacteria can be considered as a single global gene pool into which most, if not all, bacteria can dip for genes necessary for survival. In terms of antibiotic resistance, plasmids serve key roles, particularly as vehicles for resistance gene capture and their dissemination to diverse bacterial species. These various aspects of bacterial resistance to antibiotics will be explored in this presentation.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA134

Just how does a single protein recognise all of those drugs?

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Bacterial multidrug resistance (mdr) is an emerging threat to human health. Mdr can arise through one or more mechanisms. One, which is common to all domains, is the efflux of chemically and structurally dissimilar drugs by multidrug efflux transporters. How these multidrug efflux pumps recognise such different compounds is unknown, in great part due to the lack of high resolution structural data. Fortunately, many of the genes of these transporters are regulated by drug sensing transcription repressors or activators. Like the pumps they regulate, these transcription regulators are able to bind myriad structurally and chemically dissimilar drugs, nearly all of which are substrates for the efflux transporters that they regulate. In order to understand the structural basis of multidrug binding and recognition, we have determined the structures of several multidrug binding transcription regulators, including QacR and BmrR. QacR is a repressor that controls the transcription of the Staphylococcus aureus qacA gene, which encodes a multidrug efflux transporter that pumps out quaternary ammonium compounds (qacs). The substrates of the pump are monovalent and bivalent cationic aromatic/aliphatic disinfectants, antibiotics and antiseptics. When the QacA pump is overwhelmed, its substrates enter the cytoplasm whereby they bind to QacR and act as inducers, thereby allowing the derepression of the qacA gene and ultimately leading to more copies of the efflux pump. The structural mechanism of such "polyspecificity" that QacR utilises to recognise these chemically and structurally dissimilar inducers will be described. Briefly, the multidrug binding pocket can be described as a larger pocket composed of several "minipockets". As might be anticipated, the multidrug binding pocket is lined with negatively charged residues, which play some role in neutralization of the cationic drug. However, that role is not as cardinal as might be expected. What is clear is the abundance of aromatic residues in the binding pocket and their role in drug binding will be described. Moreover, the multidrug binding mechanism of QacR will be compared and contrasted with that of BmrR, an activator of the bmr gene, which encodes a multidrug efflux transporter in Bacillus subtilis. Unlike QacR, BmrR appears to be more limited in the class of drugs that it binds, i.e., BmrR binds only aromatic/aliphatic monovalent cations. Regardless of the differences between QacR and BmrR and between QacR and other multi xenobiotic binding transcription factors, for which structural data are available, similar structural and chemical principles are employed by each of these multidrug binding proteins and will be described.

This work is supported by a grant (AI048593) from the National Institutes of Health.

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SA135

Structure and function of tripartite pumps for bacterial toxin export and multidrug efflux

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Bacterial pathogens use TolC-dependent machineries to export toxins and enzymes by bypassing the periplasm via a tripartite apparatus in which the outer membrane TolC protein is recruited by a substrate-engaged inner membrane translocase of a traffic ATPase and an accessory or 'adaptor' protein. This establishes a contiguous export channel from the cytosol to the outside. Our work has focused on export of the large hemolysin toxin by uropathogenic and enterohemorrhagic E. coli, and I will summarize our view of this process. Closely related TolC-dependent tripartite machines are ubiquitous in the the cell envelopes of bacteria like Escherichia coli and Pseudomonas aeruginosa and these expel antibacterial drugs and other small noxious chemicals, so helping the bacteria survive. These multidrug efflux pumps are important to the growing threat of bacterial resistance to chemotherapy. They function similarly by recruitment of a TolC family protein by energised drug-laden translocases in the inner membrane, though in this case the adaptor is typically coupled to a proton antiporter. We have crystallised and solved the 3D structures of the TolC protein, revealing a trimeric exit duct anchored in the outer membrane and projecting across the periplasm, and also the periplasmic adaptor protein that recruits TolC. These structures reveal how the tripartite pumps assemble to a contiguous trans-envelope pores for virulence protein export and multidrug resistance, and we have now established how the recruitment and pump assembly is effected by interaction of TolC-adaptor coiled-coil interfaces in the periplasm. We have described how the periplasmic exit duct entrance can be opened in the assembled pumps to allow exit of toxins and drugs, and also shown how the entrance constriction can be blocked by potential inhibitors. This suggests it may be possible to develop new drugs to target the pumps and counteract multidrug resistance.

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SA136

Muscle fibre proliferation and remodelling

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During the human life span the muscle mass undergo major changes. Furthermore the muscles are highly adaptive to usage; training might induce muscle hypertrophy and increased strength or increased endurance. The muscles have also a remarkable ability to recover after muscle damage. Of special importance is that the muscle fibres are not able to divide. The nuclei are post mitotic, thus the fibres are not either able to increase their myonuclei. The key structure related to muscle fibre proliferation and remodelling is the satellite cells. These cells, considered being a kind of stem cell, are located in between the muscle fibre plasma- and basement membranes. They are of crucial importance and are involved in both the adaptive and repair events of the muscle fibre. A number of factors affect and regulate the satellite cells.

In the past electron microscopic analysis was needed to reveal the satellite cells, however nowadays a number of markers can be used to identify and quantify them using immunohistochemical methods and light microscopy. As the satellite cells seem to be heterogeneous within a muscle and between muscles there is no golden standard how to quantify them *in situ*. Furthermore other kind of stem cells be localized in the interstitium between muscle fibres and might act as additional candidates for muscle maintenance. Methodological considerations must therefore be taken into account when comparing results from different laboratories. The satellite cells can easily be investigated in cell cultures and their proliferative capacity can be estimated by their number of divisions they can perform and how their telomeres become shortened. One has also to remember that results from studies on animal muscles differ substantially from those on humans.

During the years we have evaluated a number of markers to identify satellite cells. Our current view is that several markers on the same section are needed for a reliable estimation of satellite and stem cells in human muscles. Depending on if two or four markers are used we observe a marked difference in the number of satellite cells in relation to number of nuclei per fibre. Correlation of the number of SC in situ, to estimates from cell culture (number of divisions) and degree of telomere shortening have shown that a concomitant decrease of satellite cells and proliferative capacity occur in degenerative muscle disorders, during ageing and in highly exercised muscles. With regard to muscle fibre proliferation we have shown that there is a highly significant correlation between the number of muscle fiber nuclei in a cross section and the cross section area (Eriksson *et al* 2005). Related to the concept of nuclear domains i.e that one nucleus can govern a certain area of the cytoplasm with information for protein synthesis, the new nuclei are supposed to come from the satellite cells. Using strength trained elite athletes, some of which had also used anabolic steroids, we have achieved new information on possible hyperplasia of muscle fibres and on the aetiology and pathogenesis of so called split fibres (Eriksson *et al* 2006). Interestingly, previously anabolic steroid users had, although they stopped training, a significant higher amount of nuclei per fiber. This suggests that they would still have an advan-

tage for increased protein synthesis if they would start training and compete again (Eriksson 2006). Upon inactivity, human as animal muscle fibres atrophy, however in humans we have not seen any loss in number of nuclei as are reported for animal muscles. Furthermore contrary to animal muscles, human muscle do not as easily become damaged upon exercise and muscle fibre necrosis are limited. Myofibrillar disorganisation as observed with electronmicroscopy, thought to represent damage and part of the cause to delayed onset muscle soreness, has recently be shown to be a process of myofibrillar remodelling (Yu *et al* 2004). The field of satellite and stem cells is growing fast and one can anticipate a dramatic increase of knowledge in the near future.

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Supported by grants from Swedish Research Council 12x-3934, Swedish National Centre for Research in Sports (CIF), Association Francaise Contre les Myopathies (AMF) project 11949 and the Medical Faculty at Umeå University,

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SA137

The role of nutrition in stimulating muscle protein accretion at the molecular level

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The increase in muscle protein synthesis that occurs in response to consumption of a complete meal by a fasted individual is a result not only of increased availability of the substrates used for synthesizing protein, i.e. amino acids, but also to nutrient- and hormone-induced activation of intracellular signaling pathways that regulate mRNA translation. Arguably, one of the most important nutrient-regulated signaling pathways in muscle involves a complex of proteins that includes the protein kinase referred to as the mammalian target of rapamycin (mTOR), the regulatory associated protein of mTOR (raptor), the Ras homolog enriched in brain (Rheb), the proline-rich Akt substrate (PRAS)40, LST8 (a.k.a. GβL), and possibly other as yet unidentified proteins. Together, these proteins form the TOR complex 1 (TORC1). Nutrients such as the branched-chain amino acid leucine alter the conformation and/or composition of the TORC1 complex, resulting in increased mTOR protein kinase activity. For example, the amino acid-induced activation of TORC1 correlates with decreased amounts of raptor and PRAS40 present in mTOR immunoprecipitates, suggesting that amino acids alter the interaction of mTOR with both proteins. Amino acids also increase the proportion of Rheb present in the GTP bound form, an event that is critical for maximal TORC1 activation. However, the mechanism(s) through which amino acids act to alter either TORC1 conformation or Rheb association with GTP is incompletely defined. Upregulated nutrient signaling through TORC1 leads to increased phosphorylation of several proteins that play important roles in regulating the mRNA binding step in translation initiation including the eukaryotic initiation factor (eIF)4E binding protein (4E-BP)1 and the ribosomal protein S6 kinase S6K1. S6K1 subsequently phosphorylates eIF4B and eukaryotic elongation factor (eEF)2 kinase. Phosphorylation of 4E-BP1 by mTOR results in its release from the inactive 4E-BP1/eIF4E complex allowing the mRNA cap binding protein eIF4E to associate with eIF4G and eIF4A to form the active eIF4F complex. Phosphorylation of eIF4B, an activator of the RNA helicase activity of eIF4A, promotes its association with the eIF4F complex. Depending on the cell type, amino acid-induced assembly of the eIF4F complex can lead to increased global rates of protein synthesis and also to changes in the selection of mRNAs for translation, thereby altering the pattern of gene expression at the protein level. For example, activation of TORC1 preferentially increases the translation of mRNAs encoding proteins such as eEF1A, eEF2, and many ribosomal proteins, thereby increasing the capacity of the cell to synthesize protein. Our recent studies have identified a new target for TORC1 signaling, the catalytic ε-subunit of the guanine nucleotide exchange factor, eIF2B. In these studies, we found that leucine addition to leucine-deprived cells causes a redistribution of the eIF2Bε mRNA from an untranslated, non-polysomal fraction into polysomes, leading to increased incorporation of [35S]methionine into eIF2Bε protein. The shift of eIF2Bε mRNA into polysomes and increased synthesis of eIF2Bε protein are both prevented by pre-treatment with the specific mTOR inhibitor rapamycin. Because eIF2B is an important regulator of global rates of protein synthesis, an increase in its expression is likely an important component in the increased capacity for mRNA translation associated with mTOR activation.

In addition to amino acids, TORC1 activity can be regulated through altered provision of fatty acids, particularly in muscle composed primarily of slow-twitch fibers. For example, in heart, blocking the uptake of fatty acids or inhibiting their oxidation leads to decreased signaling through TORC1. The mechanism through which fatty acids regulate TORC1 in heart involves activation of the AMP-activated protein kinase (AMPK). In this regard, reduced fatty acid oxidation leads to an increase in the AMP:ATP ratio resulting in activation of AMPK. AMPK represses TORC1 activity through multiple mechanisms, including direct phosphorylation of mTOR. Our studies suggest that the action of AMPK on TORC1 is dominant to many positive inputs, including those induced by various hormones and nutrients.

In summary, TORC1 represents a nexus through which nutrients and hormones act to acutely regulate mRNA translation. Moreover, by specifically increasing the translation of mRNAs encoding proteins involved in mRNA translation, activation of TORC1 upregulates the capacity to synthesize protein.

Supported by NIH grant DK15658.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C57

Focal adhesion kinase controls load-dependent transcript expression in slow oxidative rat muscle

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The present report tested the hypothesis that focal adhesion kinase (FAK) is a myocellular transducer of mechanical signals towards downstream transcript expression of protein turnover and oxidative metabolism in myofibres. This was approached in an integrative setting combining somatic transgenesis with high-throughput transcript profiling and morphometry in a physiological model for load-dependent atrophy and hypertrophy of anti-gravitational soleus muscle. Focal adhesion signalling in myofibres was perturbed via targeted overexpression of FAK and its inhibitor FRNK (FAK-related non-kinase) in soleus muscle fibres. Experimental bias was supervised by quantitative assessment of transcript level changes versus non-transfected or empty-transfected paired controls. The results points out that sarcolemmal and sarcoplasmic FAK pools separately control gene ontologies underlying load-dependent growth and differentiation of muscle fibres towards a fatigue-resistant phenotype. These findings expose FAK as a key upstream element of the mechano-dependent control of the slow oxidative muscle phenotype and highlight the resolution power of the outlined setting for system biological investigations in vivo.

This work was supported by Swiss National Foundation Grant 310000-112139 and the "Association Francaise contre les Myopathies" (AFM)

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C58

Vps34 is activated by an acute bout of resistance exercise

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Resistance exercise training results in a progressive increase in muscle mass and force production. Following an acute bout of resistance exercise, the rate of protein synthesis increases proportionally with the increase in protein degradation, correlating at 3 hours in the starved state. Amino acids taken immediately before or immediately after exercise increase the post exercise rate of protein synthesis. Therefore a protein that controls protein degradation and amino acid sensitivity would be a potential candidate for controlling the activation of protein synthesis following resistance exercise. One such candidate is the class 3 phosphatidylinositol 3OH -kinase (PI3K) Vps34. Vps34 controls both autophagy and amino acid signalling to mTOR (mammalian target of rapamycin) and its downstream target p70 S6 kinase (S6K1). The aim of this study was to determine the role of Vps34 in mTOR activation following resistance exercise. As the prevalence of Vps34 has yet to be described, we determined first its tissue distribution. Vps34 is a ubiquitous protein with high levels in brain and skeletal muscle. Interestingly, Vps34 levels were highest in muscles with a greater percentage of slow oxidative fibres. To determine whether Vps34 is modulated by exercise. Vps34 and S6K1 activity were measured in the distal hindlimb muscles of rats 30 mins, 3 hours, 6 hours and 18 hours after acute unilateral resistance exercise with the contralateral muscles serving as a control. In the tibialis anterior (TA) we found a 366.30±112.08 ($p=0.037$), 124.67±15.96 ($p=0.012$) and 129.18±0.1-fold ($p=0.013$) increase in S6K1 activity at the 30 min, 3 hour and 6 hour time points respectively. This was in contrast to the soleus, which showed no significant increase in S6K1 activity at any time point and the plantaris, which only showed a 33.14±2.29 ($p<0.001$) and 47.00±6.65 ($p=0.02$) percentage increase in activity at the 30 minute and 3 hour time point respectively. Unlike S6K1, Vps34 activity increased only at the 3 and 6 hour time point in the TA muscle showing a 100.86±18.19 ($p=0.01$) and 36.01±8.79 ($p=0.02$) percent increase respectively. A 24.48±7.92 ($p=0.02$) percent increase of activity was observed in the plantaris 3 hours post exercise giving a similar pattern of activity between Vps34 and S6K1. S6K1 activity was still high 18 hours post exercise when Vps34 had returned to basal in the TA. The increase in Vps34 activity we observe at 3 hours correlates with the increase in protein degradation and synthesis following resistance exercise. We propose that the increase in Vps34 activity results in an increase in the intracellular pool of free amino acids activating mTOR and sustaining the high activity of S6K1.

This work was supported by a project grant from the Wellcome Trust 077426/Z/05/Z

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SA138

Exercise and nutrient controlled mechanisms involved in maintenance of the musculoskeletal mass

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We have known for many years that physical activity and adequate nutrition are important for the maintenance of the mass of muscle, tendon and bone in human beings. However the underlying mechanisms are only slowly being relieved partly because human beings often show significant biological differences in the organisation and effectiveness of the control mechanisms, highlighting the need for experimental studies in people.

Work from our laboratory and that of colleagues has demonstrated that amino acids are major drivers of anabolism in muscle and bone but that tendon is unresponsive to nutritional influences. The amino acid induced effects in muscle, which appear

to be mainly due to their effects on stimulation of protein synthesis are independent of the actions of insulin and growth hormone. Insulin seems to be of major importance in the regulation of protein breakdown in muscle with a very steep dose response relationship between availability of insulin and inhibition of breakdown. Both in muscle and bone amino acids are responsible for rapid alterations in transcription of genes associated with anabolism as well as proteins associated with anabolic signalling pathways. Short term alteration of availability of glucose and lipid have no influence on muscle or bone maintenance (except in the context of insulin secretion) but evidence is growing that polyunsaturated fatty acids may have an anabolic role in muscle.

Exercise is anabolic in itself but the ability to increase muscle protein balance depends upon the availability of amino acids. Exercise will stimulate the ability to make protein for up to 48 h after exercise and although it is well understood that different modes of exercise (e.g. resistance vs. endurance) cause stimulation of different suites of genes leading to characteristic muscle adaptation, somewhat surprisingly, any kind of exercise appears to stimulate the propensity for bulk muscle protein synthesis. There are no major differences in the effects of so called "concentric" (shortening) or "eccentric" (lengthening) exercise on the acute increases in muscle protein synthesis although it is known that the latter provides a better stimulus for muscle growth. However the expression of genes associated with muscle building shows dramatic differences between these two modes, which are likely to be important for muscle building. Tendon despite being impervious to alterations of nutrition shows marked increase in collagen synthesis as a result of alteration of activity; this is also true of collagen synthesis in muscle. There is growing evidence that although growth hormone has no effect on muscle myofibrillar and sarcoplasmic synthesis it may markedly stimulate collagen synthesis in muscle and tendon.

Ageing and a wide variety of chronic disease processes cause decreases in the ability of muscle to make protein in response to adequate nutrition, a phenomenon which we call anabolic resistance and which appears to have its basis in alterations both in gene transcription and capacity and activity of signalling pathways.

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SA139

The ubiquitin-proteasome system in muscle wasting

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Increased proteolysis contributes to the muscle wasting seen in several pathological conditions (e.g. cancer, sepsis, diabetes, burn injury, trauma, etc) and during fasting. Enhanced proteolysis in skeletal muscle, the major protein reservoir in the body, is a key metabolic adaptation providing the organism with free amino acids for energy production via gluconeogenesis and direct oxidation. Net mobilisation of muscle protein in pathological situations also provides free amino acids for acute phase protein synthesis in the liver, and for protein synthesis in vital organs (e.g. brain and heart). However, sustained muscle wasting rapidly results in increased morbidity and mortality. The lysosomal, Ca2+-activated and ubiquitin-proteasome-dependent pathways and the caspases are the most important processes responsible for the breakdown of the bulk of skeletal muscle protein. The activation of the ubiquitin-proteasome system (UPS) is mainly responsible for the muscle wasting that occurs in various animal models of cachexia. This activation involves alterations in the ubiquitination, deubiquitination and proteolytic machineries (1). Other proteolytic enzymes act upstream (possibly caspase-3, m-calpain, and/or cathepsin L) and downstream (tripeptidyl-peptidase II and aminopeptidases) of the UPS, for the complete breakdown of the myofibrillar proteins into free amino acids (1). Recent studies have identified a few critical proteins that seem necessary for muscle wasting (i.e. the muscle-specific MuRF-1 and MAFbx/atrogin-1 ubiquitin-protein ligases E3s)(2). The characterization of their signalling pathways is leading to new pharmacological approaches that can be useful to block or partially prevent muscle wasting (3). I discuss the nature of the substrates of the UPS, and the evidence that the UPS is activated in weight-losing patients.

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Studies in the laboratory of the author are supported by le Ministere de la Recherche, la Fondation pour la Recherche Medicale et l'Association Francaise contre les Myopathies.

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SA140

Ribosome display and protein in situ array: Cell-free discovery tools for identification of protein binders

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Cell-free protein technologies are becoming alternatives to cell-based methods for proteomic and biotechnology applications. We have developed two powerful protein technologies by novel exploitation of cell-free expression systems. They are termed (i) ribosome display (1, 2) and (ii) protein in situ array (3). Both technologies rapidly convert genetic information into proteins without the need for E. coli cloning. While ribosome display permits direct selection and identification of protein binders from large PCR libraries, protein in situ array produces immobilised proteins in parallel through a single-step on-chip synthesis. An integration of these technologies would allow library vs library screening for high-throughput identification of protein interacting partners in vitro.

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Research in the Technology Research Group, Babraham Institute, is supported by BBSRC, UK.

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SA141

Intramolecular vs. intermolecular interactions in the proapoptotic protein ASPP2: a proposed mechanism for regulation of p53-mediated apoptosis

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The tumour suppressor protein p53 responds to oncogenic stress by inducing cell cycle arrest or apoptosis of potentially malignant cells, thereby preventing the malignant transformation (1). p53 is mutated in around 50% of human cancers. In the other 50% of tumours, where wild-type p53 is intact, the regulation of the p53 pathway is abnormal. A key question in cancer research is how the selection for the p53 response is being made. ASPP2 is a pro-apoptotic protein that stimulates the p53-mediated apoptotic response (2,3). It was previously identified in its shorter forms as 53BP2 and Bbp. ASPP2 is a 1128 amino acids protein, consisting of several structural and functional domains. The C-terminal ankyrin repeats and SH3 domain mediate its binding to p53 (4), as well as to numerous other proteins involved in apoptosis such as NF-kB and Bcl-2. However, the interactions of ASPP2 with other proteins have not been yet characterized structurally and biophysically, and the role of these proteins in regulation of the ASPP2 function is yet unknown. In addition, ASPP2 contains a proline-rich domain between residues 693-918, whose structure and function are unclear.

We have used a combination of peptide array screening with biochemical and biophysical methods to gain insight into the protein interaction network of ASPP2 and the roles of its domains in mediating these interactions. To achieve this, we expressed three recombinant ASPP2 fragments: The Ank-SH3 domains only, the proline-rich domain only and a construct containing all three domains. To identify the ASPP2-binding sites in its target proteins, we designed peptide arrays consisting of overlapping peptides derived from a series of ASPP2 binding proteins. Screening of the ASPP2 constructs for binding the peptide arrays identified the precise binding sites in its target proteins, and revealed that only the Ank-SH3 domains, but not the proine-rich domain of ASPP2, mediate protein-protein interactions. Comparison of the ASPP2 binding sites in the three anti-apoptotic Bcl family members Bcl2, Bcl-XL and Bcl-W revealed that all of them bind ASPP2 via their N-terminal BH4 domain, which is essential for their anti-apoptotic activity. We propose that ASPP2 might promote apoptosis by inhibition of this anti-apoptotic BH4 domain.

Next, we set to study the structure and function of the proline rich domain of ASPP2. Using biophysical methods including circular dichroism, analytical gel filtration and analytical ultracentrifugation we showed that the proline-rich domain of ASPP2 belongs to the family of natively unfolded proteins (5). SH3 domains are known to bind proline-rich ligands, so we tested whether such an interaction exists between the proline-rich and SH3 domains of ASPP2. Indeed, ASPP2 proline-rich domain bound the Ank-SH3 domains with micromolar affinity as shown by fluorescence spectroscopy and pull-down assays. Using peptide arrays we identified the precise Ank-SH3 binding sites in the proline-rich domain of ASPP2 (5).

In summary, our results show that only the Ank-SH3 domains of ASPP2, but not the proline-rich domain, mediate the intermolecular interactions of ASPP2 with its partner proteins. Instead, the proline-rich domain makes an intramolecular interaction with the Ank-SH3 domain of ASPP2. Natively unfolded protein domains usually mediate interactions with numerous partner proteins. The proline rich domain of ASPP2 is a unique case, where such an unstructured domain mediates an intramolecular interaction, while the structured Ank-SH3 mediates the known intermolecular protein-protein interactions (5).

Based on our findings, we propose a model for regulation of ASPP2 and of p53-mediated apoptosis. According to our model, the intramolecular interaction between the ASPP2 domains has an important role in regulating the intermolecular interactions of ASPP2 with its partner proteins. When the proline-rich domain is bound to the Ank-SH3 domain, it masks it and makes it unable to bind intermolecularly to its partner proteins. Following a yet unknown regulation mechanism, the intramolecular domain-domain interaction is released and the Ank-SH3 domains become available to bind their target proteins such as p53 or the Bcl family proteins, and promote apoptosis (5).

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The study was funded by a career development award from the Human Frontier Sceince Program Organization (for Assaf Friedler).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA142

Prediction and analysis of protein interaction sites: binding hot-spots in protein–protein and protein–ligand interfaces

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Protein assemblies are currently poorly represented in structural databases and their elucidation is a key goal in biology.

In particular, the ability to control protein-protein interactions therapeutically is of great current interest due to the many important processes involving these interactions. Recently, we have analysed clefts on protein surfaces, likely to correspond to binding 'hot-spots' according to properties thought to be important in stabilizing the native complex. This includes, sequence conservation and measures of physical properties including hydrophobicity, desolvation, electrostatic and van der Waals potentials. The resulting differences between predicting binding-sites at protein–protein and protein–ligand interfaces are striking. Generally, the prediction accuracy for protein–protein interfaces is lower. The talk will describe a number of structural bioinformatics tools for prediction and characterisation of protein binding sites, and discus the potential for targeting protein-protein interactions with small molecule drugs.

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SA143

Capturing protein interactions in the secretory pathway of living cells

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Studies on protein interaction networks in yeast have revealed that most biological processes require protein complexes rather than single proteins, and by extrapolation this also applies for mammalian cells. Interaction proteomics is thus an important approach to elucidate the functional role of the proteomes. Mass spectrometry of isolated protein complexes, protein microarrays, and library-based screenings, such as the yeast two-hybrid system, are the most popular current methods in interaction proteomics. While highly successful in the analysis of cytosolic and nuclear protein interactions, these approaches are of limited value to detect protein interactions on the luminal side of the organelles of the secretory pathway, since such interactions tend to be of low affinities and occur in an oxidative and ionic environment that is considerably different from that of cytosol and nucleus. To circumvent these problems we have developed a luminal yellow fluorescent protein (YFP) fragment complementation assay (PCA) to capture protein interactions inside the secretory pathway.

The basic concept of PCA relies on engineering reporter protein fragments that exhibit no functional activity by themselves and do not spontaneously fold. The fragments are fused to two interacting proteins. The interaction of the hybrid proteins brings the two reporter fragments into proximity leading to their folding into the active 3D structure of the complete reporter protein. The proof of concept of the luminal YFP PCA was tested by fusing YFP fragments to the homoligomeric cargo receptor lectin ERGIC-53, its interaction partner MCFD2 and to ERGIC-53's interacting cargo glycoprotein cathepsin Z. YFP PCA analysis revealed the oligomerization of ERGIC-53 and its interaction with MCFD2, as well as its lectin-mediated interaction with cathepsin Z. Using the YFP PCA we discovered a carbohydrate-dependent interaction between ERGIC-53 and the cathepsin Z-related protein cathepsin C. The high specificity of the assay was demonstrated by the finding that inactivation of ERGIC-53's lectin activity by a point mutation selectively impaired YFP complementation of the carbohydrate-dependent interaction with cathepsin Z and cathepsin C. These results suggest that YFP PCA can detect weak and transient interactions inside the secretory pathway and hence is a powerful approach to study luminal processes involved in protein secretion, including carbohydrate-mediated protein-protein interactions of low affinity.

To search for additional proteins interacting with ERGIC-53 a genome-wide YFP PCA screening procedure was developed. A cDNA-YFP1 fusion library was constructed, expressed in COS cells and screened using YFP2-ERGIC-53 as bait. Cells expressing complemented YFP were isolated by FACS and library plasmids were recovered and analyzed. This new YFP PCA approach for the genome-wide capturing of protein interactions in living cells will be presented.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA144

Role of BMPR-II mutation in pulmonary arterial hypertension

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Idiopathic pulmonary arterial hypertension (PAH) is a devastating condition, which untreated leads to death from right heart failure within three years from diagnosis. Although new treatments have been developed over the past few years, there remains a need for therapies which modigy disease progression and reverse the process of pulmonary vascular remodelling. Approximately 10% of cases of PAH are familial. The disease segregates as an autosomal dominant condition with reduced penetrance. In 2000, disease causing heterozygous germline mutations were identified in the gene encoding the bone morphogenetic protein type II receptor (BMPR-II), a receptor member of the transforming growth factor-β. Mutations in the BMPR-II gene have now been identified in more than 70% of families with PAH, as well as 10-26% of sporadic cases of idiopathic PAH. Mutations in BMPR-II may be missense, nonsense or frameshift mutations. About 30% of mutations are missense producing a change in highly conserved amino acids in critical functional regions of the receptor. Such mutations occur in the kinase and ligand binding domain of the receptor, or cause accumulation of the receptor in the endoplasmic reticulum. The latter mutations may be amenable to therapeutic approaches aimed at encouraging receptor trafficking to the cell surface. The majority of mutations lead

to truncation of the receptor or allelic loss due to nonsense mediated RNA decay. The wild type BMPR-II receptor exists as a heterodimer at the cell surface forming complexes with type I BMP receptors. On BMP ligand binding, the constitutively active serine-threonine kinase domain of the BMPR-II phosphorylates the type I receptor. The activated type I receptor then phosphorylates downstream signalling intermediaries termed Smad proteins. BMPR-II mutation is associated with reduced activation of Smad proteins in the majority of cases. However, surprisingly, the regulation of many BMP-induced genes are not altered by BMPR-II mutation. This probabaly reflects the presence of alternative Smad-independent pathways of BMP signalling, for example via mitogen activated protein kinases (MAPK). BMPR-II mutation also reduces MAPK signalling in response to BMPs. Our group have identified that BMPR-II mutation in pulmonary artery smooth muscle cells leads to a failure of the antiproliferative and proapoptotic effects of BMPs. This effect may be mediated by dysregulation of BMP-induced transcription factors in the inhibitor of DNA binding (Id) family. BMPs exert diverse and tissue specific effects. In pulmonary artery endothelial cells, BMPs are promote growth and survival, in contrast to their effects in smooth muscle cells. The presence of BMPR-II dysfunction in endothelial cells promotes apoptosis. The combination of increased endothelial apoptosis and enhanced smooth muscle cell survival is likely to contribute to the pathogenesis of PAH. A further important functional consequence of BMPR-II mutation is an altered growth response to TGF-β. Thus smooth muscle cells with dysfunctional BMPR-II are resistant to the growth suppressive effects of TGF-β. We have confirmed that reduced function of BMPR-II and increased TGF-β signalling are both features of widely used rodent models of PAH, triggered by chronic hypoxia or exposure to the plant alkaloid monocrotaline. These observations support the use of anti-TGF-β therapy in PAH. Studies in knockout mice have confirmed that additional stimuli are necessary to promote the development of PAH on a background of BMPR-II deficiency. Thus heterozygous BMPR-II knockout mice only develop more severe pulmonary hypertension than wild type controls when exposed to additional insults. We have shown that increasing circulating serotonin levels is one means by which the pulmonary hypertensive phenotype can be uncovered in the mouse. This fits with the observations in man that disease gene penetrance is usually less than 50% in familial PAH and that additional triggers are necessary for disease manifestation.

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Funded by the British Heart Foundation, Wellcome Trust and MRC

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA145

Potassium channels in the regulation of pulmonary artery smooth muscle cell proliferation and apoptosis

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A proper balance between apoptosis and proliferation is necessary for the normal functioning and development of tissue. Disturbing this equilibrium can lead to disease states, such as hypertension. K^+ channels play a major role in regulating pulmonary vascular tone by controlling the contractility of pulmonary artery smooth muscle cells (PASMC). K^+ channels are also fundamental to sustaining normal pulmonary vascular tissue homeostasis in this cell population, as they play a role in both apoptosis and cell proliferation.

There are two apoptotic pathways: the death receptor (extrinsic) and mitochondrial (intrinsic) pathways. In the intrinsic pathway, cytochrome c, released from mitochondria, is an essential component of the apoptosome which activates effector caspases in the cytoplasm. In the extrinsic pathway, activated death receptors trigger the initiation caspase activity. Effector caspases, which serve as proteases and nuclease activators, are the final mediators of apoptosis.

The Na^+/K^+ pump maintains a high level of intracellular K^+ ($[K^+]_i$). Because ionic movements into and out of the cell largely determine cell volume, and because K^+ permeability is high at rest, K^+ currents are important determinants of cell volume. Water is passively transported across the cell membrane in response to the ionic flux to maintain hydrostatic pressure gradients. Apoptotic volume decrease (AVD) is an early hallmark and prerequisite of programmed cell death. Its earliest phase is marked by efflux of K^+ and Cl^-, and outward transportation of water. As K^+ efflux increases through opened K^+ channels in early stages of AVD, Cl^- ions follow, moving down their electrochemical gradient. Water exits the cell through aquaporins to maintain the osmotic pressure balance between the intracellular and extracellular compartments, thus achieving cell shrinkage. Various subfamilies of K^+ channels have been implicated in AVD, including voltage-gated (KV) and Ca^{2+}-activated (KCa) K^+ channels, while other subfamilies, including two-pore domain (K2P), inward-rectifier (KIR) and ATP-sensitive (KATP) K^+ channels have been found to play a role in early apoptotic K^+ efflux or AVD in apoptosis. It has also been shown that increased extracellular K^+, which leads to maintaining high $[K^+]_i$ by decreasing the driving force for K^+ efflux, or pharmacological block of K^+ channels can

inhibit apoptosis prior to cytochrome c release, can inhibit caspase activation, and can inhibit cytochrome c release. This supports an important role for K^+ efflux during the early stages of apoptosis.

Physiological levels of intracellular K^+ have also been found to play a protective role against apoptosis in that it inhibits caspase and nuclease activity. Both K^+ channel blockers and high extracellular [K^+] have been shown to attenuate apoptosis. Therefore, K^+ loss or efflux, in addition to leading to AVD, creates a permissible environment for caspase and nuclease activity by relieving the inhibition on these apoptotic mediators. Overall, K^+ efflux contributes to apoptosis in two ways: by causing AVD and by releasing inhibition on endogenous caspases and nucleases.

In addition to its role in apoptosis, K^+ channels also play an important role in cell proliferation, mainly through their regulation of resting membrane potential (E_m) which in turn regulates intracellular Ca^{2+} levels. At rest, cells have a negative E_m, which is established by the electrogenic Na^+/K^+ ATPase that extrudes 3 K^+ for every 2 Na+ ions brought into the cell. Furthermore, K^+ permeability is relatively high at rest, so K^+ flows out of the cell down its electrochemical gradient. Decrease of K^+ channel activity (due to downregulated K^+ channel expression and/or to decreased K^+ channel conductance or open probability) causes membrane depolarization. This in turn would lead to an opening of L- and T-type voltage-dependent Ca^{2+} channels (VDCC) and an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). The increased $[Ca^{2+}]_{cyt}$ causes smooth muscle cell contraction through Ca^{2+}/calmodulin-mediated activation of myosin light chain kinase. Intracellular Ca^{2+} is also an important second messenger for cell migration and proliferation; Ca^{2+} influx through VDCCs triggers various transcriptional factors (e.g. CREB, NF-AT, NF-kB, c-Jun, c-Fos) that are involved in cell proliferation and protein synthesis. In addition, Ca^{2+} is also required for cell cycle progression, including the G0 to G1 transition, DNA synthesis and mitosis. Recently, another role of K^+ in promoting cell survival has been found. In response to membrane permeabilization by bacterial toxins, a decrease in cytoplasmic K^+ promotes membrane biogenesis which promotes cell survival by facilitating membrane repair.

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Research and data presented here were supported in part by NIH grants (HL064945, HL66012, and HL054043).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C59

Effects of dexfenfluramine on proliferation of pulmonary arterial fibroblasts from mice over-expressing the 5HT transporter

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Ingestion of the anorexigenic drug dexfenfluramine (Dfen) is a risk factor for development of pulmonary arterial hypertension (PAH). We have previously shown that Dfen induces PAH in wildtype (WT) mice whilst protecting against the exaggerated hypoxia-induced PAH observed in mice over-expressing the 5HT transporter (5HTT+ mice)[1,2]. In the present study we investigated the effects of Dfen on proliferation of pulmonary arterial fibroblasts (PAFs) from WT and 5HTT+ mice.

Mice (C57BL/6, 5-6 months, 25-35g, n=4) were euthanised using sodium pentobarbitone (2g/kg), pulmonary arteries dissected out, and PAFs cultured as described previously[3]. PAFs were grown to 60% confluency, quiesced for 24 hours, and exposed to normoxia or hypoxia (5%O_2) in the presence of Dfen (0.3-10μM) for a further 24 hours. Proliferation was measured by [3H]thymidine incorporation. The effects of Dfen (3μM) were also assessed in the presence or absence of $5HT_{2A}$ receptor antagonist ketanserin (30nM), $5HT_{2B}$ receptor antagonist SB204741 (300nM) or 5HTT inhibitor citalopram (100nM). Values shown are the mean±s.e.m. for 4 replicate plates from the same experiment. Experiments were repeated at least in triplicate and the results shown typical of those obtained. Statistical comparisons were made by one-way ANOVA with a Dunnetts multiple comparison test.

Dfen had no effect on proliferation of WT PAFs under normoxia (disintegrations per minute (DPMs): 0.3μM 31.9±6.5, 1μM 42.6±10.0, 3μM 35.7±9.8, 10μM 24.6±2.7, $P>0.05$ cf. control: 26.9±3.4) or hypoxia (DPMs: 0.3μM 32.3±6.1, 1μM: 37.8±7.9, 3μM: 46.8±7.7, 10μM 38.3±2.7 DPM, $P>0.05$ cf. control: 40.4±7.6). Neither did Dfen have any effect on proliferation of 5HTT+ PAFs under normoxic conditions (DPMs: 0.3μM 28.0±4.0, 1μM: 22.6±6.0, 3μM: 22.7±5.2, 10μM 22.5±3.8 DPM, $P>0.05$ cf. control: 27.1±1.2). However, hypoxic exposure led to proliferation of PAFs from 5HTT+ mice (713.5±23.6 DPM cf 81.6±16.8 DPM in normoxia, $P<0.01$), an effect which was inhibited by Dfen (3μM; 71.4±22.6DPM, $P<0.01$). The hypoxic response was restored when PAFs were pre-incubated with citalopram before addition of Dfen (806.5±93.2 DPM, $P<0.01$ cf Dfen alone). Neither ketanserin nor SB204741 had any effect on the Dfen-induced inhibition of the hypoxic proliferation of 5HTT+ PAFs (67.3±11.2 DPM and 92.0±18.0 DPM respectively, $P>0.05$).

In conclusion, Dfen has no effect on proliferation of normoxic cells from WT or 5HTT+ mice, or on hypoxic WT cells. However, Dfen inhibits hypoxic-induced proliferation of PAFs from 5HTT+ mice through a 5HTT mediated mechanism. This may explain why Dfen can protect against the exaggerated hypoxia-induced PAH observed in 5HTT+ mice.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C60

Unexpected effects of KCNQ channel modulators on pulmonary artery

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The KCNQ channel blockers, linopirdine and XE991, were recently shown to produce pulmonary vasoconstriction.[1] Retigabine and flupirtine are KCNQ channel openers that have been shown to activate recombinant and native neuronal KCNQ currents and are being developed as treatment for epilepsy. We have now investigated the actions of KCNQ channel modulators on a range of arteries and the resting K^+ conductance of rat pulmonary artery smooth muscle cells (PASMCs), as well as the expression of KCNQ channel subunits in pulmonary artery.

Male Sprague-Dawley rats (250-300g) were sacrificed in accordance with Schedule 1 of the Animal (Scientific Procedure) Act 1986. Intrapulmonary arteries (IPA 300-400μm) were dissected and mounted on a wire myograph for isometric tension studies. mRNA was isolated, reverse transcribed to cDNA and amplified using gene-specific primers for KCNQ1-5, following established protocols. Whole-cell, patch-clamp recording was used to record current under voltage clamp and membrane potential under current clamp conditions, from PASMCs.[2] KCNQ subunit protein expression was analysed by immunostaining and western blot. Data are expressed as mean ± s.e.m. of n animals/cells and compared using Student's t-test.

While the KCNQ blockers constricted IPA, they did not affect other systemic vessels, including cerebral, carotid, coronary, mesenteric, renal, femoral and tail arteries. In isolated PASMCs, linopirdine (10μM) and XE991 (5μM) inhibited a background K^+ current by 38±4% (n=11) and 36±5% (n=15) at 0mV and depolarized the membrane by 14±2 mV (n=5) and 15±6 mV (n=6), respectively. The KCNQ channel opener flupirtine relaxed IPA pre-constricted with 10μM phenylephrine with IC_{50} = 14±1μM (n=5). Retigabine was significantly more potent than flupirtine, often producing relaxation below 100nM ($p\langle 0.05$). The IC_{50} was 2.5±0.2 μM (n=5) and 3.1±1 μM (n=3) against phenylephrine and $PGF2_\alpha$-induced constriction, respectively. Retigabine was less effective at relaxing vessels constricted with 50mM K^+, where 6±1% (n=6) relaxation was seen at 10μM and 40±5% (n=6) at 100μM, and was ineffective on vessels constricted with 130mM K^+, suggesting its action via K^+ channel opening. Furthermore, relaxation was preserved in the presence of glibenclamide, implying no involvement of K_{ATP} channels. KCNQ openers shifted the concentration-response curves for the blockers to the right, suggesting an action on the same channels. Expression studies indicated that mRNA and protein for KCNQ1, 4 and 5 subunits are expressed in IPA.

Taken together these results suggest that KCNQ channels are functionally expressed in PASMCs. Inhibition by KCNQ blockers promotes depolarisation and contraction, while KCNQ openers promote relaxation. Therefore KCNQ openers might have potential as therapeutic pulmonary vasodilators.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA146

Extracellular ATP: a critical modulator of hypoxia-induced pulmonary artery adventitial fibroblast phenotype

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Nucleotides such as ATP and UTP are emerging as a ubiquitous family of extracellular signaling molecules. Nucleotides are released from cells by a variety of pathophysiologic stimuli and have been shown to play a key role in transducing mitogenic, contractile, metabolic, and secretory signals in a variety of cells through release and subsequent binding to the P2 (P2X and P2Y) family of purinergic receptors. However, little is known regarding the role of ATP in hypoxia-induced vascular cell responses. In a neonatal model of hypoxic pulmonary hypertension, we showed that Pulmonary Artery (PA) adventitial fibroblasts proliferate both *in vivo* and *in vitro* in response to hypoxic conditions. We therefore examined the hypothesis that hypoxia-induced adventitial fibroblast proliferation would be mediated by hypoxia-induced changes in ATP release and/or its extracellular degradation. We found that acute hypoxia (3%O2 10-60 min) increased extracellular ATP concentrations in adventitial fibroblasts and that chronic hypoxia (3%O2, 14-30 days) markedly attenuated the rate of extracellular ATP hydrolysis by ectonucleotidase(s) suggesting that at least two different cellular mechanisms may contribute to elevated extracellular ATP levels. Exogenous ATP (100 μM) stimulated thymidine incorporation and increased the phosphorylation of Akt, Erk1/2, mTOR, and p70S6K in fibroblasts as did UTP, UDP, ADP, ADPβS, MeSATP, αβMeATP, BzATP and some other agonists, indicating that both P2Y and P2X purinoceptors mediate mitogenic responses. PCR analysis revealed that adventitial fibroblasts express P2Y1,2,6 as well as P2X2,4,6,7 receptor subtypes. The rank order of potency of various agonists to activate each individual kinase pathway (ERK1/2, PI3K/Akt or mTOR/p70S6K) indicates that in these cells, purinergic receptors are coupled to the proliferative responses in a pathway-specific manner. Importantly, in this ATP-activated signaling network, a translational pathway, involving mTOR, p70S6K and S6 ribosomal protein, plays a central role in integrating Erk1/2 and PI3K/Akt pathways. We also found that ATP (100μM) and hypoxia (3%O2), induced expression and activation of the Egr-1 transcription factor, and

both stimuli acted, in part, through Gαi-initiated ERK1/2 signaling pathway. Apyrase (2.5U/ml), as well as the non-selective P2 receptor antagonists, suramin, cibacron blue 3GA, and PPADS (all used at 100μM) attenuated hypoxia- and ATP-induced DNA synthesis, indicating an activation and a functional role of P2Y/P2X purinoceptors in hypoxia-induced proliferative responses. In addition, suramin, cibacron blue 3GA and apyrase, markedly attenuated hypoxia-induced ERK1/2 activation and Egr-1 expression. Collectively, our findings demonstrate that PA adventitial fibroblasts can be considered as endogenous source and a target of extracellular nucleotides within the vascular wall and that a hypoxia-induced autocrine loop of ATP signaling plays a critical role in the regulation of fibroblast proliferation under hypoxic conditions.

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NIH Grants (SCOR: HL-57144; SCCOR:HL-084923; PPG: HL-14985)

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SA147

Interactions between serotonin, BMPR2 and potassium channels in the development of pulmonary hypertension: an overview

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Genetics predict that haploinsufficiency of the BMPR2 receptor and over activity of the serotonin transporter are involved in the pathogenesis of pulmonary hypertension. Intriguingly, recent evidence suggests that the downstream signalling pathways of these proteins interact and co-operate to promote pulmonary vascular remodelling. There is also evidence that these pathways impinge on potassium channel function. This symposium has unravelled and discussed these novel interactions in the setting of pulmonary vascular remodelling.

The serotonin system has been implicated in the development of pulmonary arterial hypertension (PAH) since the 1960s when the diet pill aminorex, an indirect serotinergic agent, was associated with the development of PAH. Serotonin is synthesised by the pulmonary vascular endothelium via the activity of tryptophan hydroxylase 1 (TPH1) and likely acts on underlying pulmonary artery smooth muscle cells (PASMCs) and fibroblasts in a paracrine fashion. It can cause vasoconstriction by activation of the 5-HT1B (1) or 5-HT2A receptor or it can enter the PASMCs via the serotonin transporter (SERT) to induce proliferation (2). Over-expression of SERT has been associated with the clinical development of PAH and we have recently shown that mice over-expressing SERT (SERT+ mice) are predisposed to hypoxia-induced PAH (3). In fibroblasts derived from SERT+ mice, we have demonstrated that activation of the 5-HT2A receptor induces downstream stimulation of p38 map kinase and proliferation which provides a mechanism for this phenotype. Clearly polymorphisms in the BMPR2 receptor alone do not account for the expression of the PAH phenotype and a 'second hit' is required and serotonin is an attractive candidate. Indeed, serotonin infusion can uncover a PAH phenotype in BMPR2+/- mice via inhibition of SMAD signalling (4). Serotonin can also inhibit Kv1.5 channel activity which would facilitate the vascular effects of inhibition of these channels. Serotonin can also induce mitogen activated protein kinases in pulmonary fibroblasts and this effect can be inhibited by inhibition of rho kinase, another potentially important mediator of pulmonary arterial proliferation and contraction.

Recently, over-expression of TPH1 has been observed in pulmonary endothelial cells in patients with PAH and we have recently shown the development of hypoxia-induced PAH is ablated in mice deficient in TPH1 (Tph1-/- mice)(5). This is due to inhibition of pulmonary vascular remodelling rather than a decrease in pulmonary vascular reactivity. Furthermore, TPH1 expression in pulmonary endothelial cells is induced by hypoxia in mice. Hence evidence suggests that serotonin could play a role in critical proliferative pathways, inactivation of potassium channels, the expression of a PAH phenotype associated with BMPR2 haploinsifficiency as well as hypoxia-induced pulmonary vascular remodelling. The SERT, the 5-HT1B receptor and TPH1 are all potential novel therapeutic targets for the treatment of PAH.

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This work was funded by the BHF and BBSRC.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA148

Computational drug design

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The Protein Structure Initiatives have provided an increasing resource of high quality crystal structures of therapeutic targets for drug design; this resource is expanding at an exponential rate. Most of this data is held electronically in the public domain and provides an opportunity for the biotechnology and pharmaceutical companies to exploit this information to generate candidate ligands that interfere with the function of the protein. The challenge for drug discovery is to industrialize the design process to identify a good selection of primary scaffold structures that offer excellent starting points for medicinal chemistry to provide good lead compounds. Computational methods offer an in silico approach to performing design in situ within the site. The huge benefit of de novo methods is that they can be used to

assess in silico large numbers of potential structures for their fit to the site before any synthesis is embarked upon. De novo design offers an overwhelming advantage to companies seeking to gain a patent estate for the most promising compounds.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA149

Fragment based drug discovery using rational drug design

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Over the last two decades there has been considerable interest in new approaches to drug discovery that offer improvements in the process of identifying new therapeutic agents. Technologies such as high-throughput screening and combinatorial chemistry have taken hold in most pharmaceutical companies and allow significantly larger numbers of compounds to be screened against the target of interest. Despite these developments, the industry has failed to generate the level of productivity that it has strived to achieve. All aspects of the drug discovery process therefore remain the focus of improvements with the application of new technologies.

One area that has continued to receive significant interest is lead discovery chemistry as the quality of lead compounds is thought to have a major impact on the attrition rates in drug development. Many groups have reported on the importance of compound libraries for lead generation to become more lead-like rather than drug-like. Such an approach takes into account the increase in molecular weight and lipophilicity that typically occurs as a lead molecule is optimised into a potential drug.

More recently, interest has grown in a new approach for lead generation that involves screening libraries of compounds that are significantly smaller (MW 100-200) and functionally simpler than drug molecules, often referred to as 'Fragment-based' discovery [refs 1-5]. This new approach is believed to have many advantages over conventional screening such as more efficient sampling of chemical space using fewer compounds and a more rapid hit-to-lead optimisation phase. Fragment-based drug discovery also provides significant challenges, largely due to the fact that fragments typically exhibit low affinity binding (100uM-mM) and are therefore difficult to detect using bioassay-based screening methods. However, biophysical methods such as X-ray crystallography and NMR are ideal detect such low affinity binders. Although fragments often have low affinity, they usually exhibit high 'ligand efficiency', i.e., high values for the ratio of free energy of binding to the number of heavy atoms. It is important that when a fragment hit is identified, optimisation into a useful lead compound is performed with carefully designed iterations consistent with maintaining good ligand efficiency.

In this talk I will describe the discovery and development of novel lead compounds for the key cancer targets, Cyclin-dependent kinases and Aurora kinase, using Fragment-based methods. The application of high throughput X-ray crystallography and other biophysical techniques to screen fragment libraries against CDK2 and Aurora kinase will be outlined. The advantages of using structure-based methods to guide the 'Fragment-to-lead' phase will be discussed. The process of lead optimization and subsequent development into drug candidates will be described. The profiles of the resulting clinical candidates, AT7519 (CDK inhibitor) and AT9283 (Aurora kinase inhibitor), both of which were derived from fragment hits and are now in phase 1 clinical trials, will be discussed.

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Astex Employees.

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SA150

Ligand discovery using the virtual screening program LIDAEUS

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A variety of different protein targets have been used to test a virtual screening approach for docking small molecule ligands. Over 18 isoforms of the cyclophilin family are present in the worm C.elegans. A store of over 5 million available molecules stored in a relational database is used to identify potential ligands. Over five chemically distinct families of inhibitor have been identified. Subsequent chemical modification and testing show theses families are biologically active and cause phenotypic changes in the worm similar to those caused by cyclosporine.

A similar approach has been applied to a series of cell cycle regulators including CDK,cyclin and PCNA resulting in novel and specific inhibitors. Biophysical binding studies using surface plasmon resonance and ITC have been used to characterise the protein-ligand interactions and provide experimental measures of binding energies. X-ray structures of selected complexes have been determined to provide experimental poses.

The program LIDAEUS has been parallelised to run on the supercomputer BlueGene which allows significantly higher throughput. In massively parallel docking runs it is possible to store top scoring poses for millions of compounds docked into all available classes of protein binding pockets. Such data can be used to generate novel chemical descriptors and provides new ways of classifying chemical families.

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SA151

Development and application of FITTED to the docking of ligands to flexible and solvated proteins

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To achieve optimal binding, enzyme inhibitors, receptor antagonists and agonists, or DNA/RNA binders must exhibit optimal molecular recognition with the macromolecular biological target structure, a principle that has been the basis for the development of computational drug design methods. Predicting the correct binding mode of a ligand in a protein invokes the prior positioning of the ligand by a search engine that ensures an efficient and unbiased sampling. However, most of the developed models do not account for conformational changes in the protein upon binding or presence of key water molecules. As a consequence, when activity is correlated to induced fit effects or bridging water molecules, usual approaches often lead to misleading results. The binding mode of interest must next be identified and the binding affinity predicted. For this purpose, a variety of scoring functions have been developed. However, the available scoring functions rank (to some extent) compounds according to their biological activity but their predictiveness still relies heavily on the target under study. To account for side-chain or backbone adjustments, presence of water molecules and to address the scoring function predictiveness, we have developed new strategies [1,2], now implemented in the current version of FITTED [3]. The development of FITTED and its application to a variety of enzymes, receptors, and RNA aptamers will be presented.

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SA152

Prediction of drug metabolism by cytochromes P450

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The cytochromes P450 (CYPs) comprise a vast superfamily of enzymes found in virtually all life forms. In mammals, xenobiotic metabolising CYPs provide crucial protection from the harmful effects of exposure to a wide variety of chemicals, including environmental toxins and therapeutic drugs. Elucidating the structural features of CYPs that contribute to their metabolism of structurally diverse substrates impacts on the rational design of improved therapeutic drugs and specific inhibitors. Models capable of predicting the possible involvement of CYPs in the metabolism of drugs or drug candidates are thus important tools in drug discovery and development. Ideally, functional information would be obtained from crystal structures of all the CYPs of interest. Initially only crystal structures of distantly related bacterial CYPs were available – comparative modelling techniques were used to bridge the gap and produce structural models of human CYPs, and thereby obtain some useful functional information. A significant step forward in the reliability of these models came seven years ago with the first crystal structure of a mammalian CYP, rabbit CYP2C5, followed by the structures of five human enzymes, CYP2A6, CYP2C8, CYP2C9, CYP2D6 and CYP3A4, and a second rabbit enzyme, CYP2B4. The evolution of a CYP2D6 model, leading to the validation of the model as an *in silico* tool for predicting binding and metabolism, will be presented as a case study. This work has led directly to the successful design of CYP2D6 mutants with novel activity – including creating a testosterone hydroxylase, converting quinidine from inhibitor to substrate, creating a diclofenac (anionic, cf. the preference of 2D6 for basic nitrogen containing compounds) hydroxylase and creating a dextromethorphan O-demethylase. Our modelling-derived hypothesis-driven integrated interdisciplinary studies have given key insight into the molecular determinants of CYP2D6 and other important drug metabolising enzymes.

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SA153

Overview: gases and biology

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Mammalian cells are constantly bathed in, and enveloped by, a mixture of naturally occurring gases. Whilst the biological significance of oxygen (O2) and carbon dioxide (CO2) in regulating cell function has been recognized for many centuries the possibility that other naturally occurring gaseous compounds may be synthesized from, and affect the function of, cells is a more recent phenomenon.

To date, several biologically active gases have been identified and characterised. These include nitric oxide (NO) synthesized from L-arginine by nitric oxide synthase (NOS1, NOS2, NOS3) and carbon monoxide (CO) synthesized from haem by the enzyme, haem oxygenase (HO1 and HO2). NOS and HO enzymes are widely distributed in the body. NOS1, NOS3 and HO2 are constitutive enzymes whilst NOS2 and HO1 are induced in target tissues by a number of pathophysiological triggers. Recently a third gaseous mediator, hydrogen sulphide (H2S), has been added to this list. H2S is synthesized from L-cysteine by cystathionine β synthetase (CBS) and cystathionine γ lyase (CSE). Both CBS and CSE, previously considered as constitutive enzymes involved in the transsulphuration interconversion of L-methionine and L-cysteine/L-homocysteine, can also be induced in cells/tissues in certain disease states. Other potential biologically active gases include ammonia and sulphur dioxide (SO2) - a recently discovered natural product of endothelial cell metabolism. NO, CO and H2S exhibit a number of biological effects in common. In particular, all three gases dilate blood vessels and have been proposed to play physiological roles in the control of tissue vascular perfusion and pathophysiological significance with respect to, for example, vascular diseases such as hypertension, angina, myocardial infarction as well as inflammatory states such as shock and arthritis. Furthermore, all three gases exert complex effects on cell survival. Thus, NO can either cause or protect against cell death whilst CO is generally protective. H2S promotes cell death perhaps by inhibiting cytochrome c oxidase or by promoting apoptosis following activation of p38 MAP kinase or as a result of a genotoxic effect and concomitant p53 activation. All three gases also play a prominent part in inflammation and tissue injury. Once again, the effect of NO is complex with both pro- and anti-inflammatory activity reported depending on the model of inflammation and the time course of the inflammatory response. Intriguingly, H2S may also exhibit both pro- and anti-inflammatory activity. For example, both CSE inhibitors such as DL-propargylglycine (PAG) and slow releasing H2S donors such as S-diclofenac reduce lipopolysaccharide-evoked endotoxic shock (Li et al., 2005; Li et al., 2007). Numerous examples of 'cross talk' between these three gases have been identified. Such interactions can take place at the level of transcriptional control of synthesizing enzymes, actions on transduction mechanisms mediating biological effects and direct chemical reactions. For example, NO downregulates HO1 and CSE expression in isolated cells and in intact animals. In turn, H2S inhibits NO production and NF-kB activation in lipopolysaccharide-stimulated macrophages by a mechanism which involves activation/upregulation of HO-1 and release of CO (Oh et al., 2006). In stark contrast, H2S has been reported to decrease HO-1 expression in cultured smooth muscle cells. Aside from these interactions at the transcriptional and/or translational level there are additional reports that NO either augments or reduces the vasorelaxant effect of H2S and vice versa. In addition, we have recently reported that H2S interacts with NO to form an as yet unidentified nitrosothiol moiety. This mechanism may perhaps explain the ability of H2S to further contract isolated precontracted rat aortic rings and to rapidly reverse the relaxation of aortic rings in response to both acetylcholine and sodium nitroprusside (SNP). Interestingly, mixing H2S with SNP completely abolished the vasodepressor effect of SNP in anaesthetised rats suggesting that the interaction takes place both in vitro and in vivo (Ali et al., 2006). As progress in our understanding of the biological significance of NO, CO and H2S increases at a rapid rate it is becoming more clear that a complete understanding of the effect of these gases on cell/tissue function demands a truly integrated approach to their study. Bearing in mind the interactions between these gases already identified and the possibility that other gases may also be involved we may have to concede that it is now no longer possible to 'get the full picture' simply by evaluating the effect of a single gas on any particular biological system.

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We wish to thank the Office of Life Science of the National University of Singapore for financial support.

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SA154

Soluble guanylate cyclase: coordinating gaseous signalling?

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Soluble guanylate cyclase (sGC) facilitates the conversion of guanosine-5'-triphosphate to cyclic guanosine-3',5'-monophosphate (cGMP) and regulates many aspects of cell function through activation of specific kinases, ion channels and phosphodiesterases. The role of sGC as the principal receptor for nitric oxide (NO) is well-established and known to be fundamental to the physiological regulation of numerous organ systems. Consequently, dysfunctional NO/sGC signalling has been implicated in the aetiology of an array of pathologies. Yet, NO may not represent the sole endogenous ligand for sGC. This is illustrated by the fact that triple NO synthase knockout mice are viable whereas genetic deletion of the β_1 subunit of sGC is lethal, implying that animals unable to synthesise NO can still utilise cGMP-dependent pathways. Accordingly, recent evidence suggests that alternate, endogenously-generated gaseous signalling species, such as carbon monoxide (CO) and hydrogen sulphide (H_2S), may also exert their biological effects (at least in part) via activation of sGC.

In comparison to NO, the biological chemistry of CO is simple in that it forms coordination complexes with metalloproteins, but will not react with O_2, and is therefore relatively stable in most cellular environments. The majority of endogenous CO synthesis originates from the action of haem oxygenase (HO) enzymes that oxidise 'free' haem resulting in the generation of CO, iron and biliverdin. Indeed, it is the complexity and chemical difficulty in generating CO in this manner strongly suggests that CO is deliberately synthesised, rather than a just a noxious waste product. Despite the fact that the degree of activation of purified sGC by CO is miniscule compared to NO (~4-fold versus ~200-fold), many biological effects of CO appear to be mediated via sGC, since they are sensitive to the selective inhibitor, ODQ. This holds true for the majority of smooth muscle relaxant, neurotransmitter, anti-inflammatory and anti-platelet properties of CO. Moreover, synergistic interaction of allosteric sGC activators (e.g. YC-1, BAY 41-2272) with CO implies that under some circumstances sGC may become hyperresponsive to CO. This may be most apparent during pathological episodes characterised by oxidative stress (when NO bioactivity is negated due to reactions with O_2-derived species) in which CO will retain chemical stability and may help maintain sGC-dependent signalling. Under these conditions, oxidation of specific cysteine residues in the enzyme that contribute to the allosteric sGC activator binding site, will potentially increase the responsiveness of the enzyme to CO.

H_2S is the simplest thiol species present in biological systems and physiological levels of H_2S are relatively high (compared to NO and CO), reaching ~150μM. The major routes of H_2S biosynthesis are via the enzymes cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CSE), that are highly-expressed in the CNS and vasculature, respectively. Whilst H_2S is unlikely to form a complex with the haem-iron of sGC and activate the enzyme in a manner akin to NO and CO, data suggest that H_2S interacts with NO-dependent signalling, both at the level of sGC and NO synthase. Moreover, the appreciated importance of reduced cysteine residues in sGC in maintaining NO sensitivity, may suggest that H_2S can act as a physiological reductant to preserve cGMP-dependent signalling. This is supported by the reports that CBS and CSE activity is augmented by NO, suggesting that NO and H_2S are generated concomitantly. Further still, direct interaction of H_2S and NO under appropriate conditions, may result in the formation of the simplest S-nitrosothiol (HSNO), that could act as a means to protect NO from oxidative destruction.

Considered together, the above observations give rise to the possibility that NO, CO and H_2S coordinate to preserve the (principally) cytoprotective effects of cGMP. In turn, this suggests that sGC is ideally placed to act as an endogenous 'redox sensor' and adapt its sensitivity to these gaseous signalling species to optimise cGMP-dependent signalling. A better understanding of the actions and interactions of NO, CO and H_2S is likely to identify novel targets for therapeutic intervention.

AJ Hobbs is the recipient of a Wellcome Trust Senior Research Fellowship.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA155

Bioactivity and therapeutic potential of carbon monoxide-releasing molecules (CO-RMs)

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Heme oxygenase-derived carbon monoxide (CO) serves as signaling mediator in a wide array of physiological functions to the extent that the beneficial effects elicited by CO gas when administered to mammalian organisms may be exploited for therapeutic purposes (1). In this context, the development of carbon monoxide-releasing molecules (CO-RMs) represents an ideal approach for the delivery of controlled amounts of CO for the treatment of various pathological disorders (2). Transition metal carbonyls and boranocarbonates have been chemically engineered to provide water-soluble compounds that release CO with a fast (CORM-3) or slow (CORM-A1) kinetic (3, 4). The configuration and bioactive features of a range of CO carriers containing manganese, ruthenium, boron and iron are also being investigated in our laboratory to better understand the chemical reactivity of CO-RMs in biological systems. The results collected to date indicate that CO-RMs are pharmacologically active as they possess vasodilatory, hypotensive and anti-inflammatory properties as well as cytoprotective activities. Most recently, we have focused on the idea that CO-RMs could be used clinically to maintain the integrity of organs for transplantation as they have been demonstrated to improve significantly the function of isolated kidneys preserved in cold storage solutions and exert remarkable anti-ischemic effects. Although the mechanism of action of CO-RMs remains to be fully elucidated, we have proposed that a dynamic interaction of CO with specific intracellular metal centers may be the common denominator for the

diversified beneficial effects mediated by this gaseous molecule (5). Thus, CO-RMs may help to identify new cellular targets that are responsive to CO and facilitate the therapeutic delivery of this gas in a safe, measurable and controllable fashion.

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This work has been supported by grants from the Research Kidney UK and the Henry Smith Charity

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C61

Cyclooxygenase-2-derived prostaglandins mediate vascular dysfunction of resistance arteries of endothelial nitric oxide synthase knockout mice in sepsis

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Lipopolysaccharide (LPS)-induced upregulation of endothelial nitric oxide synthase (eNOS) activity is obligatory in the pathogenic expression/function of inducible NOS (iNOS) in sepsis(1). Indeed, LPS-induced iNOS expression, NO synthesis and aortic dysfunction are profoundly depressed in eNOS knockout (KO) mice (2). In contrast we have previously shown that vascular dysfunction in resistance arteries of eNOSKO mice is substantially enhanced and associated with increased plasma cyclooxygenase (COX) metabolites(3). Therefore, we investigated the effect of the COX-2 selective and non-selective inhibitors, L-745,337 and indomethacin (indo) respectively, on LPS-induced vascular dysfunction in eNOSKO mice.

Male (25-30g) eNOSKO mice were treated with indo or L-745,337 (5mg/kg, i.p.) 15min prior to saline or LPS (12.5mg/kg, i.v., 4h). Blood samples were collected by terminal cardiac puncture under halothane anaesthesia for 6-keto-$PGF_{1\alpha}$, thromboxane (TX)B_2 and PGE_2 measurements using ELISA. Mesenteries were removed for Western blotting for COX-1/2 measurement or 3rd order arteries dissected and mounted in tension myographs. Following normalisation concentration-response curves to the TXA_2 mimetic, U-46619 (U19; 0.001-3μM), or phenylephrine (PE; 0.001-30μM) were constructed. Alternatively arteries were precontracted with U19 (~EC_{80}) and relaxation response curves to the NO donor spermine-NONOate (SNO; 0.001-3μM) determined.

Whilst COX-2 expression was barely detectable in saline-treated mice profound expression was equally evident following LPS treatment in mesenteries of wild type and eNOSKO mice (n≥3), COX-1 was unaffected (n≥5). Responses to PE, U19 and SNO were suppressed by LPS (n=5-6, $p<0.001$, table 1); an effect significantly attenuated by L-745,337 (n=5-6, $p<0.05$), whilst indomethacin had no significant effect (n=4-5). LPS-induced vascular hyporesponsivess was associated with an elevation of plasma 6-keto-$PGF_{1\alpha}$(≈5 fold),TXB_2(≈3 fold) and PGE_2(≈34 fold) levels (n=7-9). These were abolished with indo treatment; however, L-745,337 suppressed TXB_2(≈4 fold, n=8, $p<0.05$) and PGE_2 (≈14 fold; n=9, $p<0.05$) only.

Together the data suggests a compensatory role for COX-2 derived prostaglandins, in the absence of eNOS, in mediating LPS-induced vascular dysfunction of resistance arteries. COX-2 activity may be important when endothelial dysfunction is prevalent and may explain the poor outcome of NOS inhibitors in the treatment of sepsis.

Table 1

	Saline		LPS		LPS+L-745,337	
Agonist	pEC50	Max	pEC50	Max	pEC50	Max
PE	6.1±0.04	9.0±0.2mN	4.9±0.2	3.8±0.7mN	5.2±0.1	5.8±0.6mN
U19	8.5±0.2	9.3±0.5mN	7.1±0.2	7.4±0.7mN	7.2±0.1	8.4±0.3mN
SNO	6.8±0.1	103±2%	5.9±0.1	81±3%	6.3±0.12	80±4%

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S.F. is supported by The Wellcome Trust.

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SA156

Peroxisomes, phytanic acid alpha oxidation and Refsum disease: from enzymology to studies on the toxicity of phytanic acid in patient' cells, mutant mice and omega-oxidation

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Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a 3-methyl branched-chain fatty acid (FA), which accumulates in tissues and body fluids of Refsum patients. 3-Methyl FAs like phytanic acid, first need to undergo alpha-oxidation to produce the corresponding 2-methyl FA plus CO2. In contrast to 3-methyl FAs, 2-methyl FAs like pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), the product of phytanic acid alpha-oxidation, can be beta-oxidized. The mechanism of alpha-oxidation has been resolved in recent years and involves 4 reactions, including: (1.) activation of phytanic acid to phytanoyl-CoA; (2.) 2-hydroxylation of phytanoyl-CoA; (3.) cleavage of 2-hydroxyphytanoyl-CoA to formyl-CoA and pristanal, and (4.) oxidation of pristanal to pristanic acid. The enzymology of the alpha-oxidation pathway has been worked out in some detail, especially

for phytanoyl-CoA hydroxylase and 2-hydroxyphytanoyl-CoA lyase, catalyzing the second and third step of alpha-oxidation. Both enzymes are localized in peroxisomes, which explains why alpha-oxidation is strictly peroxisomal. In most patients suffering from Refsum disease (RD) phytanoyl-CoA hydroxylase is deficient, due to mutations in the structural gene (PHYH/PAHX). In search for an alternative therapy for RD we have recently focused on an alternative mechanism for phytanic acid degradation, i.e. omega-oxidation, and have found that the first step in the omega-oxidation pathway is catalyzed by different CYP450s, notably 4F3A, 4F3B, 4A11, and 4F2, in decreasing order of catalytic efficiency. Future studies are aimed at the identification of compounds able to induce the expression of the different CYP450s, in order to increase the capacity to omega-oxidize phytanic acid and thereby lower phytanic acid in RD patients. We will first test candidate compounds like fibrates, which are able to upregulate CYP4A11 expression in a mouse model for RD, which we have generated recently.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA157

Structural and mechanistic studies on peroxisomal and related oxygenases

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Phytanic acid occurs in the human diet as a metabolite of the fatty acid side chain of chlorophyll. Due to the presence of an epimeric beta-methyl group phytanic acid cannot be metabolised via the well-characterised beta-oxidation pathway. Instead it is metabolised in the peroxisomes in a 'preliminary' alpha-oxidation pathway to give pristanic acid that is subsequently degraded via beta-oxidation. Phytanoyl-CoA 2-hydroxylase (PAHX), a ferrous iron and 2-oxoglutarate (2OG) dependent oxygenase, catalyses a vital step in the alpha oxidation pathway comprising hydroxylation of both epimers of phyatnoyl CoA. Mutations to PAHX ablate its role in the metabolism of phytanoyl CoA, and results in the accumulation of phytanic acid, which in turn causes symptoms leading to Refsum Disease. The structure and function of PAHX will be discussed and comparisons made with related human enzymes involved in oxygen sensing and signalling roles.

Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2)

McDonough MA, Li V, Flashman E, Chowdhury R, Mohr C, Lienard BMR, Zondlo J, Oldham NJ, Clifton IJ, Lewis J, McNeill LA, Kurzeja RJM, Hewitson KS, Yang E, Jordan S, Syed RS, Schofield CJ PNAS 103: 9814-9819, 2006

Structural studies on 2-oxoglutarate oxygenases and related double-stranded beta-helix fold proteins

Clifton IJ, McDonough MA, Ehrismann D, Kershaw NJ, Granatino N, Schofield CJ Journal Of Inorganic Biochemistry 100: 644-669, 2006

Posttranslational hydroxylation of ankyrin repeats in I kappa B proteins by the hypoxia-inducible factor (HIF) asparaginyl hydroxylase, factor inhibiting HIF (FIH)

Cockman ME, Lancaster DE, Stolze IP, Hewitson KS, McDonough MA, Coleman ML, Coles CH Yu XH), Hay RT, Ley SC, Pugh CW Oldham NJ, Schofield CJ, Ratcliffe PJ PNAS 103: 14767-14772, 2006

Structure of human phytanoyl-CoA 2-hydroxylase identifies molecular mechanisms of Refsum disease

McDonough MA, Kavanagh KL, Butler D, Searls T, Oppermann U, Schofield CJ J Biol Chem 280: 41101-41110, 2005

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA158

The role of 2-hydroxyacyl-CoA lyase 1, a thiamine pyrophosphate dependent peroxisomal enzyme, in the metabolism of 3-methyl-branched fatty acids and 2-hydroxy straight chain fatty acids

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Molecular Cell Biology, Katholieke Universiteit Leuven, Leuven, Belgium

α-Oxidation is the process by which fatty acids are degraded by one carbon unit. It is the obligatory degradation pathway for 3-methyl-branched fatty acids, such as phytanic acid, before these can enter the β-oxidation sequence because the 3-methyl-substitution renders the third step of β-oxidation impossible.

Elucidation of the exact phytanic acid α-oxidation sequence was greatly facilitated by the detection of formate as one of its products in 1993. The pathway consists of an initial activation, subsequent 2-hydroxylation by a dioxygenase, splitting of the 2-hydroxy-3-methylacyl-CoA intermediate and subsequent dehydrogenation (1). We described that the carbon unit that is split off in the third step is formyl-CoA and the other product is a 2-methyl-branched aldehyde with one carbon less (1). The responsible enzyme was purified, sequenced, and characterized (2). It appeared to be a homotetrameric enzyme with a peroxisomal targeting sequence (PTS1) and it is the first thiamine pyrophosphate (TPP) dependent enzyme described in mammalian peroxisomes.

As the substrate for this lyase contains a 3-methyl-branch, a 2-hydroxy-group and a CoA-ester, we wondered which of these constituents are necessary in order to be a substrate for the lyase. Substrate competition experiments with 2-hydroxyhexadecanoyl-CoA, 2-hydroxyoctadecanoyl-CoA, 2-hydroxyhexadecanoic acid, 2-hydroxy-3-methyl-hexadecanoyl-CoA, 3-methylhexadecanoyl-CoA, 3-methylhexadecanoic acid and 2-methyl- and 3-hydroxy-derivatives led to the conclusion that both the 2-hydroxy moiety and the CoA-ester function, but not the 3-methyl-substitution, are obligatory for the substrates of this enzyme. Further studies showed a subcellular distribution of lyase activity with 2-hydroxyoctadecanoyl-CoA, which concurred perfectly well with the profile seen for lyase activity with 2-hydroxy-3-methylhexadecanoyl-CoA as a substrate. Incubations of 2-hydroxyoctadecanoyl-CoA with the recombinant human

lyase led to the production of formyl-CoA / formate and heptadecanal. All these data led to the conclusion that besides 2-hydroxy-3-methylacyl-CoA also 2-hydroxy long chain fatty acyl-CoAs are substrates for the lyase (3). Consequently, the previously named 2-hydroxyphytanoyl-CoA lyase (2-HPCL) is now called 2-hydroxyacyl-CoA lyase 1, abbreviated as HACL1 (4).
As HACL1 is TPP-dependent, we studied the impact of thiamine deficiencies on the α-oxidation process *in vivo* (rats on thiamine deficient diets and treated with thiamine antimetabolites) and in cultured fibroblasts from patients with thiamine responsive megaloblastic anemia and controls. Our data pointed to an impact of thiamine deficiency on the α-oxidation process (5).
The fact that HACL1 is the first peroxisomal enzyme described in mammals that is TPP-dependent, prompted us to investigate its presence and import in the peroxisome, as studies on the intraperoxisomal TPP were never carried out and no data are available about its import. We investigated the subcellular localization of this vitamin in rat liver. In addition to the already known cytosolic and mitochondrial pools, the presence of TPP in peroxisomes was established. The peroxisomal concentration pointed to the existence of a peroxisomal pool of TPP (unpublished data).
How TPP enters the peroxisome remains to be explored. Peroxisomes are surrounded by a single membrane, and permeability for small molecules and solutes through so called peroxisomal pores has been shown previously, but also transporters have been described. Does TPP enter the peroxisome in its diphosphorylated form or as thiamine? In the latter case, it should be diphosphorylated in the peroxisome. Conversion of thiamine to TPP occurs by a thiamine pyrophosphokinase. This enzyme has been described in the cytosol. We reinvestigated its subcellular localization, in order to establish if it is also present in peroxisomes. However, its localization was exclusively cytosolic, without any activity in peroxisomes (unpublished data). In addition, the enzyme has no peroxisome targeting signal. On the basis of these findings we conclude that TPP as such is imported in the peroxisome.

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This work was supported by grants from the 'Geconcerteerde onderzoeksacties van de Vlaamse Gemeenschap' (GOA 2004/08), the 'FWO-Vlaanderen' (G.0115.02) and the FP6 European Union Project 'Peroxisome' (LSHG-CT-2004-512018). P.F. and M.S. were supported by a fellowship from the 'FWO-Vlaanderen'. The authors would like to thank Wendy Geens, Luc Govaert, Stanny Asselberghs and Chantal Brees for technical help.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA159

Clinical aspects of peroxisomal fatty acid metabolism

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Peroxisomes are involved in the synthesis and degradation of complex fatty acids {1). They contain enzyme pathways mostly analogous to those present in mitochondria for beta-oxidation as well as a specific pathway responsible for alpha-oxidation. An alternative omega-oxidation pathway for metabolism of fatty acids exists which involves both microsomes and peroxisomes.
Biochemical defects in the assembly of peroxisomes can either have multiple biochemical effects if they affect the common PTS-1 signal domain used for protein import into the organelle or be more restricted in their phenotype if they affect the smaller number of enzymes imported through the PTS-2 system.Individual enzyme defects within the 60-200 proteins present in the peroxisome have also been characterised in many cases. The common spine for fatty acid metabolism in the peroxisome is the beta-oxidation pathway and it receives inputs at various points including from alpha-oxidation and also from racemisation of organic molecules including both fatty acids and bile acids(2). The chain length specificity of alpha-oxidation and maybe beta-oxidation is likely provided by the use of sterol carrier protein-2 which seems to act as a specific carrier molecule for these pathways (2). Short end-products are then exported through a carnitine-based transport pathway for further metabolism in the mitochondria.
Clinically peroxisomal diseases are associated with neurological signs affecting the central nervous system (e.g. blindness, deafness, ataxia), the peripheral nerves (neuropathies) and defects in growth and development (intellectual impairment, chondrodysplasia and osteogenesis). One example of where the role of the different peroxisomal pathways has been clarified is Refsum disease- a defect in alpha-oxidation of phytanic acid derived from food sources. The adult-onset phenotype comprises blindness, anosmia and associated neurological symptoms whose presence and severity depends on both the length of exposure and plasma levels of phytanic acid. The clinical severity of the phenotype depends not only on environmental factors (e.g. dietary intake of phytanic acid) but also probably on the relative remaining activity of the alpha- and omega-oxidation pathways.
Refsum disease is caused both by mutations in enzymes in the alpha-oxidation pathway and by defects in the PTS-2 signal driven import of these proteins (3). Different mutations in the Peroxin-7 carrier for proteins with PTS-2 signalling sequences give rise to a variety of phenotypes ranging from rhizomelic chondrodysplasia to Refsum disease. In many ways this is analogous to the mutations in the PTS-1 import pathway which give rise to phenotypes ranging from Zellweger disease to infantile Refsum disease but whose greater severity leads to clinical presentation at neonatal-childhood ages. All the mutations described to date in phytanoyl-coA hydroxylase, the principal enzyme affected in Refsum disease have been shown to be completely inactivate this 2-oxoglutarate-dependent oxygenase (4). While some mutations can be partially rescued in vitro with other chemical co-substrates as would be predicted from the alterations in the binding pocket, this approach has not yet been successful in vivo.

A number of partial phenocopies of Refsum disease have been described through defects in enzymes further down the pathway including in alpha methyl-acyl CoA racemase though some remain still to be identified. Defective alpha-oxidation results in accumulation of phytanic acid that due to its structure cannot be beta-oxidised. The mechanism of phytanic acid neurotoxicity has recently been partially elucidated with the finding that it acts to inhibit complex I metabolism in the mitochondrion in a manner analogous to rotenone. An alternative variable capacity pathway for phytanic acid metabolism exists through omega-oxidation. The enzymes involved in this pathway have recently been characterised and include a microsomal P450 system cytochrome allied to peroxisomal beta-oxidation (5).

This presentation will review the clinical-biochemical correlations of peroxisomal fatty acid metabolism and how these may be relevant to neurological disease.

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C62

Characterisation of recombinant alpha-methylacyl-CoA racemase, a novel prostate cancer target

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Branched-chain fatty acids and related molecules are important components of the human diet and are also used as drug molecules. The presence of a 2-methyl group on the carbon chain prevents 'normal' beta-oxidation in mitochondria, and these compounds are (initially) metabolised in peroxisomes. Important examples include bile acids (derived from cholesterol), phytanic acid (a dietary source of 2-methyl acids), and ibuprofen. These acids can exist as either the (R)- or (S)- isomers, but beta-oxidation is only possible for the (2S)-isomers. Alpha-methylacyl-CoA racemase (AMACR) catalyses chiral inversion of these acids (as their CoA esters), and thus regulates entry of metabolites into the beta-oxidation pathway. AMACR 1A is over-expressed in prostate, breast, colon and other cancers and is used as a prostate cancer marker, and several other splice variants are over-expressed in prostate cancer in addition to AMACR 1A. Reducing expression of AMACR 1A prevents proliferation of prostate cancer cells, suggesting it is a novel anti-cancer drug target. Despite this importance little is known about the biochemistry of the enzyme.

Human AMACR 1A was over-expressed in E. coli using the pET30 Xa vector and purified on around a 20 mg scale by metal-chelate chromatography. A chiral synthesis of substrate was developed from decanoic acid. The protected acid was specifically methylated using Evan's chemistry before deprotection and conversion to the CoA ester. Enzyme assays derivatised the CoA product mixture followed by gas chromatographic analysis. AMACR 1A can catalyse chiral inversion of the synthetic substrate 2-methyldecanoyl-CoA in both directions with an equilibrium ratio (2R/2S) of ~ 2:1. The enzyme has been characterised with respect to its catalytic properties. The results will facilitate biochemical studies on this important enzyme and its development as a drug target.

This work was supported by EU grant (QLG3-CT-2002-00696) and Cancer Research UK

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SA160

Mouse models for B-Raf-induced cancers

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Oncogenic mutations in the *BRAF* gene are detected in ~7% of human cancer samples with a particularly high frequency of mutation in malignant melanomas, papillary thyroid cancer, colorectal cancer, and serous ovarian cancer (1). Over 40 different missense *B-RAF* mutations have been found and these are clustered in either the glycine-rich P loop or the activation segment of the kinase domain. The vast majority of the mutations (>90%) represent a single nucleotide change resulting in a valine to glutamic acid substitution at residue 600 within the activation segment (^{V600E}B-RAF). It has been shown that ^{V600E}B-RAF is able to stimulate endogenous MEK and ERK1/2 phosphorylation leading to an increase in cell proliferation, cell survival, transformation, tumourigenicity, invasion and vascular development. Many of these hallmarks of cancer can be reversed by treatment of cells with siRNA to *B-RAF*, indicating that B-RAF is an attractive therapeutic target. However, ^{V600E}B-RAF mutations are detected in benign naevi and premalignant colon polyps which would suggest that this oncogene is not sufficient to induce human tumourigenesis on its own and may require mutations in other genes, particularly tumour suppressor genes (TSGs), to unleash its tumourigenic effects.

In a recent publication (2), a more detailed characterisation of the *B-RAF* mutations detected in human cancer samples was performed. The mutants were grouped into three different classes depending on their level of activity in COS cell transfections. High activity mutants were classed as those having more basal kinase activity than ^{WT}B-Raf stimulated by ^{G12V}RAS with ^{V600E}B-RAF being an example of this class. Intermediate activity mutants were classed as those having more basal kinase activity than ^{WT}B-Raf but less than ^{WT}B-Raf stimulated by ^{G12V}RAS. Impaired activity mutants were classed as those having ~30-80% less basal

kinase activity than ^{WT}B-Raf. The high and intermediate activity mutants all stimulated endogenous MEK and ERK1/2 phosphorylation and all impaired activity mutants except one also stimulated ERK phosphorylation via activation of endogenous C-RAF. The one remaining impaired activity mutant, ^{D594V}B-RAF, did not stimulate ERK phosphorylation or C-RAF activity suggesting that its role in human oncogenesis is complex.

Many questions need to be addressed in understanding the role of oncogenic *B-RAF* in human cancer development. For example, we need to gain complete insight into the role of oncogenic *B-RAF* in cancer development *in vivo*, whether it collaborates with TSGs or other oncogenes, and what downstream signalling pathways are activated by this oncogene. It is also important to understand how each of the three classes of *B-RAF* mutants contribute to tumourigenesis and the role of C-RAF in mediating their effects. In order to address these various questions, we have used gene targeting in mice to generate conditional knockin alleles, using Cre-lox technology, that are characteristic of each class of *B-RAF* mutant found in human cancer (3). In these alleles, expression of oncogenic *B-raf* is dependent on the presence of the Cre recombinase; in its absence wild-type *B-raf* is expressed. Our research is aimed at using specific Cre activation methods to induce oncogenic *B-raf* expression in various adult somatic tissues, with particular emphasis on melanocytes and colonocytes. Progress on our work with these mouse models will be discussed.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA161

Control of DNA damage tolerance by ubiquitin and SUMO

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Posttranslational protein modifiers of the ubiquitin family play a crucial role in many aspects of genome stability. In particular, tolerance to replication-blocking DNA lesions in eukaryotes is achieved by the ubiquitylation of the sliding clamp protein PCNA, an essential processivity factor for replicative DNA polymerases (1). While polyubiquitylation of PCNA facilitates an error-free damage avoidance mechanism that makes use of the genetic information encoded by the undamaged sister chromatid, we have shown that monoubiquitylation of the clamp activates specialised, damage-tolerant polymerases for mutagenic lesion bypass (2). Thus, differential modification of PCNA influences not only the efficiency, but also the accuracy of DNA replication in the presence of DNA-damaging agents. During S phase in the absence of exogenous DNA damage PCNA from budding yeast is modified by the ubiquitin-like protein SUMO, which in turn recruits a helicase, Srs2, to replication fork, thereby preventing unscheduled recombination events (3). I will describe the mechanisms by which the two modifiers cooperate in Saccharomyces cerevisiae and change the properties of the clamp, and I will discuss our observations that give insight into the cellular signals necessary for PCNA modifications.

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I would like to thank the members of my lab, Aleksandra B. Bielen, Shuhua Chen, Adelina A. Davis, Diana Huttner, Efterpi Papouli, Joanne L. Parker and Philipp Stelter, who have contributed to this work.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C63

A role for DNA-PK in a checkpoint linking histone gene expression with DNA replication

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Packaging of DNA into chromatin is essential for appropriate gene expression and the distribution of genetic material during cell division. Throughout the cell cycle, chromatin structure is maintained by balanced DNA replication and histone synthesis during S phase. DNA replication and histone synthesis are coupled and ongoing DNA replication is required to maintain histone gene expression.

Cell cycle checkpoints control the timing and order of cell cycle events following genomic insult. Activation of checkpoint pathways by replicational stress or DNA damage inhibits the progression of the cell division cycle, by inactivating the cell cycle machinery. ATR, ATM, Chk1, and Chk2 are checkpoint kinases involved in cell cycle arrest in mammalian cells. ATM and ATR belong to the phosphatidylinositol 3-kinase-like kinase (PIKK) family. ATM activation is mainly triggered by the formation of DNA double-strand breaks, whereas ATR is activated by aberrant DNA structures induced by UV light, DNA synthesis inhibitors or chemotherapeutics. A further member of the PIKK family is DNA-activated protein kinase (DNA-PK). DNA-PK is primarily required for DNA double-strand break repair by nonhomologous end joining.

Here we investigate the role of check point kinases in coupling DNA replication with histone synthesis, which occurs mainly via the control of histone mRNA stability. Exposure of aphidicolin- or hydroxyurea-treated cells to kinase inhibitors, caffeine and LY294002, uncouples DNA replication from histone mRNA stability, by altering the efficiency of histone mRNA decay triggered

by replicational stress. Specific interference with caffeine-sensitive checkpoint kinases alone has no effect on the efficiency of histone mRNA decay, indicating that ATR/ATM signalling alone cannot account for the linkage between replication and histone mRNA stability. LY294002 potentiates the ability of caffeine to uncouple histone mRNA stability from DNA replication, but only in cells containing functional DNA-PK, indicating that DNA-PK is the target of LY294002 in this process. DNA-PK is activated during replicational stress, and signalling through DNA-PK is enhanced when ATR/ATM signalling is abrogated, suggesting strongly that DNA-PK plays a role in this process. Our data show for the first time a role for DNA-PK in an intra S-phase checkpoint pathway coordinating DNA replication with histone protein synthesis. This role of DNA-PK was revealed when caffeine sensitive checkpoint kinases were inhibited, indicating that several signalling pathways link these two important processes.

This works was supported by funding from CR-UK (C.S.), AICR (C.S), Wellcome Trust (B.M and C.S) and Tenovus Scotland (BM).

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C64

Identification of peptide aptamers that modulate mutant P53 oncogenic functions

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The transcription factor p53A is a key tumour suppressor gene often lost in human tumours. In response to different cellular stresses, such as DNA damage, it can induce temporary growth arrest and DNA repair, irreversible growth arrest or apoptosis [1-3].

Mutations in the central DNA-binding domain (DBD) of the p53 gene are the most common genetic alterations in cancer; they indeed occur in about half of all human tumours [4]. P53 mutants are defective in sequence-specific DNA binding, in fact, the six most frequent cancer-associated mutations in p53 ("hotspot mutations") map to the DBD [4]. Frequently mutated residues are important also for protein stability and involved in protein binding. Inactivation of p53 due to mutations is then not only due to the loss of DNA binding but also to the loss of the ability to bind partner proteins, probably followed by the acquisition of new partners. Competition among the different proteins and DNA for a single site in p53 could be a factor in regulation of mutant p53 activity and a target for the modulation of its functions.

Given the active role of p53 mutants in promoting tumorigenicity, many efforts have been made to inactivate their function or restore wild-type activity, employing various strategies (peptide 46, CDB3, PRIMA-1, CP-31398, single chain antibody ME1), which are effective in vivo, but may not have a well characterized mechanism of action [5-9].

Here we describe the docking of small peptide aptamers with R273H p53 mutant. These peptides specifically bind the core region of different mutant p53 proteins and modulate mutant p53 functions.

The results obtained have direct therapeutic implications, as the peptide aptamer molecules, specifically targeting crucial disease proteins, should possess therapeutic potential as leading structures for both drug design and the development of protein drugs.

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SA162

Keeping DNA damage and replication in Chk to counter cancer

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DNA damage, blocks to DNA replication, decatenation defects, and mitotic spindle perturbations all trigger "checkpoint" responses which delay cell cycle progression, promote repair, and protect genome integrity in all eukaryotes from yeasts to humans. The Chk1 protein kinase is a key regulator of multiple checkpoint responses in vertebrate cells, however the underlying biochemical mechanisms are poorly understood. We are using a combination of genetics and biochemistry in mice and cultured cells to address two principal questions: 1) How do checkpoints work at the molecular level, and 2) Are checkpoints required for the evolution of tumour cells during carcinogenesis and do they help them to survive exposure to radiation and genotoxic anti-cancer drugs? Our aim is to identify rational strategies through which pharmacological inhibition of checkpoints can be exploited for therapeutic purposes.

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Work in the authors laboratory is supported by Cancer Research UK (CR-UK) and the Association for International Cancer Research (AICR)

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SA163

Cancer genetics: mouse models of cancer

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The capacity to model cancer within the mouse has advanced significantly in recent years. Perhaps the most notable technical gains have been in the development of techniques that allow the temporal and spatial control of gene expression, such that it is now possible to regulate target genes in the tissue of choice and at a given time. We have used these approaches to study tumorigenesis in the murine intestine and mammary gland. Loss of function of the tumour suppressor gene Adenomatous Polyposis Coli (Apc), has been associated with the development of both human and murine neoplasias, principally those of the intestinal epithelium. However, as Apc has been implicated in multiple cellular functions, the precise mechanisms underlying these associations remain somewhat unclear. We describe here the use of an inducible strategy to co-ordinately delete genes from the adult murine epithelium. This approach has allowed us to characterise in detail the direct consequences of inactivation of gene function. For Apc, these include failure in the differentiation programme, failure to migrate, aberrant proliferation and the aberrant induction of apoptosis. We have then been able to use this approach to directly test the efficacy of therapeutics, such as curcumin, upon these endpoints. Transcriptome analysis of this model has also identified potential new targets for therapeutic intervention, such as Sparc; deficiency of which we have now shown suppresses adenoma formation. Finally, we have been able to address how other genes modulate the consequences of Apc loss. Thus, we show that little effect following loss of CyclinD1, Tcf1 and p53; but marked differences following loss of either c-Myc or Mbd2. The models are therefore allowing us to define the earliest events associated with carcinogenesis in the intestine. Outside the intestine, we have used parallel approaches to generate murine models of mammary neoplasia, and we are now using these to accelerate the therapeutic assessment of novel agents, such as inhibitors of Parp activity.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA164

From blood doping to EPO

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To enhance physical performance some athletes have used various methods to increase red cell mass. The methods include 1. blood doping or the transfusion of autologous or homologous blood or erythrocytes, 2. administration of EPO (erythropoietin) and 3. administration of synthetic oxygen carriers. These methods have been introduced in medical practice to improve physical function and quality of life in patients with low blood count from acute blood loss, cancer or renal disease.

The methods, which lead to marked improved aerobic performance, are all prohibited under the World Anti-Doping Agency's (WADA) List of Prohibited Substances and Methods.

Blood doping has been in used over the last half-century but became less common when EPO was introduced in the 1980s. Since the introduction of tests for exogenous EPO it is likely that blood doping has become more common again.

Blood doping and administration of EPO or synthetic oxygen carriers have severe potential side effects including hypertension, heart attack, stroke and embolism.

Detection is possible for EPO and synthetic oxygen carriers. In 2004, a test was introduced for homologous blood transfusion. There is intense research ongoing to improve the specificity and sensitivity of the various detection methods. In many sports, individual haemoglobin levels are continuously monitored and maximal safety levels for competition have been introduced.

With the advancement of gene therapy and gene transfer technologies it is likely that EPO gene doping will be used in the future. Detection research projects focus on identifying "fingerprints" that may differ from makers of endogenous EPO.

The effects of EPO may be broader than erythropoiesis alone. E.g., EPO has been shown to influence angiogenesis which may improve local substrate delivery in heart and skeletal muscle.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA165

Regulation of muscle mass by growth hormone and IGF-I

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The growth hormone (GH) - insulin like growth factor I (IGF-I) axis plays a major role in the regulation of post natal growth. Overexpression of GH and IGF-I in mice leads to an overall increase in body size whereas animals deficient in GH or IGF-I are much smaller than their wild type counterparts. In humans, GH deficiency in adulthood is associated with decreases in lean body mass, of which skeletal muscle is a major component, and muscle strength. Long term GH administration is effective in increasing muscle mass and strength in these GH deficient patients.

The effects of GH are classically thought to be mediated by IGF-I. Indeed, IGF-I expression in many tissues, including human skeletal muscle, is regulated by GH. Both GH and IGF-I exert their effects on growth and metabolism by interacting with specific receptors which are ubiquitously expressed in the tissues of the body. The mechanisms by which GH and IGF-I regulate muscle mass are complex and involve processes such as protein synthesis and the proliferation, differentiation, and survival of satellite cells. A key consideration is whether GH exerts its effects on skeletal muscle independently of IGF-I, as it is now recognized that GH has direct actions mediated by the GH receptor in many tissues including muscle. Further complexity arises from the fact that various isoforms of GH and IGF-I exist, but the precise biological role of each of these isoforms remains to be established. In addition, a number of other proteins, in particular IGF-I binding proteins (IGFBPs), affect the bioavailability and activity of their ligands.

GH is currently administered as a therapeutic agent for the treatment of muscle wasting secondary to AIDS and cancer, and therapeutic approaches for IGF-I are being developed. The effectiveness of these agents in preventing muscle loss and/or increasing muscle mass in disease states has led to their use by normal individuals attempting to manipulate their muscle mass. There is, unfortunately, substantial anecdotal evidence for the widespread use of GH, and possibly IGF-I, by body builders as well as other athletes, a practice known as doping. Despite this, most of the data obtained under controlled experimental conditions does not show performance or muscle mass gains following GH administration in adults with normal GH endocrinology. Nevertheless, the use of GH as well as IGF-I is banned by the World Anti Doping Agency. As a result, considerable resources are being invested in the development of methods to detection of the use of recombinant human GH (rhGH). This is challenging because rh GH is essentially identical to the endogenously produced 22kD isoform of the hormone. Current strategies for the detection of rhGH administration rely on alterations in the ratio of 22kDa to other naturally occurring isoforms and the use of indirect markers of GH administration such as IGF-I and metabolites of bone and collagen turnover.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C65

Pre-supplementation with vitamins C and E does not reduce indices of exercise-induced muscle damage in men

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Reactive oxygen species production has been implicated in exercise-induced muscle damage following eccentric exercise. Studies on the protective effects of dietary antioxidant supplementation (AS) on muscle damage have been equivocal. However, there is some evidence that AS may attenuate indices of muscle damage (Jakeman *et al.*, 1993; Shafat *et al.*, 2004). The aim of the current study was to investigate the effect of pre- and post-supplementation with vitamins C and E on symptoms of eccentric muscle damage in males. An AS regimen was designed to maximise the antioxidant content of the exercising muscles. The study employed a randomised, double-blind, placebo-controlled, crossover design. Six males (24±4 years, 175.8±8.3 cm, 74.0±6.4 kg, mean±S.D.) were randomly allocated into two groups, vitamin supplementation (V) or placebo (P). The V group received 2x500 mg of vitamin C and 2x600 IU of α-tocopherol daily and the P group received similar tablets for 38 days (28 days pre-, 10 days post-exercise). On day 29 volunteers performed 150 maximal eccentric contractions of the knee extensors (50 sets of 3 at one minute intervals) using a randomly selected leg at a velocity of 0.52 rad.s^{-1}, while lying prone on an isokinetic dynamometer. Seated maximal voluntary isometric contraction force (at 90° knee angle) and electrically evoked force at a frequency of 20 Hz and 50 Hz were recorded before and immediately after exercise, and on days 1, 3, 5, 7 and 10 post-exercise. On the same occasions and on day two, muscle soreness was recorded for the quadriceps muscle group using self palpation at 6 points, with results recorded on a ten-point soreness scale. After a 69-day washout period the subjects received the alternate supplement for 38 days, performing the eccentric exercise protocol on the contralateral leg. Maximal voluntary isometric force (Fig. 1) and 20:50 Hz force ratio (Fig. 2) decreased significantly in both groups ($P<0.001$, RM-ANOVA; post-hoc analysis of means and confidence intervals), with no difference seen between treatments. Muscle soreness increased significantly ($P<0.01$, RM-ANOVA) peaking on day 2 following both treatments, and gradually returning to baseline by day 10. These results suggest that prophylactic supplementation with vitamins C and E does not ameliorate the functional deficit caused by eccentric exercise in men.

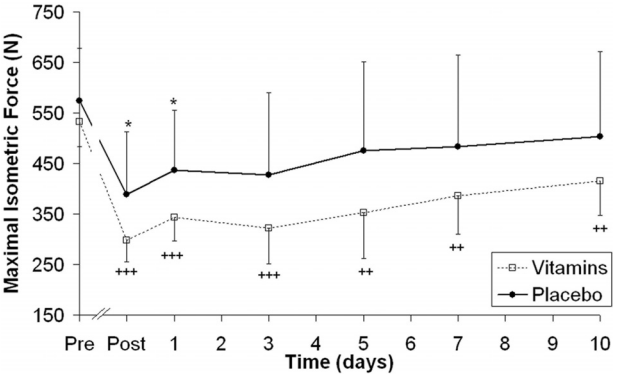

Figure 1. Mean ± S.D. of maximal isometric force after eccentric exercise. +++ $P<0.001$, ++ $P<0.01$ compared to pre-test for V (n=6). * $P<0.05$ compared to pre-test for P (n=6).

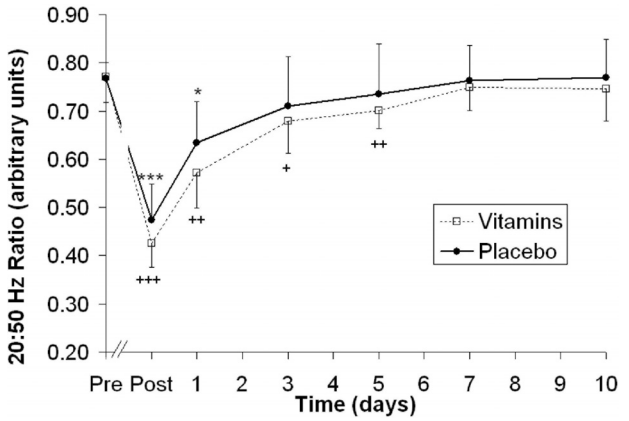

Figure 2. Mean ± S.D. of 20:50 Hz ratio after eccentric exercise. +++ P<0.001, ++ P<0.01, + P<0.05 compared to pre-test for V (n=6). *** P<0.001, * P<0.05 compared to pre-test for P (n=6).

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C66

Stretch-induced activation of anabolic pathways in C2C12 cells involves ERK activation

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Mechanical strain has been demonstrated to be a potent stimulus of skeletal muscle hypertrophy. Furthermore others and we have also shown that mechanical stimuli can modulate muscle phenotype through specific changes in gene expression (1). The mechanisms by which alterations in mechanical environment are transduced into anabolic stimuli and specific changes in gene expression have still to be elucidated. We have recently produced evidence for the calcineurin/NFAT pathway and p38 playing a crucial role in stretch-induced alteration of phenotype in skeletal muscle cells (2,3). Studies have indicated, however, that the calcineurin pathway in not integral to muscle hypertrophy (4,5). In the present study we examined the role of other intracellular signalling pathways upon stretch induced anabolism. The inhibitor of mTor, Rapamycin (Rap) and the MEK/ERK inhibitor PD98059 (PD) both caused a marked reduction in stretch induced elevation of protein synthesis in C2C12 myotubes. We therefore examined the effects of static stretch upon components of both the Akt/mTor and ERK pathways that are known to regulate translation and protein synthesis. Passive stretch, suprisingly, had no effect upon the levels of activated Akt or mTor. In contrast the MAP Kinase ERK1/2 showed significant and rapid activation in response to stretch. We also observed that p70S6K a serine/threonine kinase involved in regulating translation was activated in response to stretch. Another key step in the translational control of protein synthesis occurs at the level of the eukaryotic initiation factor 4F (eIF4F) a major regulatory part of which eIF4E was shown to be activated by static stretch. eIF4E function is regulated in part by its association with the repressor protein 4E-BP1. We observed that this factor was activated in response to stretch, as was Mnk1 an activator of the eIF4F complex. We have also carried out studies on the effects of Rap and PD upon stretch-induced activation of the components of the stretch-induced pathway regulating protein synthesis. Interestingly Rap alone had no effect upon activated ERK levels but in combination with stretch produced a marked reduction. Surprisingly neither PD nor Rap had a significant effect upon 4E-BP1 levels with or without stretch. PD alone caused a fall in activated ERK levels but this was greatly exacerbated when combined with stretch. However PD in combination with stretch also produced a significant reduction in activated mTor. These data together suggest that the ERK pathway is the major regulator of protein synthesis in response to stretch but suggest that a more complex interaction with the mTor pathway may take place.

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This work was funded by a grant from the B.B.S.R.C.

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SA166

Testosterone, muscle and satellite cells

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Nowadays it is well known that testosterone induce a spectacular hypertrophy of skeletal muscle. Due to their strong myotrophic action and despite their serious and irreversible side effects, anabolic steroids are widely used among athletes and also subjects who simply want to improve their appearance. Randomized, placebo-controlled studies clearly show that the administration of supra-physiological doses of testosterone in untrained and trained subjects produces a significant increase in muscle strength and in the cross-sectional area of skeletal muscle (1). Important mechanisms behind the myotrophic effects of testosterone on skeletal muscle were uncovered for the first time in a population of power lifters who have reported the use of testosterone (100 to 500 mg/week) in combination to a wide variety of anabolic steroids for a period of 9 ± 3,3 years (2). Long-term steroid usage accentuates the degree of fibre hypertrophy in already well-trained power-lifters (2). In steroid users, the elevated myonuclear content together with the strong correlation between fibre area and the number of myonuclei ($r = 0.86$; $p < 0.0001$) suggested for the first time that a main mechanism by which testosterone exerts its myotrophic effect is to enhance the proliferation of satellite cells which is followed by myonuclear

accretion (2). All the findings observed in skeletal muscle of long-term steroid users were subsequently confirmed in well-controlled short-term studies where subjects were given supra-physiological doses of testosterone (1). Satellite cells in skeletal muscle represent a population of stem cells located between the plasma membrane and the basal lamina and are capable of entering the cell cycle to generate daughter cells, which can either become new myonuclei or fuse together to form new myofibres that can fuse with existing muscle fibres (1). In this respect, skeletal muscle of long-term steroid users is characterised by a high frequency of fibres with centrally located myonuclei and also fibres in instance of fusing together (2). It is important to note that myonuclear addition would occur only when existing myonuclei become unable to sustain the growth of the muscle fibre (ceiling size concept) (1). In addition to the alterations in the nuclear machinery, testosterone also increases net protein synthesis and reutilization of intracellular amino acids in skeletal muscle (3). When satellite cells proliferate, some daughter cells may escape differentiation and remain quiescent (1). In this respect, in long-term steroid users, the number of satellite cells is similar to that counted in well-trained athletes but is higher to that found in untrained subjects (2). However, further studies are warranted to examine the regenerative capacity of long-term steroid users. In addition to its effects on satellite cells, it is also suggested that testosterone favours the commitment of pluripotent precursor cells into myotubes and decreases the number of adipocytes by down regulating key transcription factors involved in the adipogenic differentiation (4). The effects of testosterone on skeletal muscle are thought to be mediated via androgen receptors. When the hormone-receptor complex is translocated to the hormone responsive element within the nucleus it induces an increase in the rate of transcription. In normal resting skeletal muscle, androgen receptors are expressed in some but not all myonuclei (2), and within the same subject, differences in androgen receptor content exist between the trapezius and the vastus lateralis muscles (2). The amplitude of changes in androgen receptor content following training is also muscle dependent (1). Androgenic-anabolic steroids may either up-regulate or down regulate androgen receptor content. Long-term self-administration of steroids in humans is associated with increased androgen receptor-containing myonuclei in the trapezius but not in the vastus lateralis (2) indicating that in humans, the effects of testosterone can vary between muscles. In this respect, testosterone action might also be mediated through an androgen receptor independent pathway as it has been shown that testosterone stimulates G protein–linked membrane receptor at the plasma membrane resulting in a calcium dependent phosphorylation of a member of the MAPK family (5). Clearly, further studies are warranted to better understand the molecular pathways behind the physiological and supra-physiological action of testosterone on skeletal muscle.

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SA167

Gene doping: potential and limitations

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Treatment of skeletal and cardiac muscle using gene transfer is a major goal for diseases such as Duchenne muscular dystrophy (1). However, recent developments have raised fears about the potential for abuse in athletic competition. Some of the uses of gene transfer are relatively easy to detect in standard samples, for example the glycosylation pattern of erythropoietin produced from skeletal muscle differs from that produced by the kidney (2). In contrast, local genetic modification of skeletal muscle may be much harder to detect without taking invasive biopsies which are not currently part of the testing regime for athletes. Improvements in viral vector technology have the potential to genetically modify large amounts of muscle to change phenotype or improve repair and the hypertrophic response to training. With current technologies one can produce increased gene expression that supports increased muscle mass, e.g. insulin-like growth factor-1, IGF-1 (3), or local production of agents that block the action of the negative regulator of muscle mass, myostatin. The presentation will review the limits currently in place that reduce the potential to exploit gene transfer/inhibition technology, as well as possible methods to detect such genetic manipulation. Provided the products do not leak out into the general circulation, this type of modification will be hard to detect indirectly except by looking for antibodies to the delivery system, for example an adeno-associated viral vector. An alternative approach will be to examine the metabolic profile of the individual looking for abnormal patterns of different proteins and/or metabolites, such as is used for the detection of testosterone abuse.

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SA168

Proteinases and signalling - to PAR or Bogey: Pathophysiological and therapeutic implications via PARs and more

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Proteinases like thrombin, trypsin and tissue kallikreins are now known to regulate cell signaling by cleaving and activating a novel

family of G-protein-coupled Proteinase-activated Receptors (PARs 1 to 4) via exposure of a tethered receptor-triggering ligand (Pharmacol. Rev. 54:203, 2002). On their own, short synthetic PAR-selective PAR-activating peptides mimicking the tethered ligand sequences (PAR-APs) can activate PARs 1, 2 and 4 and cause physiological responses both in vitro and in vivo. Using the PAR-APs as probes in vivo, it has been found that PAR activation can affect the vascular, renal, respiratory, gastrointestinal, musculoskeletal and nervous systems (both CNS & PNS) and can promote cancer metastasis and invasion. In general, responses triggered by PARs 1, 2 and 4 are in keeping with an innate immune inflammatory response, ranging from vasodilatation to intestinal inflammation, increased cytokine production and increased nociception. Further, PARs have been implicated in a number of disease states including cancer and inflammation of the cardiovascular, respiratory, musculoskeletal, gastrointestinal and nervous systems. In addition to activating PARs, proteinases can cause hormone-like effects by other signaling mechanisms that may be as important as the activation of PARs. Thus, the working hypothesis of the presentation will be that the PARs themselves, their activating serine proteinases or their associated signaling pathways can be considered as attractive targets for therapeutic drug development; and that proteinases in general must now be considered as 'hormone-like' messengers that can signal either via PARs or other mechanisms.

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Supported by the Canadian Institutes of Health Research and an Alliance Project Grant from Servier International.

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SA169

β-arrestin-dependent, G-protein-independent PAR-2 signaling

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β-arrestins are pleiotropic molecules that mediate signal desensitization, G-protein-independent signaling, scaffolding of signaling molecules, and chemotaxis(1). Protease-activated-receptor-2 (PAR-2), a G-protein-coupled receptor, which has been proposed as a therapeutic target for inflammation and cancer, requires the scaffolding function of β-arrestins for ERK1/2 activation and chemotaxis(2-5). We have hypothesized that PAR-2 can trigger specific responses by differential activation of two pathways, one through classic Gαq/Ca2+ signaling and one through β-arrestins, and we hypothesized that the latter involves scaffolding of proteins involved in cell migration and proliferation. Our most recent studies have elucidated 3 different G-protein independent signaling pathways downstream of PAR-2: 1) PAR-2 spatially controls the activity of an actin filament severing protein (cofilin) through the formation of a β-arrestin-dependent scaffolding complex comprised of cofilin and its upstream regulators (LIM Kinase and the phosphatase Chronophin). β-arrestin-dependent cofilin activation is independent of Gαq/Ca2+ signaling and is essential for directed cell migration. 2) PAR-2 can promote either Gαq-dependent activation or β-arrestin-dependent inhibition of PI3K, in a cell-type specific fashion. 3) PAR-2 evoked ERK1/2 activation is mediated through both G-protein and β-arrestin-dependent pathways-leading to distinct cellular outcomes. While G-protein-dependent ERK1/2 activation leads to proliferation, β-arrestin-dependent ERK1/2 activation leads to cell migration. These studies have major implications for PAR-2 as a therapeutic target. First, they point to the importance of evaluating both G-protein dependent and β-arrestin-dependent signaling when screening receptor agonists and antagonists. Second, they raise the possibility that pathway-specific agonists and antagonists could be developed. Third, they suggest that the outcome of PAR-2 activation may vary between tissues and cell types, depending on the expression patterns of β-arrestins, Gαq, or other components of each pathway.

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These data are the result of the hard work of Dr. Lan Ge, Maria Zoudilova, Puneet Kumar, Mike Lau, Maneesh Mathur, Youly Ly and Dr. Ping Wang (students and post-docs in the DeFea lab). We thank Dr. Morley Hollenberg for antibodies and advice, Dr. Robin Plevin for PAR2 knockout mice, Dr. Bob Lefkowitz for β-arrestin knockout mice and cells, Dr. Gary Bokoch for cofilin reagents, and Dr. Christian Lytle for help with animal studies and Calcium assays.

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SA170

Gastro-intestinal roles for proteinase-activated receptors

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Proteinase-activated receptors (PARs), a family of G protein-coupled receptors consisting of four members, are now known

to mediate cellular actions of several proteinases in the mammalian body. In the gastrointestinal (GI) tract, PARs, particularly PAR1, a receptor for thrombin, and PAR2, a receptor activated by trypsin, tryptase, etc., are widely distributed, modulating various functions in health and disease.

One of the major functions of PARs in the GI systems is to regulate glandular exocrine secretion. PAR2-activating peptides cause prompt salivation in laboratory animals, an effect disappearing in PAR2-deficient animals. Since PAR2-related peptides also trigger tear secretion through PAR2 and non-PAR2 mechanisms, PAR2 could be a therapeutic target for treatment of secretion disorders. Activation of PAR2 also stimulates exocrine secretion of pancreatic juice and enzymes in vivo as well as in vitro. In the gastric mucosa, PAR2-activating peptides trigger secretion of mucus via excitation of capsaicin-sensitive sensory neurons (Kawabata A *et al.* 2001) and secretion of pepsinogen by chief cells, but suppress acid secretion. Regulation of intestinal ion transport by PAR2 and PAR1 has also been well described. Thus, PARs, particularly PAR2, appear to play critical roles in regulation of exocrine secretion. Regulation of smooth muscle tone by PAR1, PAR2 and PAR4 has also been well documented in the esophageal, gastric and intestinal tissues.

The roles played by PARs in GI diseases are a little complicated. We have shown that PAR2 agonists protect against gastric mucosal injury through stimulation of sensory neurons (Kawabata A *et al.* 2001), while PAR1 agonists exert gastric mucosal protection through stimulation of prostanoid formation (Kawabata A *et al.* 2004). In a rat normal gastric epithelial cell line, PAR1 stimulation activates multiple signaling pathways, leading to delayed formation of prostaglandin E2 (Sekiguchi F *et al.* 2007. Both anti- and pro-inflammatory roles for endogenous proteinases and/or PAR1/PAR2 have been demonstrated in distinct animal models for colitis. Most interestingly, PAR2 present in the sensory neurons or epithelial cells appears to participate in the pathogenesis of irritable bowel syndrome (IBS). Some studies have emphasized the benefit of therapeutic use of proteinase inhibitors and antagonists of PAR2 or PAR1 for treatment of the gastroesophageal reflux disease, inflammatory bowel disease, IBS, etc. In the pancreas, PAR2 is expressed by both sensory neurons and acinar cells. Stimulation of PAR2 in the sensory neurons causes pancreatic pain, and inhibitors of PAR2-activating proteinases can reverse the referred pain accompanying the established pancreatitis. In contrast, at the early stage of pancreatitis, PAR2 present in the acinar cells might mediate secretion of pancreatic proteinases into the duodenal lumen followed by decreased content of proteinases in the pancreas, leading to protection against the development of pancreatitis and related pain (Kawabata A *et al.* 2006; Singh VP *et al.* 2007). PAR2 is thus considered pro-inflammatory/nociceptive and also protective/antinociceptive.

Taken together, it is no doubt that activation of PARs, particularly PAR2 and PAR1, plays critical roles in the GI tract in health and disease, regardless of favorable/unfavorable outcomes.

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SA171

The role proteinase-activated receptors in lung inflammation and fibrosis

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There is strong evidence that the extravascular activation of coagulation proteinases contributes to both inflammation and fibrosis in response to tissue injury in a number of organs, including the lung. Extravascular intra-alveolar accumulation of fibrin, often evident as hyaline membranes, is commonly observed in the lungs of patients with pulmonary fibrosis, in acute lung injury (ALI) and in the acute respiratory distress syndrome (ARDS), in which rapid fibroproliferation and matrix synthesis can lead to the development of extensive fibrotic lesions. Excessive procoagulant activity and intra-alveolar fibrin deposition observed in these conditions is thought to arise from an imbalance between locally produced pro- and anti-coagulant factors, in combination with leakage of plasma proteins (including fibrinogen) into the alveolar space. We and others have shown that modulation of procoagulant activity within the alveolar compartment attenuates lung inflammation and fibrosis in response to bleomycin-induced lung injury in experimental animals.

In addition to their critical role in blood coagulation, it is now well recognised that coagulation proteinases exert potent pro-inflammatory and pro-fibrotic effects via proteolytic activation of proteinase-activated receptors (PARs). The PARs currently comprise four members, PAR1 to PAR4, which are activated by a unique mechanism involving the unmasking of a tethered ligand by limited proteolysis. Collectively, the proteinases of the coagulation cascade can target all four family members. Thrombin is considered to be a major activator of PAR1, PAR3 and PAR4; whereas factor Xa, on its own or as part of the tissue factor-factorVIIa-factor Xa ternary complex, activates either PAR1 or PAR2 depending on cell-type.

Current in vitro evidence suggests that PAR1 is one of the major receptors by which coagulation proteinases exert a range of potent pro-inflammatory and pro-fibrotic effects. In vivo evidence for the potential importance of this receptor was obtained in studies performed in our laboratory demonstrating that PAR1 deficiency affords protection from microvascular leak, inflammatory cell recruitment and lung fibrosis in response to bleomycin injury. This protection was accompanied by significant reductions in pulmonary levels of the potent PAR1-inducible mediators, MCP-1 (CCL2), TGF-beta1 and CTGF/FISP12. We have further shown that PAR1 is highly expressed in inflammatory and fibroproliferative lesions in lung sections obtained from patients with fibrotic lung disease. Recent unpublished work using microarray analysis to generate a transcriptional profile of bleomycin injury in mice revealed a large number of chemokines and their receptors which are regulated during the fibrotic phase of lung injury. To our surprise, expression of the gene for coagulation FX was dramatically upregulated, suggesting that part of the procoagulant activity in

lung fibrosis may be due to locally produced coagulation zymogens. Immunohistochemistry and real-time RT-PCR analysis of laser-capture microdissected lung biopsies from patients with pulmonary fibrosis confirmed that FX mRNA and protein are significantly upregulated in this condition. Subsequent in vitro studies further demonstrated that FXa was a potent inducer of fibroblast to myofibroblast differentiation via activation of PAR1 but not PAR2. Myofibroblasts are the major effector cells in pulmonary fibrosis and are responsible for the elaboration of excessive extracellular matrix proteins within the lung parenchyma. In conclusion, these data place PAR1 as one of the critical receptors involved in orchestrating the interplay between coagulation, inflammation and remodelling in response to lung injury. We propose that strategies aimed at blocking this receptor may prove useful for a number of respiratory conditions associated with excessive activation of the coagulation proteinases within both intra- and extra-vascular compartments.

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This work was supported by research grants from the Wellcome Trust, the Medical Research Council, UK and the British Lung Foundation.

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C67

A protease-activated receptor-2 (PAR-2)-interacting protein, Jab1, is involved in PAR-2-induced activation of AP-1, and PAR-2-induced release of chemokine GRO/CINC-1 from rat astrocytes via differential JNK activation protects brain tissue

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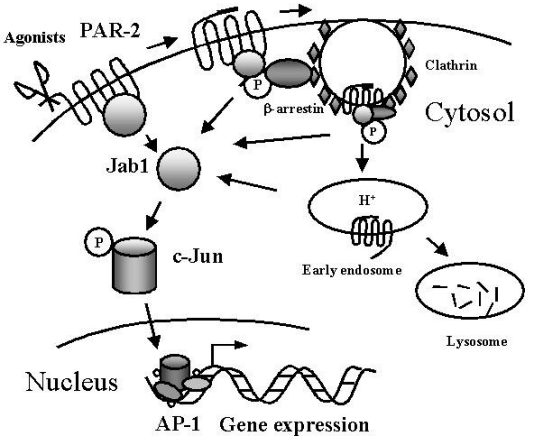
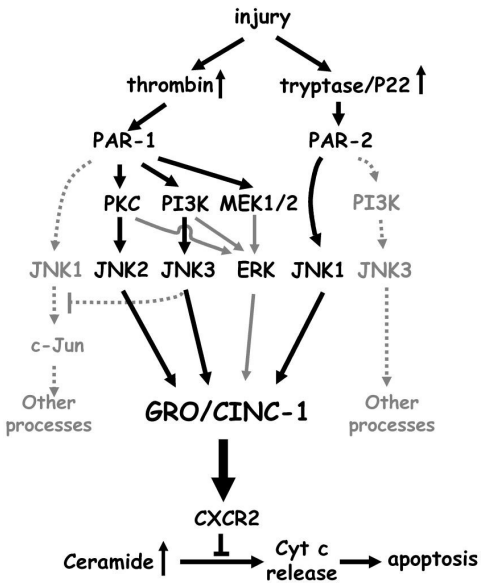
Protease-activated receptor-2 (PAR-2), a receptor for trypsin and tryptase, has important physiological /pathological functions. PAR-2-mediated intracellular signal transductions are hardly known. Here, we identified an interacting partner of human PAR-2, the Jun activation domain-binding protein 1 (Jab1). The interaction was shown by GST pull-down in vitro, and by co-immunoprecipitation assays in vivo. Jab1 was shown to be colocalized with PAR-2 in transfected HEK293 cells and in primary human astrocytes by double immunofluorescence staining. Multiple intracellular domains of PAR-2 are required for the interaction with Jab1. Agonist stimulation of PAR-2 disrupted the interaction, which could be prevented by the inhibitor of receptor endocytosis phenylarsine oxide, but not by the lysosomal protease inhibitor ZPAD. Activation of PAR-2 induced the redistribution of Jab1 from the plasma membrane to the cytosol, but did not influence expression of Jab1. Furthermore, Jab1 mediated PAR-2-induced c-Jun activation, which was followed by increased activation of activator protein-1 (AP-1). Loss-of-function studies, using Jab1 small interfering RNA, demonstrated that Jab1 knockdown blocked PAR-2-induced AP-1 activation. Jab1 is an important effector that mediates a novel signal transduction pathway for PAR-2-dependent gene expression.
Activation of PAR-1 and PAR-2 both resulted in release of the chemokine growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1), a functional counterpart of human interleukin-8, from rat astrocytes. PAR-2-induced GRO/CINC-1 release was independent of protein kinase C, phosphatidylinositol 3 kinase and mitogen-activated protein kinase kinase 1/2 activation. c-Jun N-terminal kinase (JNK) was identified in both signaling pathways to play a pivotal role. By isoform-specific loss-of-function studies using JNK (1-3) small interfering RNA, we found that different JNK isoforms mediated GRO/CINC-1 secretion. JNK2 and JNK3 isoforms were both activated by PAR-1 and essential for chemokine GRO/CINC-1 secretion. PAR-2-induced JNK1 activation, which failed to phosphorylate c-Jun, contributed to GRO/CINC-1 release. JNK-mediated chemokine GRO/CINC-1 release occurred in a JNK isoform-dependent fashion and invoked PAR subtype-specific mechanisms. Activation of PAR-2, as well as PAR-1, rescued astrocytes from ceramide-induced apoptosis via regulating GRO/CINC-1 release. PAR-1 and PAR-2 have overlapping functions, but can activate separate pathways under certain pathological conditions, to rescue neural cells from cell death. This provides functional insights into PAR-JNK signaling and the protective actions of PARs in brain.



Model for Jab1-mediated signaling pathway for PAR-2.



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culture systems (calcium signaling), (2) to cause a PAR_4-dependent platelet aggregation, as well as a vascular relaxation in both wild-type and PAR_2 null mice, (3) like trypsin, to cause oedema in a mouse model of paw inflammation *in vivo*. We found that with different potencies, KLKs 5, 6 and 14 can activate PAR_2, a receptor widely implicated in inflammation and cancer. However, only KLK14 was able to activate PAR_4 in isolated platelet preparations and PAR_4-expressing cells. In addition, KLK14 prevented thrombin from activating PAR_1, a receptor known to play a role in tumour metastasis and invasion. Further, when administered *in vivo*, KLK14 caused a paw oedema response comparable in magnitude and time course to that generated by trypsin. The oedema was accompanied by a decreased threshold of mechanical and thermal nociception. Our data demonstrate that by activating PARs 2 and 4 and by inactivating PAR_2, widely expressed kallikreins, like KLKs 5, 6 and 14, may play a role in regulating cell signaling and local inflammatory response in many pathological settings, where trypsin or thrombin are absent.

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Supported by Canadian Institutes of Health Research and US National Institutes of Health

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C68

Kallikrein signalling: regulating inflammation via proteinase-activated receptors (PARs)

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Tissue kallikreins (KLKs) are a family of secreted serine proteinases, which are up-regulated in many cancers and at sites of inflammation. Despite their widespread expression in normal and diseased tissues, the mechanisms whereby these enzymes regulate cellular function are not clear. We have recently shown (J. Biol. Chem. 281: 32095, 2006) that *in vitro*, kallikreins can activate proteinase activated receptors (PARs), a family of G-protein coupled cell surface receptors. These receptors have been associated with inflammation, having either an anti- or pro-inflammatory role in different pathological settings. We hypothesized that like trypsin and thrombin, kallikreins 5, 6 and 14, can trigger an inflammatory response by activating PARs. We studied the ability of kallikreins: (1) to activate PARs 1, 2 and 4 in cell

SA172

A2A adenosine receptors in tissue protection from reperfusion injury

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The activation of A2A adenosine receptors (A2AR) at the start of reperfusion following tissue ischemia reduces the ongoing tissue damage that progresses for many hours. Reperfusion injury is manifested by activation of lymphocytes, macrophages, and neutrophils. Through the use of antibodies to selectively deplete lymphocyte subsets, and the use of adoptive transfer of cells into lymphocyte-deficient RAG1-KO mice, CD4+ T lymphocytes have been identified as the key cells that are inhibited by A2AR activation to block the initiation of reperfusion injury in liver, kidney and heart (Yang et al., 2006;Day et al., 2005b;Day et al., 2005a). These effects are accompanied by reductions in tissue production of several cytokines and chemokines, and a marked decrease in neutrophil accumulation between 4 and 12 hours after reperfusion. In the heart we found that there is a small but significant increase in CD3 positive cells that appear in the myocardium within minutes of reperfusion following ischemia, and the accumulation of these cells is largely inhibited by A2A

agonists. Since, necrosis during reperfusion injury is reduced to a greater extent by A2AR activation in the liver (> 75%) than in other tissues, we have used liver to study in detail the effects of A2AR activation. Most T lymphocytes are not activated during reperfusion injury via TRC-mediated mechanisms the require peptide antigen presentation via MHC on antigen presenting cells. However, a small subset of T cells, known as invariant NKT cells, can be rapidly activated due to glycolipid presentation on CD1d that is found on antigen presenting cells or hepatocytes. Selective blockade of CD1d-dependent iNKT cell activation reduces liver reperfusion injury to the same extent as A2AR activation (Lappas et al., 2006). In order to further study how NKT cells initiate and trigger reperfusion injury, we have used the synthetic glycolipid, alpha-galactosylceramide (alpha-GalCer) to selectively activate iNKT cells in liver. Although Rag1 KO mice are resistant to reperfusion injury, adoptive transfer of NKT cells into Rag1 KO mice reconstitutes injury. The injection of alpha-GalCer into mice also provoks liver injury and the sequential activation of NKT, NK, and T cells as assessed by intracellular accumulation of gamma-interferon (gamma-INF) and other cytokines based on FACS analysis of cells derived from enzymatically dispersed liver. We have also examined expression of A2AR mRNA in a reporter mouse that expresses eGFP behind the A2AR promoter. In these mice, eGFP expression in increased by 5-10 fold in NKT cell and NK cells within 12 hours after injection of alpha-GalCer. In NKT cells purified by cell sorting, gamma-IFN production in response to alpha-GalCer is markedly inhibited by A2A agonists in vitro. The results indicate that NKT cells are activated by an endogenous glycolipid during reperfusion injury following tissue ischemia via invariant NKT cell receptors. A2A agonists limit liver reperfusion injury by preventing NKT cell activation. The finding that A2AR mRNA is expressed on NK cells is novel. It is not yet know how A2AR activation influences NK cell function, but the results suggest that in addition to NKT cells, NK cells participate in an innate inflammatory cascade that causes reperfusion injury after ischemia. Inhibition of NKT and NK cells by A2AR activation or by depletion or inhibition of the activation of these cells by other means may be useful to lesson tissue injury following ischemia, transplantation or other inflammatory insults.

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Supported by grants from the Nathional Institutes of Health, R01-HL37946 and P01-HL073361.

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SA173

Adenosine receptors and asthma

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Adenosine is a purine nucleoside that is expressed in all cells of the body and involved in a wide range of physiological processes. The effects of adenosine are mediated predominantly through specific cell surface receptors of which four subtypes (A_1, A_{2A}, A_{2B} and A_3) have been described. It is now well recognised that extracellular levels of adenosine markedly increase under metabolically stressful conditions, such as hypoxia and inflammation, and whilst an acutely elevated level of extracellular adenosine is considered to mediate anti-inflammatory and protective effects, chronic accumulation has been associated with pathological consequences. In asthmatic subjects, it has been demonstrated that adenosine levels in bronchoalveolar lavage fluid and exhaled breath condensate are significantly higher than those present in healthy subjects and current evidence strongly suggests that it may contribute to the pathogenesis of asthma. For example, it has been recognised for many years that inhalation of adenosine 5'-monophosphate (AMP) (5'-nucleotidase in the lung rapidly hydrolyses AMP to adenosine) in asthmatic but not healthy subjects results in dose-related bronchoconstriction and has also been shown to increase airway eosinophilia. The potent bronchoconstriction induced by AMP in asthmatic subjects has been suggested to be mediated predominantly by mast cell degranulation resulting from adenosine A_{2B} receptor activation, and in light of recent evidence, possibly also through a direct effect on airway smooth muscle *via* the A_1 receptor. Furthermore, plasma adenosine levels rapidly increase following allergen challenge in asthmatic subjects, raising the possibility that endogenous adenosine may even be directly involved in the early-phase bronchoconstrictor response to allergen, a suggestion supported in allergic rabbits by use of an anti-sense oligonucleotide directed against the adenosine A_1 receptor. Moreover, it has recently been reported that the concentrations of adenosine are increased in both plasma and exhaled breath condensate during exercise-induced bronchoconstriction in subjects with asthma, thus providing further evidence that endogenous adenosine may be involved in asthma.

The accumulation of evidence implicating a role for adenosine in the pathogenesis of asthma has led to investigations into all adenosine receptor subtypes as potential therapeutic targets for the treatment of asthma. Selective A_1 receptor antagonists are currently in preclinical development since adenosine has been shown experimentally to mediate various features of asthma through this receptor such as bronchoconstriction and mucus secretion. An inhaled antisense oligonucleotide against the A_1 receptor reached Phase II of clinical development but failed to demonstrate sufficient efficacy. However, the xanthine derivative bamifylline is approved for the treatment of asthma in Europe and it has been demonstrated that it is a selective antagonist at the A_1 receptor, in contrast to theophylline, which is a non-selective adenosine receptor antagonist, suggesting that blockade of the A_1 receptor is of therapeutic benefit. The A_{2A} receptor is expressed on most inflammatory cells implicated in asthma, and as A_{2A} stimulation activates adenylate cyclase and

consequently elevates cAMP, selective A_{2A} receptor agonists have now reached clinical development. However, initial reports concerning their efficacy are inconclusive. A_{2B} receptor antagonists are also under investigation based on the rationale that inhibiting the effects of adenosine on mast cells would be beneficial, in addition to other reported pro-inflammatory effects mediated by the A_{2B} receptor on cells such as airway smooth muscle and epithelial cells. Whilst the effects in pre-clinical models are promising, their efficacy in the clinical setting has also yet to be reported. Finally, adenosine A_3 receptor stimulation has been demonstrated to mediate inhibitory effects on eosinophils since it also elevates cAMP. However, some experimental reports suggest that A_3 antagonists mediate anti-inflammatory effects, thus the rationale for A_3 receptor ligands as therapeutic agents remains to be determined.

In conclusion, establishing the precise role of adenosine in the pathogenesis of asthma and developing appropriate subtype selective agonists/antagonists represents an exciting opportunity for the development of novel therapeutics for the treatment of asthma.

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SA174

Moving out and turning tail: restricted collision of the A_{2A}-adenosine receptor revisited

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The A_{2A}-receptor is a prototypical G_s-coupled receptors, which has several unusual features:In contrast to β-adrenergic receptors or rhodopsin, which engage their signalling cascade by collision coupling, the A_{2A}-receptor has long been known to couple to adenylyl cyclase by restricted collision coupling (1) and to form a tight complex with G_s (2). The structural basis for this is unknown, but the A_{2A}-receptor has an extended carboxyl terminus (122 amino acids after the conceptual end of the 7th transmembrane spanning β-helix TM7). The bulk of this extended C-terminus is essentially dispensable for G protein-coupling and is not required to support desensitization (3). However, it is the site of attachment of several additional proteins (4), namely α-actinin, intracellular portions of the D_2-dopamine receptor, ARNO, USP4 and translin-associated protein-X. Clearly, because of space constraints, it is not possible that all these proteins bind simultaneously and there must be rules that allow for the regulated interaction: association with the D_2-dopamine receptor is, for instance, thought to be stabilized by phosphorylation of a caseine kinase-I site in the C-terminus of the A_{2A}-receptor.

The vast majority of group I (rhodopsin-like) G protein-coupled receptors carry one (or two) palmitoylated cysteine(s) within the proximal portion of their C-terminus (typically some 20 residues removed from the end of TM7). The palmitate thioester is thought to act as an additional anchor. This stabilizes the proximal segment in an α-helical conformation (referred to as helix 8 in the rhodopsin structure, which is oriented in a manner parallel to the membrane and perpendicular to helix 7). The A_{2A}-receptor does not have any cysteine residue in the proximal segment; there is only a single cysteine in position 394 in the human receptor and this is absent in other species orthologues (e.g. of rat and mouse). Thus one is tempted to speculate that the C-terminus of the A_{2A}-receptor is more flexible because it is not constrained by a lipid anchor.

Finally, desensitization of the A_{2A}-adenosine receptor is contingent on the phosphorylation of a single threonine residue, T298 (3). This is remarkable, because efficient recruitment of arrestins typically requires a cluster of phosphates, which interact with the N-terminal phosphate sensor and thereby trigger the structural rearrangement that leads to the tight interaction between arrestin and receptor.

We investigated the mode of coupling of the A_{2A}-receptor by visualizing agonist-induced changes in mobility of the YFP-tagged receptor by FRAP (= fluorescence recovery after photobleaching) microscopy. Agonist stimulation did not affect the mobility of the A_{2A}-receptor. In contrast (and as predicted from the model of receptor-mediated G protein activation), agonist challenge induced a decrease in the mobility of the D_2-receptor. When coexpressed in the same cell, the A_{2A}-receptor precluded the agonist-induced change in D_2-receptor mobility. Thus, the A_{2A}-receptor did not only undergo restricted collision coupling but it also restricted the mobility of the D_2-receptor. Restricted mobility was not due to tethering to the actin cytoskeleton and was not modulated by the presence of active or inactive ARNO, but was – in part - related to the cholesterol content of the membrane. Depletion of cholesterol increased receptor mobility, but blunted activation of adenylyl cyclase, which was accounted for by impaired formation of the ternary complex of agonist, receptor and G protein. These observations support the conclusion that the A_{2A}-receptor engages Gs and thus signals to adenylyl cyclase in cholesterol-rich domains of the membrane. The two distinct signalling pathways of the A_{2A}-receptor, cAMP accumulation and stimulation of MAP kinase (mitogen-activated protein kinase), were previously demonstrated to be independent of each other. Activation of MAP kinase is not contingent on G protein coupling. Accordingly, while disruption of cholesterol rich domains interfered with coupling to G_s, stimulation of MAP kinase by the A_{2A}receptor was not impaired. These findings are consistent with a model where the recruitment of these two pathways occurs in spatially segregated microdomains of the plasma membrane. Thus, the A_{2A}-receptor is the first example of a G protein-coupled receptor documented to select signalling pathways in a manner dependent on the lipid microenvironment of the membrane.

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Work from the authors' laboratory is supported by grants from rhe Austrian Science Fund (FWF)

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C69

Priming of STATs for cytokine-triggered polyubiquitylation and degradation upon A_{2A} adenosine receptor (A_{2A}AR) expression in vascular endothelial cells

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Here we demonstrate that in human umbilical vein ECs (HUVECs), A_{2A}AR expression constitutively suppressed tyrosine phosphorylation of STATs 1 and 3 in response to either an interleukin-6 (IL-6) trans-signalling complex or interferon-α by priming them for cytokine-triggered degradation by the proteasome. This did not simply reflect a negative effect of A_{2A}AR expression on HUVEC viability compared to controls, as determined by MTT assays. Moreover, several lines of evidence argued that the cytokine dependence of the effect reflected a requirement for JAK-mediated phosphorylation of STATs. First, pretreatment with JAK inhibitor prevented down-regulation. Second, the ability of leptin to specifically promote the tyrosine phosphorylation of STAT3 and not STAT1 was reflected in the preferential down-regulation of STAT3 induced by leptin in A_{2A}AR-expressing cells. Third, a Y705F-mutated STAT3 was resistant to down-regulation by cytokine in A_{2A}AR-expressing cells. Finally, immunoprecipitation of endogenous and recombinant STATs revealed that cytokine treatment triggered the accumulation of polyubiquitylated STATs in A_{2A}AR-expressing cells, and that this effect was abolished by Y705F mutation. Together, these observations identify a previously unappreciated mechanism by which a cyclic AMP-mobilising G-protein-coupled receptor can negatively regulate cytokine receptor signalling in the endothelium.

Funded by project grants from the BHF, BBSRC and a PhD studentship from the Health Ministry of Saudi Arabia

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C70

UK-371,104, a novel adenosine A_{2A} receptor agonist, inhibits acute mediator release in the human neutrophil: comparison with CGS-21,680 and the phosphodiesterase 4 inhibitor, cilomilast

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Agents which elevate intracellular cyclic AMP such as PDE4 inhibitors and adenosine A_{2A} receptor agonists (Thiel *et al*, 2003; Boswell-Smith *et al*, 2006) inhibit many pro-inflammatory functions of leukocytes. Here we report on the *in vitro* profile of the novel adenosine A_{2A} agonist UK-371,104 against release of a range of inflammatory mediators in the human isolated neutrophil. The pharmacological profile has been compared with the standard adenosine A_{2A} agonist CGS-21,680 (Jarvis *et al*, 1989) and the PDE4 inhibitor cilomilast (Boswell-Smith *et al*, 2006). Functional adenosine receptor selectivity of UK-371104 (9-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydro-2-furanyl]-6-[(2,2-diphenylethyl)amino]-N-[2-(1-piperidinyl)ethyl]-9H-purine-2-carboxamide) was assessed in recombinant human A_{2A}, A_{2B}, A_3 receptor transfected HEK-293 cells and A_1 transfected CHO cells. A_{2A} and A_{2B} potencies were quantified by EC_{50} for stimulation of cAMP formation and A_1 and A_3 potencies by IC_{50} for suppression of forskolin stimulated cAMP formation, measured by ELISA. UK-371,104 potencies (nM) were 20.1 (10.1-30.0), 4358 (2177-6540), 103.0 (54-102) and >1000 at A_{2A}, A_{2B}, A_1 and A_3 respectively. Data are geometric means ± 95% confidence interval (CI), n= 8-12. UK-371,104 was significantly more potent at A_{2A} compared with all other adenosine receptors ($p<0.05$ unpaired t test). Human neutrophils were isolated from healthy volunteers by density gradient centrifugation (Hatzelmann and Ullrich, 1987). All assays were carried out in 96 well format in HBSS buffer, pH7.4. Compounds were pre-incubated with neutrophils for 10 minutes, followed by stimulation with fMLP (30nM-1μM). Elastase and superoxide release were measured by chromogenic substrate cleavage and cytochrome C reduction, respectively. Leukotriene B_4 (LTB_4) biosynthesis was measured by ELISA. UK-371,104 and CGS-21,680 produced near maximal inhibition of release of all 3 mediators with similar potencies ($p>0.05$ unpaired t test), whereas cilomilast only inhibited LTB_4 biosynthesis and was significantly more potent than both A_{2A} agonists ($p<0.05$) - Table1. These results demonstrate that UK-371,104 is a selective adenosine A_{2A} agonist that elicits broad spectrum inhibition of acute mediator release in the isolated human neutrophil. Agents such as UK-371,104 might have therapeutic utility for the treatment of lung inflammation

Table 1

Compound	Superoxide		Elastase		LTB_4	
	n	IC_{50} (nM)*	n	IC_{50} (nM)*	n	IC_{50} (nM)*
UK-371,104	14	55 (40-75)	7	126 (86-186)	10	41 (16-107)
CGS-21,680	21	44 (30-65)	9	67 (33-148)	10	19 (13-34)
Cilomilast	6	>1000	10	>3000	8	5 (1-13)

*Data are geometric means ± 95%CI

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SA175

Hypoxia-adenosinergic regulation of immune response and tissue damage

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The Hypoxia-adenosinergic regulation of immune response and tissue damage will be described. This mechanism is important

in limiting the collateral damage to normal and still healthy tissues during acute inflammation caused by pathogens or treauma. This mechanism protects normal tissues by down-regulating the effector functions of overactive immune cells. Misquidedly, the same mechanism also protects cancerous tissues from anti-tumor effects of tumor-recognizing anti-tumor T cells,

The Gs protein coupled A2A and A2B adenosine receptors play critical role in the Hypoxia-adenosinergic regulation with Hypoxia Inducible Factor -1alpha being additive or –possibly-synergistic with immunosuppressive effects of extracellular adenosine on CD4+ and CD8+ T cells.

The medical implications of targeting the hypoxia-adenosinergic mechanism range from sepsis to cancer to acute and chronic liver and lung inflammation to the use of supplemental oxygen with ARDS, COPD and postoperative patients in intensive care units.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA176

Is there life after plaque rupture?

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The relationship between plaque rupture and adaptive geometric remodelling in the context of unstable atherosclerosis was assessed in proximal brachiocephalic arteries of fat-fed apolipoprotein E (apoE) knockout mice, after constant pressure perfusion-fixation. The rate of vessel expansive remodelling was similar in vessels with plaques and without plaques (+4.0±0.3 and +4.1±0.3 x 103 μm2/week respectively), suggesting that the presence of plaque is not necessary for remodelling to occur. In vessels with plaques, the degree of expansive remodelling was strongly associated with the stability of the plaque. Vessels with stable plaques (i.e. with neither buried fibrous caps nor acute plaque ruptures) showed no expansion (+0.1±1.4 x 103 μm2/week; p=0.953), whereas those with evidence of plaque rupture expanded at a rate of +3.4±0.4 x 103 μm2/week ($p<0.0001$; p=0.034 versus stable plaques). Vessels with stable plaques suffered significant loss of lumen over time (-2.4±1.1 x 103 μm2/week; p=0.034) but those with unstable plaques maintained lumen area over time (+0.2±0.2 x 103 μm2/week; p=0.362; p=0.014 versus stable plaques). Pravastatin treatment of male apoE knockout mice caused a 5-fold increase in fibrous cap thickness and although it did not influence overall rates of vessel remodelling, it significantly increased both the amount of vessel expansion and the period of time between plaque ruptures, suggesting that it increases the ability of the plaque to resist the rupturing force caused by vessel expansion. These data suggest that vessel expansion in fat-fed apoE knockout mouse brachiocephalic arteries does not require the presence of plaque. When a plaque is present, the outward remodelling force is exerted across its cap: vessels with smaller outward remodelling forces cannot overcome the strength of the cap, and the plaque remains stable. When the remodelling force is greater than the strength of the cap then the plaque ruptures. Thus plaque rupture can be viewed as a consequence of vessel remodelling. Interventions that strengthen the plaque, such as pravastatin therapy, do not alter remodelling parameters but instead allow for more outward remodelling before a rupture is caused.

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SA177

Ion channel switching and activation in vascular smooth muscle cell proliferation and neointimal hyperplasia

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Neointimal hyperplasia and atherosclerosis are cardiovascular abnormalities resulting in heart attacks and strokes – major causes of death and disability, particularly in societies with a western-style diet. Both are contributed to significantly by a phase of enhanced cell cycle activity, proliferation and migration of vascular smooth muscle cells. The lecture will describe how this activity is associated with a switch in the types of ion channel controlling trans-membrane ion transport. One striking feature is the de novo expression of the KCa3.1 (IKCa) potassium channel. This seems functionally important because an inhibitor of KCa3.1 suppresses neointimal hyperplasia in segments of human saphenous vein (Cheong et al 2005, Mol Cell 20, 45-52). Our data also show that down-regulation of the repressive REST transcription factor, which occurs in smooth muscle cells of the neointima, is a factor enabling expression of the gene encoding KCa3.1. With the discovery of REST in smooth muscle cells we find it has wider implications, including having effects on other potassium channel genes. Concomitant with the gain of KCa3.1 there is loss of the L-type voltage-dependent calcium channel

and increased expression, and function, of the voltage-independent TRPC1 calcium-permeable channel. Antibody targeting TRPC1 suppresses neointimal hyperplasia in saphenous vein (Kumar et al 2006 Circ Res 98, 557-563), consistent with a hypothesis whereby hyperpolarisation driven by potassium channel activity (e.g. KCa3.1) enhances calcium entry through TRP channels, which in turn facilitates calcium-dependent processes of the cell cycle and gene transcription. We have also found evidence for roles of ion channels in the migration of smooth muscle cells, showing that antibody targeting TRPC5 and dominant negative mutant TRPC5 inhibit migration evoked by the key phospholipid signalling molecule sphingosine-1-phosphate (Xu et al 2006 Circ Res 98, 1381-1389). TRPC5 is a polymodal channel, responding also to lysophosphatidylcholine (Flemming et al 2006 J Biol Chem 281, 4977-4982) a dominant constituent of oxidised low-density lipoprotein. In summary, we hypothesise that ion channel switching is an important factor in vascular smooth muscle cell migration and proliferation, contributing to progression of unwanted vascular adaptation such as neointimal hyperplasia. Sensitivity of some of these ion channels to key lipid factors may mean that the processes also make adverse contributions to atherosclerosis.

We are grateful for support from the Wellcome Trust and British Heart Foundation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA178

The role of stem cells in vein graft remodelling

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Autologous vein grafts remain the only surgical alternative for many types of vascular reconstruction, although the patency rate is limited due to obliterative stenosis of the grafted vessels. The vascular remodelling occurs after vein graft due to altered biomechanical stress. We demonstrated that the earliest cellular event in mouse vein grafts is cell death, i.e. apoptosis and necrosis. Following endothelial death is cell regeneration, mononuclear cell infiltration and smooth muscle cell (SMC) accumulation, which form arteriosclerotic lesions. Endothelial cell repairs by stem/progenitor cells. Vascular endothelial cells in the areas where biomechanical stress alters may have a higher rate of death. It is a key issue to know how dead endothelial cells are replaced and which cells are responsible for regenerating the endothelium. To take advantage of transgenic animals, we developed and characterised a new animal model of vein graft atherosclerosis in wild-type and apoE-deficient mice. The lesion displayed classical complex morphological features and a heterogeneous cellular composition. By using transgenic mice expressing LacZ genes controlled by specific endothelial (TIE2-LacZ), SMCs (SM-LacZ) or all types of cells (ROSA26), we performed vein isografts in two types of transgenic mice expressing β-gal in endothelial cells and wild-type mice. We demonstrated that the endothelium on vein grafts completely disappeared due to apoptosis, and were replaced by progenitor cells, of which about one-third of cells were derived from bone marrow cells. These findings indicated the contribution of progenitor cells to regenerate damaged endothelium of the vessel wall. SMCs within atherosclerotic lesions are derived from progenitor cells. We observed that SMCs in mouse vein grafts appear in the neointima earlier than in the media after massive cell death, which is an early cellular event in the grafted vessels. Furthermore, a recent study demonstrated that smooth muscle progenitors were present in circulating blood, although their origins are unknown. Concomitantly, we showed that about 60% of SMCs in atherosclerotic lesions of vein grafts were derived from the donor vessel wall and 40% from recipients, possibly from circulating blood. These findings strongly suggest the possibility of progenitor cells being the source of smooth muscle accumulation in arteriosclerotic lesions. To explore the possibility of vascular progenitor cells for both smooth muscle and endothelial cells existing in adults, we provided the first evidence that the adventitia in aortas harboured large numbers of cells expressing stem cell markers, e.g. sca-1+ (21%), c-kit+ (9%), CD34+ (15%), Flk1 (4%) cells, but not SSEA-1+ embryonic stem cells, indicating that they are adult stem/progenitor cells. Finally, the mechanisms of stem/progenitor cell differentiation toward endothelial cells seem to depend on blood flow pattern. Support this notion is the finding that laminar shear stress induce stem cell differentiation into endothelial cells in vitro, in which growth factor receptor-HDAC-p21 signal pathways may be crucial. In summary, dead endothelial cells in vein grafts could be replaced by circulating stem cells. Functions and differentiating abilities of stem cells into endothelial cells might also be influenced by risk factors and local environment, i.e. biomechanical stress-influenced vessel remodeling mediated, to which stem cells contribute significantly.

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This work is supported by the grants from British Heart Foundation and Oak Foundation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C71

Sphingosylphosphorylcholine acts as a pro-inflammatory mediator in vascular smooth muscle cells

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The naturally occurring sphingolipid, sphingosylphosphorylcholine (SPC), is a constituent of plasma lipoproteins and is also

released from activated platelets. SPC may have atheroprotective effects via an action on endothelial cells. However, on vascular smooth muscle (VSM) cells, SPC can initiate proliferation and vasoconstriction. It may therefore have a role in the development of vascular disease. We have recently shown that SPC can activate p38 mitogen-activated protein kinase (MAPK) in VSM indicating a potential role as a pro-inflammatory factor. Such a mechanism of action could contribute to a pro-atherogenic effect. The aim of the current study was to determine if SPC could induce the release of inflammatory proteins from VSM cells and delineate the underlying mechanisms.

A7r5 cells (rat aortic VSM cell line) were incubated with 10 μM SPC for 24 hours at 37oC. Medium was removed and subjected to an inflammatory antibody array consisting of antibodies to 20 chemokines and cytokines. Compared to control, binding to only one antibody was significantly increased; monocyte chemoattractant protein-1 (MCP-1). Enzyme-linked immunosorbent assays (ELISA) for MCP-1 were used to confirm the results of the array. Conditioned medium from SPC-treated A7r5 cells revealed an increase in MCP-1 release (200 μg/ml) compared to control (75 μg/ml) (n=3). Lipopolysaccharide (25 μg/ml) used as a positive control, increased MCP-1 release to 415 μg/ml. Interestingly, the structurally-related sphingolipid, sphingosine 1-phosphate, did not increase MCP-1 release from A7r5 cells. The promoter regions for the MCP-1 gene contain consensus sequences for 2 transcription factors associated with inflammatory responses, nuclear factor-κB (NF-κB) and CCAAT enhancer binding protein (c/EBP). To determine whether SPC can activate either or both of these transcription factors, electromobility shift assays (EMSA) were carried out on A7r5 cells incubated with 10 μM SPC for 1 hour. SPC-treated cells showed an increased DNA-protein binding with oligonucleotides specific for a c/EBP consensus sequence. EMSA to determine DNA-binding of activated NF-κB revealed that SPC also activates NF-κB in VSM cells. For both c/EBP and NF-κB, activation by SPC was dependent on p38MAPK phosphorylation, as observed by pre-incubation with the p38MAPK inhibitor, SB203580.

In conclusion, SPC can induce release of the inflammatory chemokine, MCP-1, from VSM cells. This may occur via activation of the pro-inflammatory transcription factors, NF-κB and c/EBP, leading to increased gene and protein expression of MCP-1. This SPC-induced inflammatory response may play a role in vascular disease.

This work was supported by The British Heart Foundation.

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C72

Effect of prostacyclin analogues on artery function and smooth muscle cell proliferation in the pig and rabbit: implications for drug-eluting stent development

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Background: Prostacyclin (PGI_2) analogues may have potential for use in a novel drug-eluting stent by virtue of an IP receptor mediated, anti-proliferative effect on smooth muscle cells (SMC). We examined the functional response of the pig coronary (PCA) and rabbit iliac artery (RIA) to PGI_2 analogues, and assessed the anti-proliferative potential of these compounds.

Methods and Results: 3-4mm length artery rings were precontracted with $PGF_{2\text{-}\alpha}$ (2μM, PCA) or phenylephrine (0.2μM, RIA). Dose response curves were generated by cumulative addition of the PGI_2 analogues, AFP-07 or cicaprost (1nM-1μM), alone or in the presence of the EP_1 or EP_3 receptor antagonists SC51322, and L-798106 respectively (1μM). In PCA, AFP-07 produced a weak biphasic response consisting of a maximum relaxation of 18 ± 3.5% (30nM), followed by a reversal of the initial relaxation. SC51322 and L-798106 potentiated the relaxation response. In RIA, AFP-07 produced a maximum relaxation of 70 ±7% (30nM), followed by partial reversal. The reversal was fully blocked by L-798106, and partially by SC51322. Cicaprost produced near complete relaxation in RIA. SMC proliferation was assessed by [^{3}H]-thymidine incorporation, following 24-hour incubation in 10% or 2% foetal calf serum (FCS). AFP-07 (0.01-1μM) and cicaprost (0.1-1μM) significantly inhibited RIASMC proliferation following 2%FCS stimulation. A reduced anti-proliferative effect was observed in RIASMC stimulated with 10%FCS, and no anti-proliferative effect of cicaprost or iloprost was observed in PCASMC stimulated with 10%FCS.

Conclusions: IP receptor agonists have significant actions on EP receptor subtypes in the coronary artery. The rabbit iliac artery is more sensitive to the relaxant, and anti-proliferative effects of PGI_2 analogues, compared to the pig coronary artery. This may, in part, be due to differences in the distribution of the prostanoid IP, EP_1 and EP_3 receptor types within each tissue.

The following compounds were kind gifts:

cicaprost (Schering AG, Germany),

AFP-07 (Asahi Glass Co., Japan),

L-798106 (GlaxoSmithKline, United Kingdom)

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA179

Molecular efficacy switches in agonists and receptors – how to swap between agonism and inverse agonism in 7TM receptors

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It is known anecdotally among medicinal chemists that small chemical modifications occasionally, in puzzling ways can turn antagonists into agonists - and the other way around. We have studied this phenomenon in a systematic manner using a multitude of terminally modified wFw tripeptides in combination with a large library of mutants of the constitutively active ghrelin receptor. Efficacy –switch epitopes was identified in the ligand, where certain types of chemical changes swapped the ligand between high potency agonism and equally high potency inverse agonism. The wFw-containing peptides – agonists as well as

inverse agonists – are affected by receptor mutations covering the whole main ligand-binding pocket with key interaction sites being an aromatic cluster in TM-VI and VII and residues on the opposing face of TM-III. Importantly, gain-of-function in respect of either increased agonist or increased inverse agonist potency or in respect of swap (up and down) between high potency versions of these properties, was obtained at a number of key positions. In particular, space generating substitutions at position III:04 shifted the efficacy of this chemotype ligands from inverse agonism toward agonism, whereas similar substitutions at position III:08 - one helical turn below - shifted the efficacy from agonism toward inverse agonism. It is suggested that the relative position of the ligand in the binding pocket between this "efficacy shift region" on TM-III and the opposing aromatic cluster in TM-VI and TM-VII leads either to agonism – i.e. in a superficial binding mode - or it leads to inverse agonism – i.e. in a more profound binding mode. This relationship between different binding modes and opposite efficacy is in accordance with the Global Toggle Switch Model for 7TM receptor activation (Annu.Rev.Pharmacol.Toxicol. (2006) 46: 481-519). Thus, it is clear that efficacy can be structurally "uncoupled" from affinity / potency and a picture of the structural basis for this is emerging which fits with efficacy-swap mutations in for example the CB1 and opioid system.

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SA180

Fragment based screening by STD NMR - from soluble targets to GPCRs

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Saturation transfer difference NMR spectroscopy (STD NMR) is based on the principle that saturation of a protein leads to a transfer of this saturation to the free ligand if there is an exchange between the bound and the free ligand. The difference of a spectrum with protein saturation and a spectrum without represents a spectrum that shows only molecules that bind to the receptor. The technology is very robust and can be applied to proteins with a molecular weight larger than 10kD. There is no upper limit to the size of the protein. Using this method, it is easy to identify ligands from a mixture of compounds. The protein can also be anchored into the membrane of liposomes or native cell walls. STD-NMR is very sensitive if one has a fast off-rate and uses a large excess of the ligand. We have been working with as little as 30 pmol (a few micrograms) of protein. By competition titrations, it is possible to arrive at very precise binding constants for the ligands. This can alternatively be achieved by a quantitative analysis of binding curves as a function of the amount of the ligands.

The use of STD NMR for assaying the interactions between ligands and cellular transmembrane receptors is also described. STD NMR can be used to analyze the binding of small molecules to large membrane integrated receptors in living cells using a newly developed variant of the STD protocol called STDD (Saturation transfer double difference) spectroscopy.

A newly developed variant of this technique allows also the determination of binding properties of oligosaccharides to receptor proteins when the receptor is a membrane integral protein. This technique called STDD (saturation transfer double difference) NMR spectroscopy also allows the determination of binding properties of carbohydrates with membrane integrated receptors in living cells.

In order for HIV to infect human cells, two human receptor proteins, CD4 and CCR5, have to interact with a highly glycosylated viral protein, the gp120.We analyzed, the interaction of a glycopeptide derived from the hypervariabel loop V3 of gp120 with the human seven helix transmembrane chemokine receptor, CCR5, is described. The receptor CCR5 is a G-protein coupled receptor (GPCR). This interaction is crucial for the HIV to infect human macrophages, which occurs during the asymptomatic early phase of HIV infections. STDD NMR and Biacore surface plasmon resonance experiments allow the characterization of the binding epitope of the gp120 derived glycopeptide in its interaction with the CCR5 receptor.

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SA181

Membrane protein complexes investigated by solid-state NMR

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Solid-state NMR (ssNMR) offers structural insight into the formation of molecular complexes for a wide range of molecular sizes and binding affinities (see, e.g.[1]). Recent instrumental and methodological progress has enabled novel possibilities for using multi-dimensional ssNMR to study molecular 3D structures and interactions in noncrystalline systems.

In our group, such methods are used to study protein folding and aggregation on the atomic level and in a time-resolved manner for proteins involved in Alzheimer's and Parkison's disease (see, e.g.,[2]).

In addition, we have developed a set of ssNMR experiments to study molecular structure, topology and complex formation in lipid bilayers. Such techniques can be used to characterize ligand binding to G-protein coupled receptors[3] or ion channels. For

example, we have shown that high affinity toxin-binding to a chimeric Kv1.3 channel involves structural rearrangements of both constituents[4]. More recently, we have refined the ligand structure in the free and channel-bound state using ssNMR. Comparison to dynamical studies in solution suggests that an intrinsic structural plasticity underlies ion channel recognition. In addition to ligand-channel studies, ssNMR experiments are possible under variable physico-chemical conditions that may provide new insight into signal transduction in seven-helix receptors[5] and ion channel activation.

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Financial support by the following funding agencies is gratefully acknowledged: MPG, DFG, EU, VW foundation and FCI.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA182

Protein kinase Cι: Oncogene, prognostic marker and therapeutic target

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Protein Kinase C (PKC) isozymes have been implicated in the control of such diverse cellular functions as proliferation, differentiation, polarity and survival. Since the discovery that PKCs are cellular receptors for the tumor promoting phorbol esters, they have been implicated in various aspects of cancer. Distinct changes in the pattern of PKC expression and activity have been associated with the transformed phenotype of cancerous cells, and disruption of the activity/expression of multiple PKC isozymes can affect the transformed phenotype. However, direct genetic evidence that any PKC isozyme is a human oncogene had been lacking. We recently demonstrated that the atypical PKC isozyme PKCι is an oncogene in non-small cell lung cancer (NSCLC) (1). PKCι expression is elevated in the vast majority of NSCLC cell lines and primary tumors and PKCι expression correlates with poor clinical outcome in NSCLC patients. Interestingly however, PKCι expression is elevated in both early and late stage disease. Therefore, PKCι expression profiling holds particular promise as a prognostic marker to identify NSCLC patients with early stage disease who are at high risk for relapse. PKCι expression in NSCLC tumors is driven by tumor-specific amplification of the PKCι gene in ~30% of NSCLC cases. PKCι gene amplification drives PKCι expression in these tumors. Functionally, genetic disruption of PKCι expression inhibits multiple aspects of the transformed phenotype including transformed growth in soft agar, invasion through Matrigel, and growth of subcutaneous tumors in nude mice (2). Genetic dissection of the oncogenic PKCι signaling pathway demonstrate that PKCι drives transformed growth by activating a PKCι→ Rac1→ Pak→Mek1,2→Erk 1,2 signaling pathway that is required for transformed growth (2). The transforming activity of PKCι requires the N-terminal Phox-Bem 1 (PB1) domain of PKCι which serves to couple PKCι to downstream effectors via adaptor molecules. We have designed and implemented a high throughput drug screen to identify compounds that can disrupt PB1-PB1 domain interactions between PKCi and the adaptor molecule Par6 (3). Our screen identified the gold compound aurothiomalate (ATM) as a potent and specific inhibitor of the PB1 interactions between PKCι and Par6 that exhibits potent anti-tumor activity against NSCLC both in vitro and in vivo. Structural analysis, site-directed mutagenesis and modeling indicate that ATM specifically targets the PB1 domain of PKCι to mediate its anti-tumor activity (4). ATM is currently in phase I clinical trials for the treatment of NSCLC. Molecular aspects of oncogenic PKCι signaling will be discussed with a particular emphasis on their implications for diagnosis of cancer, use as prognostic markers, and potential as novel therapeutic targets.

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This work was supported by grants from the National Institutes of Health, The American Lung Association/LUNGevity Scholar Award and the James and Esther King Biomedical Research Program of the Florida Departmen of Health to APF. RPR is recipient of a Ruth L. Kirschstein National Research Service Award from the National Institutes of Health.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA183

PKCδ and apoptosis

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Apoptosis, or programmed cell death, is critical for vertebrate development, the clearance of damaged or genetically altered cells and is induced by a variety of exogenous agents including irradiation, chemotherapeutic drugs and cell toxins. Phosphorylation of cellular proteins by protein kinases is widely utilized as a mechanism to relay information within the cell, and signal

transduction by this mechanism has been shown to regulate many cellular processes such including proliferation and apoptosis. We are interested in how specific members of the protein kinase C (PKC) family function to modulate apoptosis. As a model we use salivary epithelial cells either in culture, or derived from genetically modified mice that have specific defects in protein kinase C directed signal transduction. Our studies have demonstrated that PKCδ is an early and essential regulator of apoptosis in salivary epithelial cells and that PKCδ may function upstream of the mitochondria as an integrator of diverse death signals (1, 2). Conversely, PKCα activity is required for cell survival, as inhibition of PKCα using a dominant negative PKCα induces apoptosis in salivary epithelial cells (3).

Since PKCδ is a ubiquitously expressed kinase, an important question is how the pro-apoptotic function is activated in response to specific signals. Structure-function analysis of PKCδ suggests that activation of this pro-apoptotic function is regulated by multiple mechanisms. Using techniques to localize PKCδ in cells undergoing apoptosis, we have shown that PKCδ translocates to the nucleus, and we have identified a nuclear localization sequence (NLS) in the COOH-terminus of PKCδ (4). Mutations in PKCδ which prevent its nuclear translocation, also inhibit apoptosis, indicating that PKCδ functions in the nucleus to regulate the apoptotic pathway. However, PKCδ is predominantly cytoplasm in the absence of an apoptotic signal, suggesting that nuclear transport must be regulated by an additional mechanism. Our studies indicate that tyrosine phosphorylation of PKCδ on specific residues in the regulatory domain is also necessary for nuclear translocation. Mutation of these residues inhibits both nuclear accumulation of PKCδ and apoptosis, suggesting that in the absence of an apoptotic signal, the regulatory domain of PKCδ functions a cytoplasmic retention signal. While our studies indicate that both tyrosine phosphorylation and the COOH terminal (NLS) are required for apoptotic stimulus induced nuclear import of PKCδ, further studies are needed to decipher how these events are related. Finally, a third level of regulation of the pro-apoptotic activity of PKCδ is evident from studies which show that caspase cleavage of PKCδ occurs in the nucleus of apoptotic cells. Since caspase cleavage is likely a mechanism for amplifying the apoptotic signal, this amplification step can presumably only occur in cells in which PKCδ has been translocated to the nucleus.

The identification of both pro- and anti-apoptotic isoforms suggests that PKC may function as a molecular sensor, promoting cell survival under favorable conditions and executing the death of abnormal or damaged cells when needed. Our goal now is to identify nuclear phosphorylation targets of PKCδ and to further understand the mechanism by which PKCδ regulates the apoptotic pathway. Understanding the molecular basis for regulation of apoptosis by PKC isoforms may contribute to the development of therapeutic strategies to treat diseases such as cancer and neurodegenerative disorders.

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National Institute of Dental and Craniofacial Research (USA).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C79

Protein Kinase Cα is retained at the plasma membrane by the multidrug transporter P-glycoprotein

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Members of the protein Kinase C (PKC) family control a broad array of cellular functions by orchestrated phosphorylation of several different protein substrates. PKCα, a cytosolic member of the PKC family, upon exposure to phorbol esther (PMA) is translocated to the plasma membrane where it is brought into proximity of its substrates. Down-regulation of PKCα activity can occur through internalization by delivery of PKCα to the endosome compartment via a caveolae mediated process (1). We have shown previously that expression of the multidrug resistant transporter P-glycoprotein (Pgp) prevents the interaction of PKCα with caveolin-1, the major coat protein responsible for caveolae assembly, and consequently its internalization (2). We have now corroborated and extended these results. Down-regulation of PKCα-GFP is prevented when expressed in NIH-3T3-MDR-1 fibroblasts, a cell line permanently expressing Pgp. Transient expression of Pgp in NIH-3T3 and HEK293 cell lines, that do not endogenously express Pgp, confirms the finding and rules out any influence of the NIH-3T3-MDR-1 cell line selection process. Furthermore, by using mutations that abolishe Pgp transport activity, we show that Pgp function is not required for the retention of PKCα at the plasma membrane. Pgp is also a substrate of PKCα and it has been shown to be phosphorylated at eight sites(3). We show, by mutation of these sites, that PKCα-mediated phosphorylation of Pgp is not required for this phenomenon. Retention of PKCα at the plasma membrane might be due to either a direct molecular interaction with Pgp or mediated by a third molecular intermediary. Internalization of Na+/K+ATPase, a key plasma membrane transporter responsible for maintaining Na+ and K+ gradients, is dependent on PKCα phosphorylation (4). We show that retention of PKCα at the plasma membrane in a Pgp-dependent manner increases Na+/K+ATPase internalization. Since PKCα has a key role in cellular equilibrium and signal transduction, alteration of its trafficking is likely to greatly influence cell physiology as exemplified by our study on Na+/K+ATPase.

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C80

The C2 domains of classical PKCs are specific PtdIns(4,5)P_2-sensing domains

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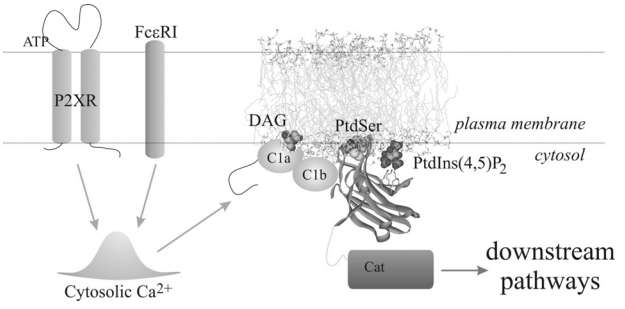
C2 domains are conserved protein modules widely distributed in many eukaryotic signaling proteins being PKCs among them (1, 2). The C2 domains of classical PKCs bind to membranes in a Ca^{2+}-dependent manner and thereby act as cellular Ca^{2+} effectors. Recent findings suggest that the C2 domain of PKCα interacts specifically with PtdIns(4,5)P_2, through its *lysine rich cluster*, with a higher affinity than that obtained with POPS-containing vesicles (3, 4). In this work, we performed a comparative study between the three C2 domains of classical PKCs. Isothermal titration calorimetry has revealed that the C2 domains of PKCα and β display a double affinity to bind to PtdIns(4,5)P_2-containing vesicles than the C2 domain of PKCγ. Comparative studies by using lipid vesicles composed of both POPS and PtdIns(4,5)P_2 as ligands revealed that the domains behave as PtdIns(4,5)P_2-binding modules rather than POPS-binding modules, demonstrating that the presence of the phosphoinositide in membranes increases the affinity of the domain about 3.5-fold in PKCγ, 4.5-fold in PKCβ and 9.5-fold in PKCα. When their Ca^{2+}-dependences for membrane binding were analyzed it was observed that in the presence of PtdIns(4,5)P_2 all C2 domains decreased their Ca^{2+} needs, although the most significant was PKCβ with a 36-fold decrease compared to 8.5- and 5-fold decreases exhibited by PKCγ and PKCα, respectively. *In vivo* experiments using differentiated PC12 cells transfected with each C2 domain fused to ECFP and stimulated with ATP demonstrated that at limiting intracellular [Ca^{2+}], the three C2 domains translocate to the plasma membrane at very similar rates, but however, the plasma membrane dissociation event is different among them, being PKCα the isoenzyme that persists for a longer time in the plasma membrane, reflecting their different Ca^{2+} needs and affinities for PtdIns(4,5)P_2.



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This work was supported by Grants from the Fundación Médica Mutua Madrileña, Fundación Ramón Areces, Dirección General de Investigación (BFU2005-02482), and Fundación Séneca, Región de Murcia (00591/P1/04).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA184

Isoform-specific functions of PKC: the platelet paradigm

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Platelets are highly active cells of the blood system, responsible for primary haemostasis and arterial thrombosis. To achieve this they have tightly regulated adhesive properties, secretory mechanisms and a highly dynamic cytoskeleton. These functions may be activated by a multitude of receptors for agonists ranging from small soluble molecules to large macromolecular structures such as collagen. In order to transduce signals to functional events, platelets express a wide array of intracellular signalling components including a wide range of protein kinases. Importantly amongst them platelets express members of the protein kinase C family, and in human platelets the most prominent isoforms to be expressed are PKCα, PKCβ, PKCδ and PKCθ (Crosby & Poole, 2002). Multiple pharmacological studies have demonstrated a critical role for the PKC family in regulating all major functional activities in platelets, but we are only now beginning to elucidate the roles played by individual isoforms in regulating these activities.

We have used combinations of pharmacological and genetic approaches to dissect out roles of individual PKC isoforms in regulating the various functional activities carried out in platelets in response to a variety of agonists. In particular, roles of the classical isoforms, PKCα and PKCβ, and a novel isoform, PKCδ, will be presented in this abstract. Importantly we reveal that the classical isoforms PKCα & β play both redundant and non-redundant roles. We show that PKCα is critical and required for dense granule secretion, with little contribution from PKCβ, whereas for aggregation there is redundancy of function between the two isoforms, where absence of one isoform may be compensated by presence of the other. In contrast to the positive contributions made by the classical isoforms, the novel isoform PKCδ plays a largely negative signalling role. This is mediated through a physical and functional interaction of PKCδ with VASP, leading to inhibition of filopodia formation, such that in the absence of PKCδ filopodia formation is no longer transient but sustained. VASP is essential for mediating signalling by PKCδ, since the effects of the PKCδ-selective inhibitor rottlerin on filopodia formation, actin polymerisation and platelet aggregation are ablated in VASP-/- platelets. The work therefore reveals a new pathway for regulation of actin and filopodia, and thereby platelet aggregation, and shows PKCδ to be a major negative regulator of these events (Pula et al. 2006).

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The author is grateful to Jeffrey Molkentin (Children's Hospital, Cincinnati, USA), Kai Schuh (University of Wuerzburg, Germany), Keiko Nakayama (Tohoku University, Japan), Keiichi I. Nakayama (Kyushu University, Japan) &

Ulrich Walter (University of Wuerzburg, Germany) for collaborations enabling this work to be performed. We are grateful to the British Heart Foundation for financial support for this work.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA185

Functional significance of manipulating PKCα signalling during lymphocyte development and leukaemogenesis

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During B and T cell development, haemopoietic progenitor cells (HPCs) must pass through a series of developmental checkpoints to ensure that the emerging mature lymphocytes are tolerant to self-antigens, while capable of recognising foreign antigens. Protein kinase Cα (PKCα) is a ubiquitously expressed serine/threonine protein kinase that has been implicated in the regulation of a variety of cellular functions including proliferation, differentiation and apoptosis in response to a diverse range of stimuli. In an effort to define the role of PKCα during lymphocyte lineage commitment and differentiation, we manipulated mouse-derived HPCs to stably express plasmids encoding kinase-active or -inactive PKCα constructs and then assessed their developmental potential by placing the cells in T cell or B cell generation systems *in vitro* and *in vivo*. In this way, we demonstrated that PKC activation is critical for T cell progenitors to successfully transit through the primary developmental checkpoint (β-selection), as expression of a plasmid-encoding kinase-inactive PKCα (PKCα-KR) in HPCs, resulted in an early block in T cell development. To address whether PKCα played a critical role during the early stages of B cell development, PKCα-KR-expressing HPCs were cultured in a B cell development system. Surprisingly, analysis of the developing cells revealed the spontaneous generation of B lineage cells that possess the key hallmark features of human CLL cells at both the phenotypic and molecular level. This finding indicates that inhibition of PKCα activity in B lineage cells serves as an oncogenic trigger, initiating the development of CLL. Taken together, these studies reveal that subversion of PKCα signalling can have distinct functional outcomes on B and T lineage cells. Our recent data defining the cellular and molecular mechanisms that control these events will be discussed.

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SA186

GPCR heterodimers: function and ligand pharmacology

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An increasing number of G-protein-coupled heptaspanning-membrane receptors (GPCRs) are reported to be expressed on the plasma membrane as dimers. Quite often dimers are constitutive in a given cell and a given cell can express both homodimers and a variety of heterodimers. From our work on homo and heteromerization of receptors present in striatal neurons it has been feasible to demonstrate some consequences of homo and heteromerization in terms of both, ligand pharmacology and receptor function. There are several examples showing that cells sense neurotransmitters (or hormones) in a different way when they express one or another set of receptors that ensemble into heterodimers (see for instance Ginés et al, 2000; Canals et al., 2003). One of such examples is given by the lack of desensitization of D1 receptors (D1R) interacting with adenosine A1 receptors (A1R) in striatal GABAergic neurons unless both receptors in the D1R/A1R heteromer are activated simultaneously. In fact signalling and/or desensitization can vary depending on whether one or the two receptors of the heteromer are activated and the resulting signal is quite often is not simply the addition of the signals given by individual activation of the receptors; even there are instances in which heteromer-mediated signalling becomes qualitatively different.

On the other hand homo or heteromerization leads to the so-called intramembrane (or horizontal) interactions, which means that the pharmacology of a given receptor usually changes (for agonists and/or antagonists): i) when it forms heteromers with another receptor and/or ii) when the partner receptor in the heteromer is activated. This is due to conformational changes in the receptors transmitted within the receptor-receptor interface at the plane of the membrane bilayer. In this regard new models considering receptor dimers have been recently developed (Albizu et al., 2006, Franco et al., 2005, 2006). Until now the approaches for fitting ligand binding data have been based on the existence of receptor monomers. From a recently devised model for receptor dimers, there exists a new approach for fitting data that gives more accurate and physiological relevant parameters (Casadó et al., 2007, data in preparation). Fitting data using the new procedure gives not only the equilibrium dissociation constants for high and low affinity binding to receptor dimers but a new parameter reflecting the molecular communication within the dimer. A comprehensive way to fit binding data from saturation isotherms and from competition assays to a dimer receptor model is now possible as it is also possible to give actual values for the concentration giving 50% reduction in radioligand binding when performing competition experiments. These values are much more reliable to establish potency orders in Pharmacology than the IC50 values reported from competition experiments using monomer-based approaches. In summary the occurrence of receptor dimers opens for GPCRs new perspectives from both the functional and the pharmacological point of view.

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This work was supported by grants SAF2005–05481 from the Spanish Ministerio de Ciencia y Tecnologia

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SA187

Novel methods for site-specific fluorescent labelling of GPCRs in living cells – use of a FLASH-light to look at GPCR acitvation

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Several different approaches have been applied in vitro to study changes of a given GPCR during agonist induced receptor activation. As a result one can reason that stimulation of G protein-coupled receptors by an agonist leads to a conformational change and hence to a transition of the receptor into an active conformation, which can subsequently couple to a G-protein. Conformational changes have been well established to occur within the transmembrane domain (TM) III and VI and could have ramifications into the 3rd intracellular loop, which is one of the coupling regions for G-proteins. Evidence is accumulating that agonists of different efficacy could induced different changes in receptor conformations.

Using a Fluorescence resonance energy transfer (FRET) based approach employing the FlAsH-tetracystein tag technology in combination with the cyan fluorescent protein (CFP), we were able to site-specifically label appropriately modified GPCR in living cells(1). Since the tag consists of minimally only six amino acids we were able to position the labeling sequence in different positions within the 3rd intracellular loop of the α2A- adrenergic receptor. Here we report, for the first time in living cells, differences in conformational changes in specific locations of the third intracellular loop when a receptor is stimulated with full or partial agonists. The positions were chosen to be underneath transmembrane domain (TM) VI, underneath TM V or in the middle of the 3rd intracellular loop, while CFP was fused to the C-terminus. All constructs were characterized with respect to ligand binding and no significant changes were observed when compared to the wild-type receptor. All receptors constructs were expressed at the cell surface and showed agonist induced changes in the FRET-ratio when stimulated with the full agonist norepinephrine. Significant differences were observed for a set of ligand with different efficacy for G-protein stimulation. The strong partial agonists, dopamin and clonidine, both induced changes in the FRET signal for all three receptor constructs tested, but with significantly smaller signal amplitude than the full agonist norepinephrine. The weak partial agonists, norphenylephrine and octopamine, only induced changes in the FRET-signal for the construct underneath TM VI. From these data it can be speculated, that strong partial agonists, can induce conformational changes within the entire 3rd intracellular loop, while weak partial agonists do only induce conformational changes close to TM VI.

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SA188

Signalling and trafficking of virally encoded chemokine receptors

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A number of human and animal herpes (HHV4-8) and pox viruses encode G -protein coupled receptors (GPCRs) with seven transmembrane (7TM) segments - most of which are clearly related to human chemokine receptors. It appears, that these receptors are used by the virus for immune evasion, cellular transformation, tissue targeting, and possibly for cell entry. In addition, many virally-encoded chemokine 7TM/GPCRs have been suggested to be causally involved in pathogenic phenotypes like Kaposi sarcoma, atherosclerosis and HIV-infection. Moreover, recent data suggest that HCMV encoded receptors are involved in tumor development.

However, to date, the role of these receptors during the viral life cycle and in viral pathogenesis is poorly understood. The majority of these receptors is found in the membranes of intracellular organelles that include components of the endocytotic pathway, i.e. multivesicular endosomes/lysosomes. It was suggested that this is the place where the viral receptors are incorporated into the viral membranes during the final stages virus assembly. I will present data on how these viral receptors are endocytosed and targeted to these intracellular lysosomal compartments. Our focus lies on one protein that has recently been identified to specifically target 7TM/GPCRs - typically by interaction with their carboxy-terminal domains – to the degradative pathways. This protein is the GPCR-associated sorting protein GASP. By addressing the signalling and post-endocytic trafficking properties of these viral receptors and their possible interaction with GASP, we hope to gain important insights in the function and pathology of these viral proteins. I will also report about the role and molecular mechanisms of the HCMV chemokine receptor US28 in cell proliferation and tumor growth in malignant melanoma cells.

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SA189

Novel aspects of GPCR regulation by phosphorylation

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GPCRs can be phosphorylated on multiple sites following agonist stimulation. We show here that a range of protein kinases (not just the GRKs) are able to mediate agonist-dependent receptor phosphorylation. Furthermore, we provide evidence that different receptor kinases phosphorylate receptors on different sites and the signalling outcome of receptor phosphorylation is dependent on which receptor kinase is employed in the phosphorylation. Finally, by use of a transgenic mouse expressing a muscarinic receptor mutant that is unable to undergo phosphorylation we investigate a novel role of receptor phosphorylation in neuronal function.

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C81

Investigating activation of human G protein-coupled receptor kinase 2 by c-SRC, using site-directed mutagenesis and real-time FRET measurements

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Arrestin binding to G protein-coupled receptors requires phosphorylation of the agonist-stimulated receptor by GRK2. Once arrestin is bound to the receptor signaling via G proteins is arrested and the receptor is said to be desensitized. The receptor can then be targeted to clathrin-coated pits to be internalized and alternative signaling pathways may be activated.
Using a previously established FRET-based assay to monitor arrestin3 binding to the β2-adrenergic receptor (β2AR) in single living cells, we show that GRK2 phosphorylation by c-Src accelerates arrestin3 binding to the β2AR, presumably by increasing GRK2 activity. HEK-293 cells were transfected with β2AR tagged with YFP, arrestin3 tagged with CFP and GRK2 with or without c-Src. A slow and fast phase of arrestin3 binding to β2AR was observed in agreement with previous work (J. Biol. Chem. 280:9528 (2005)). Cells that were not transfected with c-Src displayed a slow phase with a half-life of about 24 seconds, whereas for cells co-transfected with c-Src the half-life was reduced by approximately 50% to about 13 seconds.
PP2, a src-family kinase inhibitor, was used to show that the observed acceleration of arrestin3 binding to the receptor was caused by the catalytic activity of c-Src. Treatment with 10μM PP2 for one hour had no effect on arestin3 binding to the receptor in cells not co-transfected with c-Src, but increased the half-life of arrestin3 binding to the receptor in cells co-transfected with c-Src to abot 25 seconds, identical to that of cells without c-Src. It has previously been reported that c-Src-mediated tyrosine phosphorylation of GRK2 occurs at residues 13, 86 and 92 (Cell Signal. 18(11):2004 (2006)). In order to show that phosphorylation of GRK2 by c-Src was required for the acceleration in arrestin3 binding to β2AR we tested a phosphorylation impaired GRK2 mutant (Y13,86,92F). There was no significant difference between the half-life of the slow phase of arrestin3 binding to β2AR in cells transfected with Y13,86,92F GRK2 with or without c-Src (a half-life of 54 and 59 seconds respectively), indicating that c-Src was having no effect on the mutated GRK2. However, cells expressing the mutated GRK2 without c-Src showed a half-life approximately 3 fold slower than that of wild-type GRK2. The half-life for the slow phase of arrestin3 binding to the β2AR in cells transfected with mutated GRK2 with c-Src was in the region of 5-fold slower than that of cells transfected with wild-type GRK2 with c-Src. We conclude that the Y13,86,93F GRK2 mutant is in some way catalytically impaired.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C82

GASP and the postendocytic sorting of the dopamine receptor family

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Following agonist induced endocytosis, G protein-coupled receptors (GPCRs) may either be resensitized and recycled back to the plasma membrane or targeted to the lysosomes for degradation. It has been recently shown that the cellular fate of the mu (MOR) and delta (DOR) opioid receptors and also the D1 and D2 dopamine receptors, which are all members of the GPCR superfamily and implicated in addiction, can be controlled by a specific protein interaction of the C-termini with GASP (GPCR-associated sorting protein) (1,2). In particular, GPCRs that interact with GASP appear to be targeted to the degradative pathway, and those receptors that do not interact with GASP, recycle. The fundamental question is what determines whether a receptor recycles or degrades? In the present study, the postendocytic sorting and potential GASP binding was investigated in Human Embryonic Kidney (HEK) 293 cells stably expressing either the D3, D4 or D5 dopamine receptors. The GST pulldown assays revealed that recombinant GASP-1 produced by in vitro translation bound with much higher affinity to GST fusion proteins containing the C-tails of the D2-like receptors (D2, D3 and D4) than to the D1-like (D1 and D5) or GST alone. A similar profile was seen with GASP-2 binding. Confocal microscopy revealed that D2-like receptors are poorly endocytosed when

compared to D1-like receptors, however some internalization does occur in response to agonist. Biotin protection assays revealed that D3 was extensively proteolyzed after prolonged agonist treatment (10μM dopamine, 180mins) as were the D4 isoforms D4.2 and D4.7. In addition, transfection of siRNA oligonucleotides corresponding to regions in either GASP-1 or GASP-2 subsequently down-regulated the expression of either protein in a time dependent manner. Therefore, the affect of down-regulation of GASP on receptor trafficking will also be studied.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA190

Nitric oxide and respiratory rhythm in mammals: a new candidate for phase transition

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Nitric oxide (NO) is one of the so called atypical neurotransmitters which also include other gaseous molecules such as carbon monoxide, and as such is a highly promiscuous messenger. NO production in the brain can originate from activation of the neuronal NO synthase (nNOS) isoform contained within neurones, which is calcium-dependent and has been shown to be present in a variety of brain areas (Campese et al. 2007). A property of NO is its ability to freely cross biological membranes once produced. As a result its modulatory actions within the brain are through pre- and postsynaptic effects. These properties all make NO an excellent candidate in synchronizing neuronal discharge in a population of neurones which may be an important factor in the organization of oscillatory activity within a central pattern generator as already described. Indeed, NO has been shown to modulate numerous centrally based rhythmic activities such as fœtal swallowing, feeding in pond snails, thalamocortical neurons, locomotion and respiration.

Our interest is the role of NO within the brainstem respiratory network. Much of the literature has described a key role for NO in the ventilatory response to hypoxia but few studies have defined a putative role during normoxia. The use of knock-out mice for deleting the different isoforms of NOS have shown some or no modifications of basal ventilation even when multiple NOS knock out mice were tested (Tsuisui et al. 2006). Furthermore, in such studies the effect of NO is not clearly localized to the peripheral or central nervous system or both. To date no studies have been performed at the neuronal level in the intact respiratory network to reveal the effect(s) of NO on respiratory neurone discharge. Thus the goal of my presentation will be to describe the effects of NO on both motor output and single respiratory neurones within the medullary respiratory network studied in vitro and in situ, and to provide evidence for the biochemical pathways involved in mediating the effects of NO during normoxia.

I will show that in an in vitro rhythmic medullary slice containing the Pre-Bötzinger complex, basal respiratory network motor output activity is modulated by NO from birth with an excitatory effect on inspiratory neuronal bursting. Evidence suggests that this excitatory effect involves a NO-mediated reinforcement of the NMDA component of inspiratory discharge. Results obtained at the neuronal level from an intra-arterially perfused anaesthetised juvenile rat preparation show that NO can have both inhibitory and excitatory effects on all types of respiratory neurone. These effects can be attributed to two known and distinct biochemical pathways that involve soluble guanylate cyclase and peroxynitrite respectively. Moreover, NO modulates both GABA and NMDA triggered responses but surprisingly this was limited to two specific types of respiratory neurones which have been ascribed major roles in the onset and offset of the inspiratory and expiratory phases and seemed essential for generating the basic respiratory oscillation in adult mammals (Richter et al. 1992).

These results compliment the previously described role of NO during the ventilatory response to hypoxia by strongly supporting NO as major modulator of respiratory neuronal bursting during normoxia. Furthermore, the data indicate specific targeting of NO actions on ligand mediated responses in two types of respiratory neurones involved in respiratory phase transition mechanisms.

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This work was supported by French Foreign Office PAI Alliance no. 05815NB.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA191

TASK channels determine pH sensitivity of serotonergic raphé neurons but do not contribute to central respiratory chemosensitivity

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Central chemoreception is the mechanism by which the brain senses changes in CO2/H+ to regulate breathing. The cellular identity of chemoreceptors has not been definitively established and the molecular substrate(s) for pH-sensitivity in chemosensory neurons remain largely unknown. Here, we identify phenotypically distinct neurons within the retrotrapezoid nucleus (RTN) as chemosensors and show that they express a pH-sensitive background K+ current different from TASK channels which accounts for their chemosensitivity. Further, we show that pH sensitivity of serotonergic raphé neurons is essentially eliminated in TASK channel knockout mice even as ventilatory responses to CO2 are fully retained, effectively dissociating chemosensitivity of raphé neurons from respiratory chemosensitivity.

We recently identified a population of CO2-sensitive neurons in the RTN that appear to function as specialized chemoreceptors. These neurons are intrinsically sensitive to CO2 in vivo, are glutamatergic and send excitatory projections to the respiratory rhythm generator (1). In addition, CO2-sensitive RTN neurons

express Phox2b (2), a transcription factor mutated in a congenital central hypoventilation syndrome characterized by a specific deficit in chemosensitivity (3). In a medullary brain slice preparation we identified a group of intrinsically pH-sensitive neurons in the RTN that share morphological features with those recorded in vivo (1). In addition, we found that these RTN neurons express a weakly-rectifying pH-sensitive K+ current with properties reminiscent of the TASK channel subfamily of K2P background K+ channels.

The purpose of the current work is twofold: to confirm that pH-sensitive RTN neurons identified in vitro constitute the same group of chemoreceptors identified in vivo; and to test if TASK channels contribute to the pH sensitivity of these RTN chemoreceptors and to respiratory chemosensitivity.

We took advantage of our observation that Phox2b could serve as a marker for RTN chemosensitive neurons, as described in vivo. Chemosensitive RTN neurons were identified in vitro by making loose patch current clamp recordings of Vm; these cells were spontaneously active at pH 7.3, inhibited by pH 7.5 and activated by pH 6.9. There was no difference in pH sensitivity of RTN neurons from age-matched rat and mouse pups (P7-12). Following functional characterization, we harvested the cytoplasmic contents of pH-sensitive neurons and performed single cell RT-PCR using outside and nested primers for Phox2b (and GAPDH as a control). Of neurons for which control RNA was detected (N=8), we found that most also expressed Phox2b (N=6). Along with other common characteristics (anatomical localization, morphological features, pH sensitivity), the shared expression of Phox2b strongly suggests that pH-sensitive cells recorded in vivo and in vitro represent the same population of RTN chemosensory neurons.

We used pharmacological and genetic approaches to test if TASK channels contribute to the pH-sensitive current in RTN chemoreceptors. The inhalation anesthetic halothane, which typically activates TASK channels in vitro, inhibited a K+ conductance in pH-sensitive RTN neurons in rats and increased their firing rate. In addition, halothane had no effect on the pH-sensitive K+ current expressed by rat RTN neurons. Further, acidification activated RTN neurons normally in mice with deletions of TASK-1, TASK-3 or both subunits and the pH-sensitive background K+ current of these cells was the same in control and knockout mice. Together, these data indicate that TASK channels contribute little to the pH-sensitive resting K+ current of RTN chemoreceptors from rat or mouse. By contrast, bath acidification no longer activated serotonergic raphé neurons in slices from TASK channel knockout mice and the pH-sensitive background K+ current previously attributed to TASK channels in these neurons was eliminated. Although raphé neurons were no longer activated in vitro by acidification in TASK knockout mice, the respiratory response of these animals to CO2 was normal in the awake state, as judged by whole animal plethysmography. In conclusion, TASK channel underlie the pH sensitivity of serotonergic raphé neurons in slices but these channels are not necessary for the pH sensitivity of RTN neurons in vitro nor for central respiratory chemosensitivity in vivo. These results supports the possibility that RTN neurons are chemoreceptors and they suggest that the pH sensitivity of TASK channels and serotoninergic neurons contributes little to respiratory chemosensitivity.

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This work was supported by F32 HL80890 (DKM), HL74011 (PGG) and NS33583 (DAB) from the National Institutes of Health.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA192

Chemosensory control of respiratory activity: role of purinergic signalling

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There has been increasing interest in the role of purines in the nervous system. In addition to its known role as an intracellular energy source, ATP also functions as an extracellular signalling molecule (acting through ionotropic P2X and metabotropic P2Y receptors) in the central and peripheral nervous system and many peripheral tissues. Immunohistochemical studies demonstrate the presence of ATP receptors throughout the brainstem. The role of ATP in central mechanisms controlling respiratory activity has been studied in our laboratories over the last 6 years. Results obtained to date suggest that ATP-mediated purinergic signalling plays an important role in several brainstem mechanisms responsible for respiratory control.

(i) *The role of ATP in central CO_2 chemosensory transduction.* Central respiratory chemoreceptors are essential for maintenance of constant levels of arterial PCO_2, and pH. Our recent experimental data suggest that the purine nucleotide ATP acting via both ionotropic P2X and metabotropic P2Y receptors may play an important role in central chemosensory transduction. It was found that in response to an increase in PCO_2/[H+] (hypercapnia) chemosensitive structures located on the ventral surface of the medulla oblongata rapidly release ATP, which acts locally within the medullary respiratory network to evoke adaptive enhancement in breathing.

(ii) *The role of ATP in the hypoxic ventilatory response.* Using transgenic animal models we demonstrated that in the carotid body ATP, released by the O_2-sensitive glomus cells and acting at P2X receptors located on the afferent terminals of the carotid sinus nerve, triggers enhancement of ventilation during hypoxia. $P2X_2$ receptors are expressed by physiologically identified respiratory neurones of the medullary ventral respiratory column. Marked ventilatory depression occurs during hypoxia in mice deficient in $P2X_2$ receptors. During hypoxia ATP is released within the ventral respiratory column and acts to maintain respiratory activity in conditions when hypoxia-induced depression of respiration occurs.

(iii) *The role of ATP in afferent processing in the nucleus tractus solitarii (NTS).* It was found that during hypoxia ATP is released on the dorsal surface of the medulla in locations overlaying the NTS, however, blockade of P2 receptors in the NTS had no effect on the respiratory responses evoked by hypoxia. Rhythmic release of ATP that coincided with lung inflation was recorded in the NTS regions where afferents from the slowly adapting lung stretch receptors terminate. Application of ATP increased, while application of P2 receptor antagonists decreased discharge of the NTS second order neurones that receive afferent input from the pulmonary stretch receptors. ATP may therefore be released from

the central terminals of slowly adapting lung stretch receptors in the NTS to mediate neurotransmission in the Breuer-Hering reflex pathway. ATP is unlikely however, to be involved in mediating neurotransmission in the carotid chemoreceptor reflex.
In summary, the data obtained in our laboratory has revealed the importance of ATP-mediated purinergic signalling in the brainstem mechanisms underlying respiratory control. It emerges that ATP acts as a common mediator of peripheral and central chemosensory transduction and may also contribute to neurotransmission at a first central synapse in the Breuer-Hering reflex pathway.

Supported by the BBSRC and the Wellcome Trust.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA193

Respiratory plasticity induced by intermittent hypoxia: roles of phosphatases and reactive oxygen species

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The significance of neuroplasticity in the respiratory control system has been appreciated only in recent years. One of the best studied models of respiratory plasticity to date is respiratory long-term facilitation (LTF), a progressive increase in respiratory motor output lasting several hours following acute intermittent hypoxia (AIH, 3 to 10 episodes), but not following sustained hypoxia of similar cumulative duration. Although the functional significance of LTF remains uncertain, suggested roles include stabilizing breathing during sleep or offsetting respiratory depression during hypoxia. However, regardless of its functional significance, our perspective is that the capacity to elicit LTF may be harnessed as a therapeutic approach for multiple clinical disorders of ventilatory control. Thus, a detailed understanding of cellular and synaptic mechanisms of LTF may provide the rationale for new pharmacological approaches in the treatment of severe ventilatory control disorders including obstructive sleep apnea or respiratory insufficiency during spinal cord injury or neurodegenerative motor neuron disease (e.g. ALS).
Our understanding of the cellular/synaptic mechanisms that underlie LTF in phrenic motor output (pLTF) has increased dramatically in recent years. Our working model is that intermittent hypoxia triggers intermittent serotonin release near phrenic motoneurons, initiating pLTF by activating 5-HT2A receptors on their dendrites. Serotonin receptor activation initiates signaling cascades (e.g. PKC activation), stimulating new protein synthesis necessary for pLTF maintenance. New synthesis of brain-derived neurotrophic factor (BDNF) is necessary for pLTF since intrathecal administration of siRNAs targeting BDNF mRNA abolish increased BDNF concentrations in the ventral cervical spinal cord and pLTF. BDNF activates its high affinity receptor, TrkB, thereby phosphorylating and activating ERK MAP kinases and protein kinase B. We postulate that these kinases induce glutamate receptor trafficking, inserting more receptors in the post-synaptic membrane and strengthening synapses between (glutamatergic) brainstem respiratory neurons and phrenic motoneurons.
Cellular pathways leading to pLTF are regulated by inhibitory constraints. For example, serine/threonine protein phosphatases appear to be an important regulator of pLTF expression. Intrathecal injections of okadaic acid (a potent inhibitor of multiple serine/threonine protein phosphatases) reveal pLTF following brief exposures to sustained hypoxia, a stimulus normally unable to engage this mechanism. On the other hand, intraspinal okadaic acid alone does not facilitate phrenic motor output, demonstrating that protein phosphatase inhibiton is necessary, but not sufficient, to induce pLTF. Since intrathecal okadaic acid has no effect on pLTF expression following AIH, we postulate that the protein phosphatase contraint of pLTF is diminished by AIH, and that differential regulation of protein phosphatase activity accounts in part for pLTF pattern-sensitivity.
Differential inhibition of protein phosphatases by AIH *versus* sustained hypoxia may result form differential reactive oxygen species (ROS) formation in these conditions. Our hypothesis is that greater ROS formation during AIH (*versus* sustained hypoxia) enables pLTF through greater inhibition of relevant spinal phosphatases. Indeed, ROS are potent inhibitors of some serine/threonine protein phosphatases. In agreement, AIH-induced pLTF is impaired by administration of a superoxide dismutase mimetic or NADPH oxidase inhibitor, thereby demonstrating that ROS are necessary for pLTF expression. A link between the ROS and protein phosphatase inhibiton requirement of pLTF is provided by observations that intrathecal okadaic acid restores AIH-induced pLTF in rats treated with a superoxide dismutase mimetic. Thus, protein phosphatase inhibiton offsets the pLTF ROS requirement, an observation consistent with the idea that the primary actions of ROS in regulating pLTF are via their inhibitory actions on okadaic acid-sensitive phosphatases. ROS may also regulate other points in the cellular cascade of pLTF.
Considerable progress is being made in understanding the fundamental mechanisms of LTF, including its inhibitory constraints. Such an understanding is critical if we are utilize this knowledge in the development of therapeutic approaches to the treatment of ventilatory control disorders. Strategies utilizing pharmacological agents, or new technologies such as RNA interference, may be most effective if we target molecules that, when inhibited, confer a gain of function.

[Supported by: NIH HL080209 and HL69064]

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA193a

In vivo imaging to advance cell-based therapies for cancer

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Advances in molecular imaging have enabled the study of the nuances of disease and the subtlies of therapeutic responses. In cancer imaging has improved the study of immune cell therapies (1) and helped to reveal the nature of residual disease (2). These studies have contributed to the emerging paradigm of cancer stem cells, which suggests that the root of a cancer may be metabolically and phenotypically distinct from the bulk of the tumor (2). Therefore, therapeutic strategies with overlapping and redundant specificities covering the full range of the disease course need to be developed and applied to the treatment

of cancer. The objective of such approaches is to target the tumor cells that persist after conventional therapies as these are the cells that result in relapse and subsequent morbidity and mortality. We have used imaging to refine the combination of an immunotherapy and an oncolytic viral therapy to capture the broad specificity of each and overcome their limitations (3). We combined cytokine induced killer (CIK) cells—an ex vivo expanded and systemically deliverable immune cell with tumoricial capabilities—with oncolytic vaccinia viruses that have been modified so that their replication is selective for tumor cells. The CIK cells serve as delivery vehicles to get the virus to the tumor and infected tumor cells then upregulate the cell surface markers that the CIK cells use to recognize and kill the tumor. As such the effects of the combination therapy are synergistic. We have demonstrated safety and efficacy of this dual biotherapy in a variety preclinical models of solid tumors and hematologic cancers. Individually each agent has been shown to be safe, with minimal toxicities, and to hold promise for the treatment of cancer and for further clinical development. The preclinical imaging and efficacy data indicated that the combination therapy results in increased anti-tumor effects relative to either therapy alone and does not require any additional steps in the preparation of either therapeutic agent. After elimination of tumor cells in immunocompetent animals using this approach a strong cytotoxic T cell response was observed in the treated animals and relapse of the tumor was not observed in many of these animals even after reinoculation of these mice with new tumor cells. The study of complex diseases and combination therapies is greatly improved through the use of imaging approaches that are capable of cellular and molecular specificity in living animal models of human biology and disease.

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This work was funded by the National Cancer Institute of the National Institutes of Health.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA194

Lanthanide-based probes for MR-Molecular Imaging investigations

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Molecular and cellular imaging is a relatively young field that is rapidly changing our approach towards understanding and solving problems in in vivo diagnoses with innovative solutions. The development of high sensitive, targeting and responsive agents is a major challenge to enhance the role of MRI in the field of Molecular Imaging applications.

Understanding the relationships between structure and dynamics of Lanthanide(III) chelates has been fundamental for the development of high sensitive Gd(III) based agents.

From targeting Human Serum Albumin for the development of angiographic agents, our research efforts are now addressing the visualization of molecules (characterizing diseased states) that are present at much lower concentration(1).

The need of targeting molecules that are present at very low concentration requires the development of a novel class of contrast agents characterized by higher contrasting ability and improved targeting capabilities.Efficient targeting procedures for cellular labeling and recognition of epitopes characterizing important pathologies have been set up (2).

Furthermore, interesting insights on nano-sized structure containing Gd(III) ions have been gained to suggest that innovative approaches to high relaxivity agents may also be possible. As far as the delivery of a large number of Gd-complexes at the targeting sites is concerned, several systems are currently under intense scrutiny, including dendrimers, liposomes and other form of lipophilic aggregates.

Finally, much attention is currently devoted to CEST agents that represent an emerging class of MRI contrast media of huge potential. They act as negative agents by reducing the signal intensity of water protons through a saturation transfer mediated by chemical exchange. The great potential of CEST agents lies on the possibility of switching on and off the contrast at will, making possible the detection of more agents, each uniquely characterized by specific frequency of their mobile protons. Marked sensitivity improvements have been obtained by using as source of mobile protons the water molecules contained in the inner cavity of liposomes, properly shifted by the addition of a shift reagent.

S. Aime, et al., Design of Contrast Agents for Molecular Imaging In Vivo. in "In Vivo MR Techniques in Drug Discovery and Development" (Ed. N. Beckmann), Taylor & Francis, New York, 2006, chap. 4, pp. 47-72.

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,Cancer Res 2006, 66 (18), 9196-201.

Financial support from Bracco Imaging

S.p.A. is gratefully acknowledged. This study was funded in part by the EC-FP6-projects DiMI and EMIL and by MIUR.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA195

Real molecular imaging of metabolism

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The distribution, metabolism and excretion is of importance for all pharmacological active drugs. Non-invasive mapping of the drug injected is possible using the well known major imaging

modalities: CT, PET, SPECT and MRI. While CT, and SPECT require labelling with a heavy atom (I, Tc) it is possible to label with F or C isotopes using PET and MRI. Using PET it is possible to map the fate of the injected F atom, but using MRI it is possible to map the molecules, as such, containing i.e. a C-13 label. The sensitivity of MRI is, however, low and extended use of C-13 spectroscopy has not taken place due to the low resolution (cm3) and long imaging times (many minutes).
Recently methods to hyperpolarise the magnetic active nuclei have been introduced making it possible to perform in vivo metabolic MR imaging of some small endogeneous molecules, such as pyruvate and amino acids. It has been shown that Pyruvate and the metabolites lactate, alanine and bicarbonate can be mapped in a heart where ischemia have been introduced.
Images will be shown where the high MRI proton resolution in animals have been superimposed on the metabolic images created after injecting pyruvate in rats and pigs. Flow and perfusion images were also created!
The technique open possibilities for many interesting pharmacological studies, where the metabolism and the site for metabolism are important for the understanding of the new drug effect. Real molecular imaging has become a new important researh tool for pharmacological and physiological studies.

My former colleges at Amersham Health, Malmø Sweden and GE Healthcare.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA196

Cell tracking using Magnetic Resonance Imaging

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With the rapid development in cell biology, particularly in the area of stem cells, the interest to explore the therapeutic potentials of stem cells has increased. In order to assess the therapeutic possibilities, the dynamics of cell migration and differentiation after implantation into the host organ must be studied. This again requires access to noninvasive imaging modalities allowing monitoring in individual subjects over time. During the last few years, various approaches using different imaging techniques, particularly optical imaging but also magnetic resonance imaging have been applied for this purpose.
In order to detect the cells of interest against the host tissue background, the cells need to be labeled to produce a strong contrast. This has been achieved mostly with iron oxide nanoparticles, so-called USPIOs, which are incorporated into the cells, thus producing a strong signal loss in T2*-weighted MRI by virtue of susceptibility differences to the adjacent environment.
There are basically three fundamentally different routes of cell labeling. First, the USPIOs are injected systemically and are consequently picked up by blood borne cells, in particularly macrophages. Using such a labeling approach, inflammation foci can be demarcated after the macrophages have invaded the inflammation areas. Secondly, cells can be labeled in vitro by using various techniques (lipofection, endocytosis, electroporation, etc.) to effectively incorporate the USPIOs into the cells. Such prelabeled cells are then implanted and their dynamics (migration, proliferation) are followed with T2*-weighted MRI. Finally, a thrid approach aims to stereotactically inject free iron-oxide label into the tissue area of interest where the label is expected to be incorporated by the cells in the close neighboorhood. When specific cells migrate out of this unspecifically labeled region (e.g. progenitor cells migrating from the subventricular zone to the olfactory bulb along the rostral migratory stream), this can be observed as a newly generated T2*-weighted contrast.
The general idea of labeling cells with USPIOs requires, however, careful analysis and validation by independent techniques (mainly histology and immunohistochemistry) to minimize unambiguities and misinterpretations of seemingly cell-caused T2*-weighted contrast. There is a wide range of such confounding factors to be considered for image contrast assignment: vascular, BOLD based, macrophage activity, bleedings, pathophysiologically caused signal loss in the host tissue, transfer of contrast agents to host cells etc.
To go beyond mere localization of labeled cells by MRI to allow for observation of functional fate, new strategies must be developed. For this purpose, two approaches have been followed recently. The first deals with responsive MRI contrast agents while the second approach involves the use of transgenic cell lines generating their own contrast mechanisms under cell-specific promoters. Both these strategies will, in principle, permit to detect when cells change from one functional cell state to another (preselected) new cell state because only then the responsive contrast agent will be activated or the intrinsic contrast mechanism upregulated by activation of the corresponding promoter.
Issues dealing with the potential and limits of all these different labeling strategies, their careful image interpretations and with issues involved of generating the contrast will be discussed. For this discussion, particular focus will be laid on application in experimental neuroscience and studies of cerebral diseases.

Support by the Hertie Foundation, by the European Networks of Excellence EMIL and DiMI, and by a European grant (StemStroke) are gratefully acknowledged.

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SA197

MAGUK association signalling complexes: genetic, proteomic and bioinformatic studies of the synapse

S.G. Grant

Team 32: Genes to Cognition, Wellcome Trust Sanger Institute, Cambridge, UK

Multiprotein signalling complexes in the postsynaptic terminal of central nervous system synapses are essential for the induction of neuronal plasticity and cognitive processes in animals. The prototype complex is the N-methyl-D-aspartate receptor (NRC/MASC) complex, comprised of 185 proteins and embedded in the postsynaptic density (PSD), which is a set of complexes totalling ~1100 proteins. It is striking that 72% (5/7) of

NRC/MASC genes and 46% (18/39) of the X chromosomal PSD genes are already known to be involved in human cognitive disorders. Furthermore, of the 67 known proteins mutated in X-linked metal retardation, 18 (27%) encode postsynaptic proteins. Over 50 of the NRC/MASC genes are involved in human diseases. Evolutionary studies suggest that mammalian MASC arose from a simpler ancestral form in metazoans and unicellular eukaryotes. The organisation of MASC proteins in these species may represent a 'proto-synapse'. Developmental studies of MASC assembly using primary neurons suggests that a genetic transcriptional program may orchestrate synaptogenesis.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA198

Functional interactions between the p75 neurotrophin receptor and phosphodiesterases

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Damage to the CNS axons in stroke, neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease as well as in traumatic injury is devastating due to the limited regenerative capacity of the adult CNS. Elevation of cAMP in neurons, either by cAMP analogs or by inhibition of type four phosphodiesterases (PDEs), the enzymes responsible for the breakdown of cAMP, overcomes inhibition of axonal regeneration (1). However, the lack of selectivity among PDE4 inhibitors, such as rolipram, has limited their clinical applications (2). Failure to develop specific PDE4 inhibitors has been primarily due to selecting the catalytic domain as a therapeutic target, which is highly homologous between the different PDE4 isoforms (2). Here we show that the p75 neurotrophin receptor (p75NTR), a TNF receptor superfamily member that is upregulated upon nervous system injury (3), downregulates cAMP in neuronal cells by direct interaction with the unique C-terminus domain of phosphodiesterase 4A5 (PDE4A5). We initially observed that overexpression of p75NTR in NIH3T3 fibroblasts induces a reduction in cAMP that is rescued by the PDE4 specific inhibitor rolipram. To examine whether p75NTR regulates cAMP in neuronal cells, we isolated cerebellar granular neurons (CGNs) from p75NTR -/- and wild-type animals and showed that loss of p75NTR in neurons results in increase of cAMP. Immunostaining of postnatal mouse cerebellum with specific antibodies against different PDE4s, showed specific expression of PDE4A in CGNs. To examine the mechanism linking PDE4-mediated p75NTR downregulation of cAMP, we investigated the hypothesis whether p75NTR could directly recruit members of the PDE4 family to the membrane, a mechanism previously shown to induce cAMP degradation by adrenergic receptors (4). Co-immunoprecipitation experiments using isoform-specific antibodies showed that p75NTR specifically recruits the PDE4A5 isoform to the membrane. A membrane-targeted fluorescent reporter of PKA activity that generates a change in fluorescence resonance energy transfer (FRET) when it is phosphorylated at the membrane of living cells (5), showed reduced PKA activity at the membrane upon expression of p75NTR. Deletions of the intracellular domain of p75NTR indicated that the juxtamembrane amino acid sequence 275R-342L is necessary for the interaction with PDE4A5. To determine whether the p75NTR – PDE4A5 interaction was direct, we screened a PDE4A4/5 peptide library with the intracellular domain of p75NTR. We identified the LR1 and C-terminal unique regions of PDE4A4/5 as the interacting motifs directly interacting with p75NTR. Our results identify p75NTR as the first membrane receptor that can recruit PDE4A4/5 to the membrane and thus regulate intracellular levels of cAMP. The biological significance of targeting the p75NTR / PDE4A5 interaction in neuronal functions will be discussed.

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This work was supported by the Pharmacology NIH Training Grant 5T32-GM07752 to BDS and MAP, the DFG postdoctoral fellowship to CS, the ASPET fellowship to JRM, the Alliance for Cell Signaling and NIH grant NS27177 to Roger Y. Tsien supported J.Z., NIH grants NS21072 and HD23315 to M.V.C.; and NIH grants NS51470 and NS52189 to K.A.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA199

Proteins, synapses and memory

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Synapses in the central nervous system change their strength and structure in response to experience, particularly on the postsynaptic side. To understand how synapses work, we seek a comprehensive and quantitative description of the molecular organization of the postsynaptic density (PSD). From quantitative

mass spectrometry and electron microscopy measurements, a three dimensional view of the PSD has emerged, with stoichiometric counts of individual PSD components and a topological understanding of their protein interactions.
Postsynaptic scaffold proteins such as PSD-95, GKAP and Shank/ProSAP organize signaling complexes and regulate synaptic function and morphology. Shank proteins promote the growth of dendritic spines, the postsynaptic compartments that house excitatory synapses. Shank1 knockout mice show reduced size of dendritic spines and synapses, and surprisingly, demonstrate enhanced spatial learning with impaired long-term memory retention. We propose that Shank promotes the maturation of small, plastic dendritic spines into larger, more stable spines that mediate long-term memory storage.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA200

Dissecting out the role of DISC1 in Schizophrenia through protein-protein interactions

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Disrupted in Schizophrenia 1 (DISC1) is an increasingly well-validated schizophrenia risk gene, which is associated with cognitive deficits in both schizophrenics and the normal ageing population. In our original studies we generated a network of protein-protein interactions (PPIs) around DISC1, achieved by utilizing iterative yeast-two hybrid screens, combined with detailed pathway and functional analysis. This so-called 'DISC1 interactome' contains many novel PPIs and provides a molecular framework to explore the function of DISC1. We have characterized two of the DISC1 partnerships, with PDE4 and Ndel1, in detail in great examples of Industry-Academia collaboration. It is clear from our studies that DISC1 is able to interact with a wide range of proteins and has the profile of a key 'hub' protein within these networks. Our own studies and those of others will be discussed in the context of the overall interactome and implications for new therapies for this disease.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C83

Reaction of integrin beta1 with cytokeratin-1 in neuroblastoma NMB7 cells

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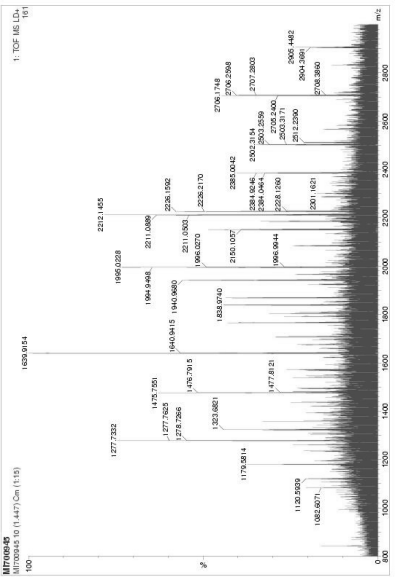
Cytokeratin-1 was isolated as a partner of Src tyrosine kinase from neuroblastoma NMB7 cells. The identity of cytokeratin-1 was further confirmed with mass spectrophotometry. This molecular complex of cytokeratin-1/Src was found as an associate with molecular scaffolder RACK1 (Receptor for Activated Protein Kinase C). Of interests, the cytokerain-1/Src/RACK1 was found to be actively reactive with membrane receptors, such as integrin beta 1. We are interested to apply them to trap signal kinase and phosphatase upon cytokine stimulation, especially during neurogenesis.



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We thank National Science Council and Academia Sinica for financial supports.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA201

Targeting of the hypoxia inducible factor pathway

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The hypoxia inducible factor (HIF) transcriptional complex is central to hypoxia signaling in cells and activates the expression of target genes involved in angiogenesis, oxygen transport, iron metabolism, glycolysis, glucose uptake, growth factor signaling,

apoptosis, invasion and metastasis. Deregulation of the HIF pathway occurs in many human cancers and has been shown to drive both angiogenesis and tumor progression and furthermore it correlates with the severity of tumor grade. Inhibition of HIF activity has been shown to significantly inhibit tumor growth in vivo. HIF is therefore an attractive therapeutic target.

The HIF-1 complex consists of α and β subunits. Both HIF-1α and HIF-1β (also known as aryl hydrocarbon nuclear receptor translocator, ARNT) are members of the basic helix-loop-helix Per/Arnt/Sim (bHLH-PAS) transcription factor family. Activation of HIF-1 is dependent on the availability of the HIF-1α subunit, while HIF-1β is usually constitutively expressed in cells. HIF-1α protein is tightly regulated in normoxia via the oxygen-dependent degradation domain (ODD). The ODD domain contains a number of prolyl residues that are recognised and hydroxylated by specific prolyl hydroxylase domain (PHD) enzymes. This results in binding of the von Hippel-Lindau protein (pVHL), an E3 ligase which targets the HIF-1α protein for degradation via the proteasome pathway.

HIF-1α protein is rapidly induced in response to hypoxia and growth factor stimulation by an increase in stability and synthesis respectively. Upon induction, HIF-1α translocates to the nucleus where it binds to HIF-1β to form the HIF-1 complex. Transactivation of HIF-1 target genes is dependent upon binding to the hypoxia responsive element (HRE) found within their promoter region. The HIF-1 complex recruits a number of co-activators, such as p300/CBP thus transactivating the expression of a multitude of target genes, the most prominent being vascular endothelial growth factor (VEGF). In human cancer, changes in microenvironmental stimuli, genetic instability and mutations leading to loss of tumor suppressor function or oncogenic activation can lead to the overexpression of HIF-1α. Indeed, it is now clear that deregulation of the HIF pathway occurs in response to many of the key genetic abnormalities that lead to cancer.

There are several sites in the HIF pathway that are potential intervention points for inhibition by small molecule inhibitors. These include inhibition of HIFα stability or protein synthesis or interference of HIF-dependent interactions. A number of small-molecule inhibitors of HIF have been described, although their exact mechanism of action remains to be understood. In addition, cell-based high-throughput screens are also being used to identify novel small molecule inhibitors of HIF. These systems generally utilize cells transfected with multiple HREs linked to a specific reporter gene construct. This allows efficient screening of large libraries of compounds for HIF-inhibitory activity. We have recently developed and validated a cell-based assay which we used to perform a high-throughput screen (HTS) to identify novel small molecule inhibitors of the HIF pathway. Extensive hit evaluation has allowed us to identify a potent inhibitor of tumor cell growth in vitro and in vivo. In addition our deconvolution analysis has identified a mechanism of action for one of our novel compounds. These studies will be presented in detail in the context of our current understanding of HIF cancer biology.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA202

Role played by BRCA1 in transcriptional regulation and response to therapy

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BRCA1 is a tumour suppressor, implicated in the hereditary predisposition to breast and ovarian cancer. Substantial data now exists to suggest a role for BRCA1 in transcriptional regulation. BRCA1 has been shown to interact with the RNA Pol-2 holoenzyme complex; to interact with multiple transcription factors such as p53 and c-Myc and play a role in chromatin remodelling. We have previously identified a range of BRCA1 transcriptional targets and linked these to specific cellular pathways regulated by BRCA1 such as interferon stimulation. Current research is focused on novel transcriptional mechanisms that underpin additional defined phenotypes associated with BRCA1 deficiency including sensitivity to DNA damage based chemotherapy and resistance to spindle poisons.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C84

O-Glycan regulation of apoptosis and proliferation in colorectal cancer cell lines

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Many proteins associated with the regulation of cell growth are glycosylated (1). O-glycosylation has not been examined and few specific inhibitors exist for study of O-glycosyation pathways (2). A series of analogues of benzyl-O-N-acetyl-D-galactosamine (benzyl-O-GalNAc) were synthesised chemically. These included phenylethyl and cyclohexyl-O-glycosides and the corresponding azides and two C-glycosides as benzyl- and phenylethyl derivatives. The compounds were tested in PC/AA/C1, PC/AA/C1/SB10C, HCA7/C29 and CaCO-2 colorectal cancer cell lines. The different cell lines showed variable susceptibility to the inhibitors with PC/AA/C1/SB10C and HCA7/C29>>PC/AA/C1>>CaCO-2. In the susceptible cell lines PC/AA/SB10C and HCA7/C29, all inhibitors induced apoptosis, tested with acridine orange/propidium bromide staining and PARP cleavage. In the same cell lines all inhibitors blocked proliferation, tested with bromdeoxyuridine labelling.

The susceptible cell lines showed aryl glycan formation with each inhibitor (3). These were extracted and analysed by MALDI TOF mass spectrometry (4). Each inhibitor showed characteristic

structures which could be mapped onto known glycosylation pathways.
Gene array analysis of PC/AA/C1/SB10C cells exposed to benzyl-O-GalNAc, benzyl-O-Gal-azide and benzyl-C-GalNAc identified apoptosis and proliferation genes as targets. The genomic response to the inhibitors thus gives support to the results obtained with growth analysis and the biochemical and chemical findings.
Target pathways relevant to cell growth and mediated by O-glycosylation have been identified by this work and form the basis for future study.

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This work was supported by grants from the Biotechnology and Biological Sciences Research Council (grant B19303) and the University of Bristol Cancer Research fund.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C85

A critical role for toll-like receptor 4 in colitis-associated adenocarcinoma development in interleukin-10-deficient mice

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Chronic inflammatory conditions such as colitis are associated with an increased risk of developing adenocarcinoma (Rutter *et al.* 2004). Current evidence suggests that colitis occurs due to an aberrant immune response to enteric flora (Mahida & Rolfe, 2004). Bacterial toxins such as endotoxin have potent proinflammatory effects through activation of toll-like receptor 4 (TLR4) (Beutler, 2004). In this study we examined the role of TLR4 signalling in a model of colitis-associated adenocarcinoma; the interleukin-10-deficient (IL-$10^{-/-}$) mouse.
Wild type (WT), IL-$10^{-/-}$ or IL-$10^{-/-}$/$TLR4^{-/-}$ mice were studied at 3 months for signs of colitis. Inflammation was assessed using macroscopic and histological scoring systems. Neoplastic changes were assessed using macroscopic mucosal polyp score and histological identification (epithelial hyperplasia, aberrant crypt foci, abnormal crypt formation, submucosal invasion of crypts, neoplastic nuclei). Chemokines were measured by Rnase protected assay (RPA). Leukocyte kinetics were measured in colonic mucosal venules of anaesthetized mice (intravenous ketamine 200 mg/kg and xylazine 10 mg/kg) using intravital microscopy. IL-$10^{-/-}$ mice had significantly increased macroscopic (3.9+0.3) and microscopic scores (5.5+0.4) over WT mice (0.6+0.03 and 0.8+0.2, respectively). In IL-$10^{-/-}$/$TLR4^{-/-}$ mice a small but significant increase in macroscopic (4.8+0.4) and histological (6.6+0.3) scores was observed over IL-$10^{-/-}$ mice. No significant difference in granulocyte infiltration (myeloperoxidase assay) was observed between the mutant mice. Interestingly, mucosal hyperplasia in the form of polyps was significantly increased the double mutant mice ($p<0.05$) and histological examination noted increased neoplasia scores (4.8+0.5 vs 3.3+0.5 respectively). These neoplastic histological changes were associated with an increased incidence of adenocarcinoma (submucosal invasion of neoplastic crypts) in the absence of TLR4 (10/19 vs 1/8 IL-$10^{-/-}$ mice). Concomitantly, IL-$10^{-/-}$/$TLR4^{-/-}$ mice had significantly lower chemokine levels (e.g. RANTES, MIP-1α,β, MCP-1, Tac-3 and IP10, $p<0.05$) than IL-$10^{-/-}$ mice. In vivo leukocyte kinetics showed enhanced rolling flux ($p<0.05$) and no difference in adherent leukocytes in the absence of TLR4.
The absence of TLR4 promotes colitis-associated adenocarcinoma in IL-$10^{-/-}$ mice. Our data suggest that TLR4-dependent chemokine synthesis plays a part in modulating colitis-associated neoplasia development perhaps through altered leukocyte recruitment.

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Canadian Institute of Health Research (operating and group grant) and Alberta Cancer Board.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA203

Characterisation of cancer stem cells in Chronic Myeloid Leukaemia

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CML is a myeloproliferative disease that originates in a haemopoietic stem cell as a result of the t(9;22) translocation, giving rise to the Philadelphia chromosome and bcr-abl oncoprotein. The disease starts in chronic phase, but as a result of genomic instability, it progresses over time to accelerated phase and then to blast crisis, becoming increasingly resistant to therapy. Bcr-abl is a constitutively active tyrosine kinase that has been targeted by tyrosine kinase inhibitors (TKIs), including imatinib (IM), nilotinib and dasatinib. We have developed various flow cytometry techniques to enable us to isolate candidate CML stem cells from chronic phase patients at diagnosis that efflux Hoechst dye, express CD34, lack CD38 and are cytokine non-responsive in culture over periods of up to 12 days in growth factors. These stem cells have been shown to regenerate bcr-abl positive haemopoiesis in immunocompromised mice upon transplantation(1). We previously demonstrated that IM was anti-proliferative for CML stem cells but did not induce apoptosis(2). Clinical experience now confirms that IM may not target CML stem cells in vivo with few patients achieving complete molecular remission and relapse occurring rapidly upon drug withdrawal. Our recent efforts have focused on understanding why CML stem cells are resistant to IM and on trying to find novel ways to induce

apoptosis of this population. We have shown that CML stem cells express very high levels of functional wild type bcr-abl - no kinase domain mutations have been detected in the stem cell population. Dasatinib, a more potent multi-targeted TKI than IM, inhibits bcr-abl activity more efficiently than IM but still does not induce apoptosis of the stem cell population (3). Most recently we have tested a number of novel drug combinations and found that farnesyltransferase inhibitors have activity against CML (4). BMS-214662 is the most effective of these and induces apoptosis of phenotypically and functionally defined CML stem cells in vitro, as a single agent and in combination with IM or dasatinib. The effect against CML stem cells is selective with little effect on normal stem cells. The drug is also effective against blast crisis CML stem cells and equally effective against wild type and mutant bcr-abl, including the most resistant mutant T315I. In association with apoptosis there is activation of caspase-8 and caspase-3, inhibition of the MAPK pathway, IAP-1, NF-KB and iNOS. Furthermore, BMS-214662 synergises with MEK1/2 inhibitors, suggesting a second mechanism other that RAS inhibition for induction of apoptosis. Our intentions are now to explore the activity of BMS-214662 in other cancer stem cell disorders and to move this pre-clinical work to a clinical trial combining dasatinib with BMS-214662 in CML.

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We would like to acknowledge support from Novartis and Bristol Myers Squibb.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA204

DNA replication repair: from molecular insights towards new approaches to anti-cancer therapy

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DNA repair and damage response pathways are activated as a tumour barrier at early stages during cancer development. Here, we describe a direct link between oncogenes that start cancer outgrowth and the tumour barrier. We show that oncogene-induced senescence is associated with signs of DNA replication stress, including prematurely terminated DNA replication forks and DNA double strand breaks. The replication lesions caused by oncogenes are tumour specific and indicate that an increase in DNA damage is associated with tumour development. Such DNA lesions are similar to those produced during radiation- or chemotherapy to kill tumour cells. A new concept for cancer therapy is to amplify endogenous tumour-specific DNA lesions, to specifically kill tumour cells. This can be achieved following inhibition of DNA repair.

Based on this concept we report that BRCA2 defective breast cancers can be specifically targeted using inhibitors of Poly(ADP-ribose) polymerase (PARP). We propose that, in the absence of PARP, spontaneous DNA single strand breaks collapse replication forks and trigger homologous recombination repair. We further show that BRCA2 deficient cells, as a result of their recombination deficiency, are acutely sensitive to PARP inhibitors, presumably because resultant collapsed forks are no longer repaired. Thus, PARP activity is essential in recombination deficient BRCA2 mutated cells. We exploit this requirement to specifically kill BRCA2 deficient tumours by PARP inhibition alone. Treatment with PARP inhibitors is likely to be highly tumour specific since only the tumours (which are BRCA2-/-) in the BRCA2+/- patients are completely defective in homologous recombination repair. The use of an inhibitor of a DNA repair enzyme alone, in the absence of an exogenous DNA damaging agent, to selectively kill a tumour represents a new concept in cancer treatment.

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SA205

Biochemistry of oxidative stress

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The terms "antioxidant", "oxidative stress", and "oxidative damage" are widely used but rarely defined. I will attempt to define what they mean, and examine the ways in which oxidative stress can affect cell behaviour and the relevance of this to human diseases, taking cancer as an example. Oxidative stress can also affect cells grown in culture, sometimes leading to over-estimation of the physiological relevance of events observed in cultured cells.

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SA206

Oxidative stress and redox regulation of lung inflammation

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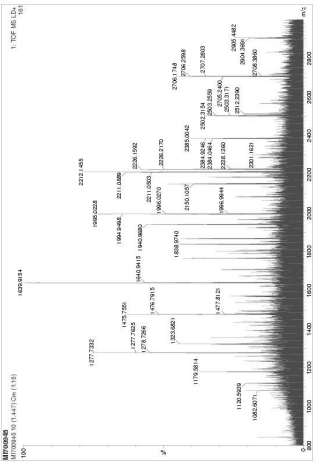
Oxidative stress and inflammation are major hallmarks of various chronic inflammatory diseases and cancer. We determined the role of oxidative stress in triggering the inflammatory response in the lungs in response to cigarette smoke by assessing the role of NADPH oxidase, and redox sensitive transcription factors Nrf2 and NF-κB in pro-inflammatory cytokine release.

1) Role of NADPH oxidase: NADPH oxidase is a multisubunit complex that generates superoxide anion in one-electron reduction of molecular oxygen using electrons supplied by NADPH. We determined the effect of targeted ablation of components of NADPH oxidase (p47phox-/- and gp91phox-/-) on inflammatory response in lungs against detrimental effects of cigarette smoke (CS). Surprisingly, CS exposure caused significant influx of neutrophils in bronchoalveolar lavage fluid (BALF) and macrophages in lung tissue of wild-type mice, which were augmented in p47phox-/- and gp91phox-/- mice compared to respective controls. Lung levels of NF-κB, and pro-inflammatory cytokines, such as KC, IL-6, MCP-1 and TNF-α were significantly increased in wild-type mice and were augmented after CS exposure in both the knockout mice. These data suggest that genetic ablation of p47phox-/- and gp91phox-/- components of NADPH oxidase assembly decreases oxidative stress but enhances susceptibility to proinflammatory effects of cigarette smoke.
2) Role of Nrf2: Nuclear erythroid related factor 2 (Nrf2) is a redox sensitive transcription factor that is involved in transcriptional regulation of many antioxidant genes. We determined the role of Nrf2 in cigarette smoke-mediated regulation of antioxidant genes in macrophages and lung epithelial cells. Treatment of human macrophages and alveolar epithelial cells to a variety of oxidants decreased Nrf2 nuclear translocation by its post-translational modification which was associated with sequestration of Nrf2 in the cytoplasm. Immunohistochemistry data on human peripheral lung tissue showed sequestration of Nrf2 predominantly in the cytoplasm of airway epithelium, alveolar type II cells and macrophages in smokers and patients with COPD compared with non-smokers. Cytoplasmic sequestration of Nrf2 was associated with increased NF-κB translocation in the nucleus. These data suggest that cigarette smoke extract caused sequestration of Nrf2 in the cytoplasm by its post-translational modifications associated with activation of NF-κB in macrophages and epithelial cells as well as in human lungs of smokers and COPD.
3) Role of NF-κB and histone acetylation: Reactive oxygen species (ROS) play a key role in enhancing the inflammation through the activation NF-κB and alteration in nuclear histone acetylation and deacetylation (chromatin remodeling) leading to sustained gene expression of pro-inflammatory mediators in the lung (Figure). Histone acetylation is reversible and is regulated by a group of acetyltransferases (HATs) which promote acetylation, and deacetylases (HDACs) which promote deacetylation. We show that oxidative stress induced by cigarette smoke enhances lung inflammation through the activation of intrinsic HAT activity of co-activator molecules, and increased histone 3 phospho-acetylation leading to increased NF-κB activation. Oxidative stress also inhibits the activity of HDACs, activates cells for NF-κB transactivation and enhances inflammatory gene expression which leads to chronic inflammatory response both in monocytes and epithelial cells in vitro and in vivo in rodent lungs exposed to cigarette smoke. Increased histone acetylation was associated increased activation of IκB kinase-α (IKKα), and interaction of NF-κB with CBP leading to increased acetylation of RelA/p65 subunit of NF-κB. Down-modulation of HDAC2 was related to increased phosphorylation of HDAC2 at serine residue. These data provide new information on oxidant-mediated regulation of inflammatory response by chromatin remodeling at molecular level.
Overall, we conclude that oxidants play an important role in triggering the inflammatory response either by activation of NF-κB and/or down-modulation of Nrf2. However, genetic ablation of components of NADPH oxidase assembly enhanced susceptibility to lung inflammation implicating a protective role of endogenous ROS against inflammation.



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SA207

Resveratrol as an antioxidant and prooxidant agent: mechanisms and clinical implications

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Resveratrol, is a phytoalexin present in a wide variety of plant species, including mulberries, peanuts and grapes, and thus is a constituent of the human diet. It exists in two isoforms; cis-resveratrol and trans-resveratrol, where the trans-isomer is the more stable form. While trans to cis isomerisation is facilitated by ultraviolet light and high pH, the cis to trans conversion is facilitated by visible light, high temperature, or low pH.

Resveratrol has been the focus of numerous in vitro and in vivo studies investigating its biological attributes, which include antioxidant and anti-inflammatory activities, anti-platelet aggregation effect, anti-atherogenic property, estrogen-like growth promoting effect, growth inhibiting activity, immunomodulation, and chemoprevention (1-3). In fact, recently, it has been demonstrated that the stilbene blocks the multistep process of carcinogenesis at various stages: tumor initiation, promotion, and progression. More recent data provide interesting insights into the effect of this compound on the lifespan of yeast and flies, implicating the potential of resveratrol as an anti-aging agent in treating age-related human diseases (1).

Over the last few years, a number of studies have provided evidence of an important role of reactive oxygen species (ROS) or reactive oxygen metabolites (ROM) in mediating the development of oxidative stress. Excessive ROS accumulation may induce the oxidative modification of cellular macromolecules (lipid, proteins and nucleic acids) with deleterious potential. Evidence has accumulated that resveratrol is both a free radical scavenger and a potent antioxidant because of its ability to promote the activities of a variety of antioxidative enzymes. The ability of the polyphenolic compounds to act as antioxidants depends on the redox properties of their phenolic hydroxyl groups and the potential for electron delocalization across the chemical structure. Nevertheless, depending on the concentration of the phytoalexin and the cell type, it has also been shown that resveratrol can exhibit prooxidant properties, leading to oxidative breakage of cellular DNA in the presence of transition metal ions such as copper. Recently it has been proposed that such prooxidant action could be a common mechanism for anticancer and chemopreventive properties of plant polyphenols (4,5). This paper is intended to provide the reader an up-date of the antioxidant and prooxidant properties of resveratrol and its clinical implications.

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SA208

What role do nitros(yl)ation products play *in vivo*? More questions than answers

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Nitric oxide (NO) is a ubiquitous messenger and effector molecule that plays important roles in regulating a perplexing number of different cell functions in prokaryotes and across the plant and animal kingdom. In mammalian cells, NO is formed either via oxidation of the amino acid, L-arginine by enzymes of the NO-synthase family or via enzymatic/non-enzymatic reduction of nitrite. While the former pathway is dependent on the availability of oxygen, much of the latter is inhibited by its presence. Together, these processes may serve the function of providing sufficient amounts of NO along the entire physiological oxygen gradient, but for exactly what purpose is not entirely clear. Depending on the tissue under investigation, the amounts of NO that can be produced from nitrite under hypoxic conditions appear to be rather high, and the level of redundancy of enzyme systems capable of reducing nitrite to NO as well as their subcellular localization are puzzling. Similarly unclear is what factors govern production and stability of nitrosation and nitrosylation products (e.g., S-nitrosothiols and NO-heme complexes) in health and disease and what defines "nitrosative stress", which roles NO, nitrite and nitrate play during sustained periods of hypoxia (conditions under which vasodilatation would seem unlikely to provide much benefit), and how inflammation and nutritional status affect these pathways. This presentation will focus on the occurrence and mechanisms of formation of S- and N-nitrosation (nitrosothiols and nitrosamines) and heme nitrosylation (NO-heme) products in blood and tissues under a number of different conditions in vivo. Particular emphasis will be placed on their usefulness as biomarkers of disease in translational studies and misconceptions as well as open questions that continue to hamper progress in this area of research.

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C86

Differential role for ROS in shock: hydroxyl radicals promote while hydrogen peroxide antagonizes hypotension and mortality

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Recently, septic shock has become the main cause of death in intensive care units, surpassing mortality caused by myocardial infarction, stroke and trauma. The mortality rate is extremely high, and the main cause of death is organ failure and refractory hypotension. To cause excessive vasorelaxation and hypotension, the endothelial lining may release vasodilators. The most significant of these are nitric oxide (NO), prostacyclin (PGI2), and the elusive endothelial-derived hyperpolarizing factor (EDHF). Although NO is critical in controlling vascular tone, inhibiting NOS in septic shock does not improve outcome, on the contrary.

The term reactive oxygen species (ROS) collectively describes a group of oxygen derivatives that includes superoxide radicals (O2.-), hydrogen peroxide (H2O2) and hydroxyl radicals (.OH). Generally, ROS are believed to cause chronic endothelial dysfunction, and to play an important role in cardiovascular diseases such as hypertension and diabetic vasculopathy. However, many studies also support the notion that endothelial cells may produce a vasodilating ROS. As these relaxations are mostly sensitive to catalase, a role for H2O2 in vasodilatation was suggested, and H2O2 was even proposed as a candidate for EDHF.

We previously reported that TNF combined with the caspase inhibitor zVAD causes hyperacute shock in mice (Cauwels et

al., 2003). Here we show that this shock does not depend on NO, prostaglandins, epoxyeicosatrienoic acids (EETs) or H2O2, and that calcium-dependent small-conductance K+ channels (SK) play a prominent role. Tempol, a potent O2.- and .OH scavenger, and a superoxide dismutase (SOD) mimetic, prevented the abrupt hypotension, whereas NOS inhibition did not. To understand the mechanism of action of tempol, we compared its effects with SOD and cell-permeable SOD. Surprisingly, only tempol could fully protect, indicating that .OH, and not O2.-, are responsible for the hyperacute shock. In addition, treatment with catalase pointed towards a possible detrimental effect of H2O2 removal. Indeed, catalase aggravated hyperacute zVAD+TNF toxicity, and when combined with tempol, it even undid the long-term protection provided by tempol. Together, our data thus indicate that H2O2 has a protective role in TNF-induced shock, and that part of the protective capacity of tempol resides in its ability to cause H2O2 accumulation. Importantly, tempol also completely protected against TNF- or LPS-induced shock.
In conclusion, our study indicates a shock-inducing effect of .OH radicals, as well as a shock-antagonizing effect of H2O2 accumulation, and provides a rationale for the therapeutic use of tempol (as .OH scavenger and H2O2 inducer), or analogous compounds, in (septic) shock.

Cauwels et al. (2003) Nat. Immunol. 4:387-393

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C87

Human $P2X_7$ receptor stimulation results in rapid NADPH oxidase activation and reactive nitrogen species generation

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There is growing evidence that $P2X_7$ receptors ($P2X_7R$) couple to rapid activation of NADPH oxidase leading to the generation of reactive oxygen or nitrogen species (RONS) (Parvathenani *et al*, 2003; Suh *et al*, 2001; Ferrari *et al*, 2000). In this study, we have investigated the action of extracellular adenosine 5' triphosphate (ATP) on the oxidative state of differentiated THP-1 macrophages loaded with the RONS-sensitive fluorescent dye dichlorofluorescin diacetate. All experiments were performed in a physiological salt saline containing (in mM) 130 NaCl, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 5 $NaHCO_3$, 1.5 KH_2PO_4, 25 HEPES, 10 glucose (pH 7.3 with NaOH). All data expressed as mean ± s.e.m. with number of experiments in parenthesis and statistical analysis was performed using Student's T-test.
Stimulation of differentiated THP-1 macrophages with extracellular ATP triggered a dose dependent burst of RONS formation within 30 s (ATP EC50 1.96 ± 0.26 mM (n = 3)) that was inhibited using the $P2X_7R$ inhibitor 1-[N,O-*bis*-(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62; IC50 368.1 ± 273.1 nM, n = 3). The addition of extracellular superoxide dismutase (≥10 U/ml, n = 4) completely blocked RONS formation suggesting that the RONS sensitive dye was measuring peroxynitrite generation and not hydrogen peroxide. ATP-induced RONS generation was inhibited using N-acetylcysteine (an antioxidant, IC50 86 ± 5.5 μM, n = 3) or diphenyleneiodonium (IC50 11.7 ± 5.6 μM, n = 3), partially inhibited with the inducible nitiric oxide synthase (iNOS) inhibitor 1400W (1 μM, 77.9 % control, n = 2, p = 0.024), but not with allopurinol. Therefore indicating NADPH oxidase and iNOS are responsible for the generation of RONS. Finally, bromoenol lactone (BEL) inhibits calcium-independent phospholipase A2 (iPLA2); incubation of cells with BEL inhibited ATP-mediated RONS generation (IC50 3.7 ± 1.5 μM, n = 3). Co-application of exogenous arachidonic acid (10 μM) with 3 mM ATP was able to recover BEL (10 μM)-inhibited RONS generation suggesting the involvement of iPLA2 in the intracellular signalling process from $P2X_7R$ to RONS generation.
To conclude, these results demonstrate that human macrophage $P2X_7Rs$ rapidly engage NADPH oxidase triggering the generation of peroxynitrite. This pathway may play an important role in host defence and inflammation.

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This work is supported by the British Heart Foundation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA209

Nuclear receptor pathways in the blood vessel wall

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The nuclear receptor family of ligand activated transcription factors constitutes 48 members in man (1). We and others have shown that the blood vessel wall and its vascular smooth muscle and vascular endothelial cell components contain a wide variety of nuclear receptors including members of the retinoic acid receptor, retinoid X receptor, farnesoid X receptor, pregnane X receptor, and peroxisome proliferator-activated receptors (PPAR)–α, -β/δ and –γ families. In recent years, in particular PPARα and PPARγ have been the focus of much attention since they are the molecular targets for the clinically used lipid lowering fibrate and the insulin sensitising glitazone classes of drugs respectively (2). Moreover, PPARα and PPARγ ligands are anti-inflammatory and anti-proliferative in vascular cells and inflammatory cells, and have been shown to reduce atherosclerosis in animal models and reduce restenosis after balloon angioplasty in animal models and in patients. At the same time there has been a great interest in the potential endogenous ligands for PPARs, which have been suggested to be a variety of lipids, oxidised lipids, eicosanoids, and a number of polyunsaturated fatty acids (2).
The PPARs are activated by a wide variety of lipids at a relatively low affinity, which has led to the suggestion that they may be general lipid sensing transcription factors within the body. However, recent data suggests that potent high affinity PPAR ligands may also exist formed from arachidonic acid. Arachidonic acid is metabolised into a group of diverse highly active

lipid mediators by 3 major pathways: the cyclo-oxygenase pathway producing predominantly prostanoids, the lipoxygenase pathways producing leukotrienes, HETEs and HODEs, and the epoxygenase pathway. Eicosatrienoic acids (EET)s are a group of short lived arachidonic acid metabolites produced by this cytochrome P450 epoxygenase pathway (3). A number of EETs and EET-metabolites can activate PPARα (4) and PPARγ (5). Furthermore, EETs released by vascular endothelial cells in response to flow are anti-inflammatory at least in part by activating PPARγ (5). Little is known however, regarding the enzymic source of these epoxygenase products. CYP2J2 is a vascular and pulmonary epoxygenase that is known to produce anti-inflammatory and anti-proliferative mediators (3). We have found that CYP2J2 activates all the PPARs and in particular PPARα. CYP2J2 activation of PPARα is anti-inflammatory, and anti-proliferative in vitro. In addition to vascular tissue, monocytes also contain CYP2J2 and when endogenous epoxygenases are inhibited, a role for CYPs in the tonic suppression of monocyte/ macrophage activation is revealed. PPARα is also known to mediate the metabolic response to fasting, by in part inducing pyruvate dehydrogenase kinase-4. In vivo in the hearts of cardiac-specific CYP2J2 transgenic mice, we found that the presence of CYP2J2 selective augments the PPARα dependent induction of pyruvate dehydrogenase kinase-4 in response to fasting. This data with that of others suggests that local epoxygenase's in particular CYP2J2 may be an important source of PPAR ligands.

In conclusion, nuclear receptors play an important function in regulating vascular function. In particular recent evidence points to a protective role in vascular cells for the lipid sensing nuclear receptors PPARα and PPARγ. We have found that at least one epoxygenase, CYP2J2 is an excellent source of PPAR ligands. The epoxygenase enzyme family by making short lived mediators in the blood vessel wall may therefore provide a critical link between lipid sensing and transcriptional control.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C88

Regulation of nitric oxide bioavailability by hydrogen sulphide: Implications for cardiovascular function and human disease

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The gaseous mediators hydrogen sulfide (H2S) and nitric oxide (NO) are synthesized in the body from L-cysteine and L-arginine respectively. Analogous to NO, H2S is enzymatically produced; from cystathionine-γ-lysase (CSE) and cystathione-β-synthase (CBS). Although the physiological significance of H2S is not yet clear its effects are similar to NO in that it exhibits endothelium-dependent vasodilator activity and may play a part in septic and haemorrhagic shock, hypertension, regulation of cardiac contractility and in inflammation. This has prompted the speculation of NO-H2S 'cross talk'. However, to date, there have been no reports of a chemical or physiological interaction between H2S and NO.

Here we show that incubation of H2S gas or the H2S donor, sodium hydrosulfide (NaHS), with a range of NO donors or NO gas in vitro and in vivo leads to the formation of a nitrosothiol molecule as determined by a combination of electron paramagnetic resonance, amperometry and measurement of nitrite [1]. In cultured RAW264.7 cells, cGMP accumulation was not observed unless NO was released with Cu2+.

In pharmacological experiments using pre-contracted rat aortic rings, NO donor-mediated vasorelaxation was inhibited by NaHS and restored after the addition of Cu2+. Furthermore, endothelium (NO)-dependent vasodilation induced by acetylcholine and histamine was attenuated by NaHS and L-cysteine but enhanced by the CSE inhibitor DL-propargylglycine. In sharp contrast, endothelium (NO)-independent vasodilation induced by isoprenaline was not inhibited by NaHS or L-cysteine. In the anaesthetised rat intravenous infusion of low concentrations of NaHS significantly increased mean arterial blood pressure whereas higher concentrations exhibited glibenclamide (KATP)-sensitive vasorelaxation [2]. The vasoconstrictor effect of low NaHS concentrations was abolished in animals pretreated with L-NAME whereas NO-mediated vasorelaxation was inhibited by H2S. Finally, using liver homogenates from lipopolysaccharide treated rats we present evidence for the endogenous formation of this nitrosothiol in vivo[1].

These findings provide the first evidence for the formation of a novel 'nitrosothiol' generated from H2S and NO which exhibits little or no vasorelaxant activity either in vitro or in vivo. The consequence of the formation of this novel molecule may be to remove endogenous NO to mediate vasoconstriction. We propose that a crucial and unappreciated role for H2S in the vascular system is the regulation of the availability of NO [2,3].

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C89

The vascular adenosine-EET pathway exerts a renal protective function

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Adenosine plays a critical role in the regulation of renal vascular tone and tubular function (1). Stimulation of adenosine $_{2A}$ receptor ($A_{2A}R$) in rat preglomerular microvessels (PGMV) is linked to production of epoxyeicosatrienoic acids (EETs) and 11,12-EET was identified as the likely candidate mediator of PGMV dilatation on activation of the $A_{2A}R$ (2). We also showed that in rat PGMV, EETs stimulate mono-ADP ribosyltransferase resulting in Gs_{α} activation and subsequent activation of adenylyl cyclase and K^{+}_{Ca2+} channel activity (3).

EETs are important modulators of cardiovascular function as they exhibit vasodilation and natriuretic properties (4). Increase in EETs is a significant component of the kidney's adaptive response to prevent blood pressure (BP) elevation in response to high salt (HS) intake. As adenosine levels are increased by salt intake and mice lacking $A_{2A}R$ exhibit elevated BP, we proposed that adenosine is the stimulus for increased epoxygenase activity in response to HS. In male Sprague-Dawley (SD) rats fed HS (4.0% NaCl) or normal salt (NS; 0.4% NaCl) diet for 7 days, renovascular responses to 2-chloroadenosine (2-CA; 10μg) were augmented and renal protein expression of $A_{2A}R$ induced in HS fed rats (5). These changes were associated with a 5-fold upregulation of CYP2C23 protein, a salt-inducible epoxygenase, and a 3-fold increase in the renal efflux of EETs and dihydroxyeicosatrienoic acids (DHTs). The responses to 2-CA were unaffected by inhibition of nitric oxide (NO) synthase or cyclooxygenases with L-NAME (200μM) and indomethacin (10μM), respectively, but a selective epoxygenase inhibitor, N-methylsulfonyl (propargyloxyphenyl) hexanamide (MS-PPOH; 12 μM), significantly reduced the response to 2-CA in HS rats, whereas lesser changes were evident in NS kidneys.

We then examined the role of the $A_{2A}R$-EET pathway in Dahl salt sensitive (DS) rats, a genetic model of salt-dependent hypertension. When male DS rats were fed NS or HS (8.0% NaCl) diet, BP increased from 124±3 to 167±2mmHg in HS-fed rats ($p<0.05$). Compared to SD rats, kidneys of DS rats exhibit reduced sensitivity to 2-CA, as reflected by a rightward shift in the dose-response (D-R) curve (IC_{50} 0.47μg vs. 7.26μg in SD and DS rats, respectively; $p<0.05$). Responses to 2-CA in DS rats were decreased by L-NAME, but not by MS-PPOH nor indomethacin. The D-R curve to 2-CA was similar in DS rats fed a NS or HS diet, as was renal efflux of EETs+DHTs (54.5±8.3 vs. 70.5±4.7 ng in response to 50μg 2-CA, respectively). HS intake in DS rats failed to upregulate renal protein expression of $A_{2A}R$ and CYP2C23. Thus, NO, not EETs, is the key mediator of 2-CA-induced dilation in DS rats. Thus, the $A_{2A}R$-EET pathway mediates the renal protective effects of adenosine and an inability to upregulate this pathway may contribute to the development of salt-induced hypertension.

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Supported by grants from the NIH DK69687 & HL34300

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SA210

Emerging roles for blood vessel wall hydrogen sulphide in vascular function

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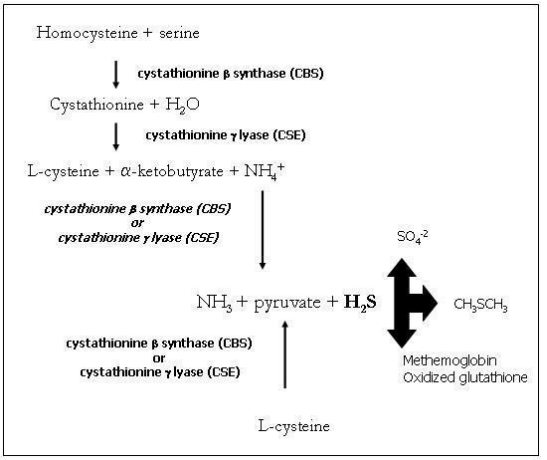
Historically hydrogen sulphide is best known as a toxic gas that has a typical smell of rotten eggs. Hydrogen sulphide can be generated endogenously from L-cysteine in a reaction catalysed by cystathionine gamma lyase (CSE) or cystathionine beta synthase (CBS) It is now known that hydrogen sulphide can directly alter smooth muscle tone .The expression of CBS and CSE appears to be tissue specific. CSE has been described to be widely expressed in peripheral vascular tissues and the heart. The actions of H2S itself have been studied in vascular tissues and uterine strips from pregnant rats, in which the predominant effect was relaxation. Similarly, it has been recently demonstrated that NaHS induces a dose-dependent relaxation of the rabbit ileum and rat vas deferens . In rat studies, bolus injection of hydrogen sulphide produced a transient decrease in mean arterial blood pressure. The possible role payed as mediator by hydrogen sulphide in vivo is further supported by the finding that the plasma levels of hydrogen sulphide in spontaneous hypertensive rats is significantly lower than that of the matching background e.g. WKY. The mechanism through which hydrogen sulphide exerts its relaxant properties is not fully understood, although it is likely mediated by the opening of K+ ATP channels. Hydrogen sulphide exerts a relaxant effect on rat aortic tissue and induces a transient reduction of blood pressure through a direct stimulation of K+ ATP channels and subsequent hyperpolarization of rat aortic vascular smooth muscle cells. Indeed, glibenclamide, a K+ ATP channel antagonist, reversed these actions, while pinacidil, a K+ ATP channel opener, mimicked the hydrogen sulphide - induced relaxation. These results have been recently confirmed using a sophisticated whole-cell and single-cell patch-clamp technique. Other potential targets of action of hydrogen sulphide on vascular smooth muscle include voltage-dependent Ca2+ channels and Ca2+-dependent K+ channels. Hydrogen sulphide has also effects on the heart where exogenous administration of NaHS (as a source of hydrogen sulphide) caused negative inotropism without affecting heart rate and coronary perfusion flow Interestingly hyperhomocysteinemia is a disease characterized by deficient expression of CBS which causes peripheral and cerebral occlusive arterial disease and the role of hydrogen sulphide in this pathology has never been addressed in depth yet it may be cause of atherosclerosis and/or thrombotic complications associated to hyperhomocysteinemia. In addition following heart transplantation the vascular diseases that develops are coupled to an increase in plasma homocysteine concentration.

In addition recently there has been a growing body of evidence suggesting a cross-talk between NO and hydrogen sulphide suggesting that the balance between these two gaseous mediators may play an important role in the vascular homeostasis.While the major reported effect of hydrogen sulphide in the context of smooth muscle is relaxation, it is noteworthy that in some tissues and in some species, hydrogen sulphide can exert powerful contractile responses.
In conclusion the current literature indicates hydrogen sulphide as a biologically active molecule that has mainly a vasodilator effect in vivo and in vitro and this is opening a new avenue in the search for new therapeutic targets in the cardiovascular field.



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SA211

What is EDHF?

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The phenomenon of endothelium-dependent hyperpolarization and relaxation and the possible existence of a factor – endothelium-derived hyperpolarizing factor (EDHF) - has been widely studied for the past 25 years. The broad consensus view is as follows:-

Classical EDHF Pathway; gap junctions or K^+?

If the endothelial synthesis of NO and prostacyclin is blocked using NO-synthase and cyclo-oxygenase inhibitors, ligands like acetylcholine (ACh) and substance P (SP) still hyperpolarize and relax vascular myocytes in an endothelium-dependent manner. These effects can be blocked by a mixture of TRAM-34 + apamin (blockers of endothelial IK_{Ca} and SK_{Ca} channels, respectively) but not by iberiotoxin + apamin. This then is the *pharmacological signature* of the (classical) EDHF pathway. ACh and SP raise endothelial $[Ca^{2+}]$, opening IK_{Ca} and SK_{Ca} channels and hyperpolarizing the endothelial cells. Controversy still surrounds the mechanism by which the endothelial hyperpolarization is transmitted to the myocytes to trigger vasodilation. *The gap junction hypothesis claims that there is no EDHF per se*; rather the hyperpolarization is transmitted to the myocytes via myo-endothelial gap junctions. The *K^+ hypothesis proposes that EDHF is K^+*. This ion, effluxing from endothelial cells through opened IK_{Ca} and SK_{Ca} channels activates myocyte K_{IR} channels and Na^+/K^+ ATPases; the resulting hyperpolarization produces the observed vasodilation.
It is likely that both hypotheses are correct, the importance of each being dependent on the vessel and its degree of tone.

Non-Classical Pathways; other hyperpolarizing factors

The widespread use of bradykinin (especially in the coronary circulation) to activate the classical EDHF pathway described above, inadvertently created confusion in the literature and amongst EDHF workers. In addition to activating the classical pathway, bradykinin also releases epoxyeicosatrienoic acids (EETs; most likely both 11,12 EET and 14,15 EET) from the endothelium. These diffuse to the myocytes and hyperpolarize these cells by activating myocyte BK_{Ca} channels. Thus, in early experiments on the EDHF pathway, the widespread use of the blocking mixture charybdotoxin (TRAM-34 was not available) + apamin was certainly effective in abolishing 'EDHF' responses mediated by bradykinin. However, it was not then realised that charybdotoxin not only blocked endothelial cell IK_{Ca} channels but also myocyte BK_{Ca} channels and thus both the classical EDHF pathway and the EETs pathway were inhibited.

What indeed is EDHF?

Based on the original observations, the classical pathway can best be described as that endothelium-dependent vasodilator pathway that is blocked by TRAM-34 + apamin. For those who feel that myo-endothelial gap junctions are the key to the resulting vasodilator response, no actual mediator is necessary. However, when vascular tone is moderate, considerable evidence identifies EDHF as endothelium-derived K^+. Indeed the involvement of K^+, locally released from neurones and skeletal muscle cells to increase blood flow within the brain and skeletal muscle, respectively, is well-established. EETs, prostacyclin (and sometimes even NO) are each liberated from the vascular endothelium and each can hyperpolarize the adjacent myocytes. They and other endothelium-derived substances could thus be described as EDHFs. However, the involvement of BK_{Ca} or of K_{ATP} in their actions means that it is inappropriate to give them the descriptor 'EDHF'.

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This work was funded by the British Heart Foundation

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SA212

Interaction of dietary and genetic factors in cancer prevention: The EPIC Study

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Secular trends in incidence, international comparisons, migrant studies and studies with twins have all shown a strong environmental cause for most cancers. It has been estimated that at least 30% cancers, and over 70% for some sites such as cancer of the large bowel, could be prevented by diet. This would mean such cancers are potentially preventable diseases and that dietary factors would be of great importance in public health. Findings from very large molecular epidemiological studies and smaller trials of intermediate risk markers allow some insights into the interaction between diet and gene variants and dietary mechanisms underlying DNA damage relevant to the development of cancer.

The European Prospective Investigation of Cancer (EPIC) is a prospective study that aims to investigate individual risk of cancer, and interaction with biomarkers of cancer risk, and polymorphisms in genes governing nutrient and xenobiotic metabolism. 500,000 men and women have been studied in detail for their dietary habits and their blood samples biobanked at –190C throughout Europe for over 10 years. In colorectal cancer, the first results from the EPIC cohort show strong inverse associations with fibre from food and strong positive effects of red and processed meat. These findings are supported by intervention studies with human volunteers under carefully controlled conditions showing that haem in red and processed meat enhances endogenous N-Nitrosation and the presence of the promutagenic DNA adduct O6 carboxymethyl guanine in exfoliated cells.

Phytoestrogens are found in plants, such as soy and their structural similarity to estradiol has prompted hypotheses that they are protective in breast cancer through inhibiting enzymes involved in oestrogen metabolism. In the largest investigation so far conducted, in 2000 women taking part in the EPIC study, the interaction between polymorphisms in HSD17B1, CYP19, SHBG, ESR1, ESR2 and NR1I2 genes, plasma hormone levels and phytoestrogen exposure was investigated. There was a significant interaction between isoflavones, SHBG levels, and SHBG D356N polymorphism (p=0.019). Even in western populations consuming comparatively low levels, phytoestrogens interact with gene variants involved in oestrogen metabolism and signalling. Such interactions may also occur in populations consuming high levels of soy and affect individual risk of cancer development.

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SA213

Determining the efficacy of dietary phytochemicals in cancer chemoprevention

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There is currently much research interest in cancer prevention, particularly the role that dietary phytochemicals might play in blocking initiation of tumours or in delaying or reversing their progress. Thousands of in vitro studies in cell culture and many animal models representing a wide range of target tissues have shown anti-cancer efficacy for a large number of diet-derived compounds. However, success in clinical trials to date has been much more limited. Prevention trials in apparently healthy populations, with development of cancer as the main endpoint, require large numbers of individuals to be followed over many years, even decades, with enormous cost implications. To enable many more agents to be tested much more quickly, validated surrogate endpoint biomarkers are required, which will accurately determine outcome at a much earlier time in the process of tumour development. Such biomarkers are currently few and far between. One possibility is to identify at risk patients who have developed premalignant lesions, such as intraepithelial neoplasia, and monitor the progress of these in response to chemopreventive treatments. Several dietary phytochemicals, including indoles and polyphenols, have shown promise in this respect, with regression of respiratory papillomatosis, cervical and prostate intraepithelial neoplasia and oral leukoplakia. However, the relationship of some of these early lesions to tumour outcome is uncertain. Identification of reliable molecular markers would be advantageous to determine earlier changes, to relate directly to the carcinogenic process and, where possible, to allow less invasive assessment of efficacy. This requires a detailed knowledge not only of the stages of carcinogenesis for a particular tumour, but also of the mechanisms of action of the preventive agent. An ideal molecular marker would be closely linked to tumour development and treatment efficacy. Measurement directly or via a closely related activity should be possible, and preferably in non-invasive clinical specimens. Such biomarkers would be valuable in several aspects of chemoprevention – as targets to identify new agents or to optimise lead compounds; as risk biomarkers for selecting suitable cohorts for chemopreventive trials, or as indicators of efficacy for determining response to mechanism-based interventions or potential toxicity. There is no shortage of candidate proteins related to oncogenic processes (drug metabolising enzymes, growth factors, transcription factors, cell cycle and apoptosis related proteins) which have been shown to be modulated by phytochemicals in vitro. However, many of these mechanistic studies have been carried out with single high doses that are not achievable in vivo. Thus some of the reported effects may not be physiologically relevant. Effects can be cell type specific and so different panels of biomarkers may be required for different target tissues or for different cancer subtypes within a single tissue. On the other hand there appears to be a certain degree of similarity in the protein targets affected by a variety of structurally unrelated phytochemicals, suggesting similar mechanisms of action. A detailed understanding of the effects of dietary agents (for example on

growth factor receptor signaling, epithelial to mesenchymal transition, cell cycle arrest and apoptosis) following extended treatment at physiologically achievable doses, with respect to agent, target tissue and cancer subtype, will help to identify useful biomarkers. Such an understanding would include identification of primary targets for phytochemicals (particular proteins such as receptors, or more general such as redox status), reasons why healthy cells are generally more resistant and comparison of in vitro with in vivo efficacy. Also of increasing importance is the investigation of combinations of phytochemicals, or their use in combination with other therapies, to increase efficacy or decrease unwanted side effects.

Work in our laboratory is funded by the MRC, the Hope Foundation and an EU Network of Excellence (ECNIS).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C90

Lycopene inhibits PDGF-BB-induced signaling and migration in human dermal fibroblasts

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In melanoma development and progression, PDGF has been suggested to modulate the microenvironment, especially stromal fibroblasts, to the benefit of melanoma growth, invasion, and metastasis. Lycopene, a natural carotenoid that is abundant in tomato, has been shown to inhibit proliferation of several types of cancer cells. However, little attention has been paid on melanoma and skin fibroblast cells. In the present study, we determined the effects of lycopene on stromal fibroblasts. We found that lycopene inhibited PDGF-induced human Hs68 skin fibroblast migration on gelatin and collagen. Further analysis showed that lycopene inhibited PDGF-BB-induced signaling in human Hs68 and cultured skin fibroblasts. PDGF-BB-induced phosphorylation of PDGF receptor, ERK1/2, p38, and JNK was attenuated by lycopene, whereas the expression of each total protein was not affected, suggesting that lycopene has relative specificity on PDGF-BB-induced signaling. Surprisingly, dot binding assay indeed demonstrated that lycopene could directly bind to human PDGF-BB in a dose-dependent manner, suggesting that lycopene acts on PDGF-BB rather than on fibroblasts. In the parallel experiments, lycopene inhibited melanoma-induced fibroblast migration in a non-contact coculture system and attenuated signaling transduction pathway in fibroblasts simulated by melanoma-derived conditioned medium. Our results suggest that lycopene is an effective inhibitor on migration of the stromal fibroblasts and this effect may contribute to its anti-tumor activity.

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C91

Targeting Neuroblastoma; the search for a better ganglioside-based molecular solution

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Neuroblastoma, the commonest solid extracranial tumour of infancy, is treated with a number of therapeutic modalities. Depending upon staging at presentation, these include resection, "differentiation therapy" with retinoid and related morphogens and conventional chemotherapy. Latterly targeted immunotherapy has been directed at a defined ganglioside GD2 specifically enriched in the plasma membrane (PM) of aberrant cells. This targeted monoclonal antibody approach, while offering promise clinically is compromised by expression of the relevant ganglioside on nerve cells among others which also bind inducing significant pain. The ameliorative opiate therapy administered is both undesirable and potentially harmful. Clearly, a more restricted target would offer the possibility of minimising this undesirable side-effect and obviate the need for morphia. Recent advances in glycolipidomics now permit the extension ESI-MS-based, small molecule analyses to these molecules with unparalleled sensitivity and discrimination. Application of these methodologies to cultured neuroblastoma cells should enable a more accurate identification of the composition of neuroblastoma PM gangliosides with the aim of tailoring the immunotherapeutic approach more rigorously in future.

Here we have used analytical and imaging approaches to define the pattern and topology of ganglioside expression at the PM. Analytically we have used Qtof mass spectrometry to characterise fragmentation patterns of major ganglioside species from standards and tissue extracts. This has allowed us to identify the diverse range of ganglioside species even those with relatively low abundance from their unique profiles and will enable us to confirm the identities of the "unusual" once we have completed analyses of control neural tissues. For imaging work we used a panel of recently commercially available monoclonal antibodies to 13 gangliosides in immunofluorscence studies of cultured Kelly and SK-N-AS human neuroblastoma cell lines. This approach should provide the complementary data that helps us to identify which gangliosides are displayed on the outer aspect of the PM in contrast to what is present within the whole cell. Together these data may provide evidence to accurately define more viable candidate ganglioside molecular targets or combinations of ganglioside targets for neuroblastoma therapy.

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SA214

COX-2 and colorectal cancer:signalling downstream of COX-2 represents potential novel targets for the prevention/treatment of colorectal cancer

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Although up to 70-80% of colon cancers are believed to be preventable, colorectal cancer is the second most common cause of cancer deaths in the industrialised world and trends towards increasing obesity suggest the potential for a further significant increase in its worldwide incidence. There is therefore an urgent need to develop novel methods for the prevention and treatment of this cancer.
Non-steroidal anti-inflammatory drugs (NSAIDs) are chemopreventive against colorectal cancer, mostly through their inhibitory effects on the cyclooxygenase isoform COX-2. COX enzymes represent the committed step in prostaglandin biosynthesis and it is predominantly increased COX-2-mediated prostaglandin-E2 (PGE2) production that has a strong association with colorectal neoplasia, by promoting cell growth, cell survival, migration, invasion and angiogenesis. COX-2 is overexpressed in the majority of colon cancers and a subset of adenomas. COX-1 and COX-2 inhibition by traditional NSAIDs, such as aspirin, although chemopreventive have some side effects. Interestingly, the use of COX-2 selective NSAIDs has also shown promise in the prevention/treatment of colorectal cancer while having a reduced impact on the gastric mucosa. However, the prolonged use of high dose COX-2 selective inhibitors is associated with a risk of cardiovascular side effects. There is therefore an urgent need to further our understanding of the downstream mechanisms by which PGE2 promotes tumorigenesis and hence identify safer, more effective strategies for the prevention of colorectal cancer. In particular, PGE2 synthases and E-prostanoid receptors (EP1-4) have recently attracted considerable interest in this area. Recent evidence suggests that the EP4 receptor may represent an important target for the prevention/treatment of colorectal cancer. It is hoped that the inhibition of the synthesis and signalling of those prostaglandins most highly associated with colorectal tumorigenesis, such as PGE2, may have advantages over COX-2 selective inhibition and therefore represent more suitable targets for long-term chemoprevention. Since COX-2 is found to be overexpressed in cancers such as breast, gastic, lung, and pancreatic, these approaches may have broad implications for the prevention/treatment of other malignancies.

Cancer Research UK

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SA215

Novel agents for cancer prevention based on nitric oxide

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Nitric oxide biology has provided the impetus for the development of anticancer agents based on their ability to release NO. The so called NO-donating NSAIDs (NO-NSAIDs) are emerging as a prototypical members of this class of compounds for the chemoprevention of cancer. They consist of a conventional NSAID to which a NO-releasing moiety is covalently attached. Recent progress in their preclinical and mechanistic evaluation has unraveled their cardinal features. Compared to their parent compounds, NO-NSAIDs are up to several hundred times more potent in inhibiting the growth of colon cancer cell lines and also quite effective in preventing colon cancer in various tumor animal models of colon and pancreatic cancer. A strong cell kinetic effect, including inhibition of proliferation, induction of cell death, and inhibition of cell cycle phase transitions underlines their chemopreventive effect.
NO-aspirin (NO-ASA) ids the NO-NSAID best studied mechanistically. The induction of oxidative stress appears crucial for its anticancer activity. Major effects have been identified on various cell signaling pathways including Wnt, NOS2, MAPK and Nrf2 signaling. Perhaps paradoxically, NO-ASA induces the expression of COX-2 while inhibiting cell growth strongly. NO-NSAIDs, particularly NO-ASA, appear to be very safe compounds, as evidenced from many animal and early human studies. An ongoing clinical trial is designed to determine whether NO-ASA can inhibit early stages of colon carcinogenesis in subjects at risk for colon cancer.

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SA216

Glycogen synthase kinase 3 as a convergence point in hypertrophic signalling

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Glycogen synthase kinase 3 α/β (GSK3α/β)were first identified over 25 years ago as as key enzymes in the regulation of carbohydrate metabolism. In their active form, these protein kinases catalyse inhibitory phosphorylations of glycogen synthase, thus cooperating in glycogen breakdown. However, subsequently, it has been recognised that they participate much more widely in the regulation of biological processes. GSK3α/β each contain a Tyr-residue [GSK3α(Tyr279)/GSK3β(Tyr216)], maturational intramolecular autophosphorylation of which facilitates and is necessary for activity. In terms of diurnal biological control, the most important regulatory event for GSK3 is thought to involve phosphorylation of GSK3α(Ser21)/GSK3β(Ser9) which inhibits their activity. However, there may also be additional, less well-defined sites of regulatory phosphorylations. Classically, phosphorylation of GSK3α(Ser21)/GSK3β(Ser9) is catalysed by protein kinase B/Akt (PKB/Akt), which is activated as a result of phosphatidylinositol 3-kinase (PI3K) signalling. Through this, insulin promotes glycogen synthesis by inhibiting GSK3, thus relieving the inhibition of glycogen synthase. In addition, activation of other protein kinases promotes phosphorylation of GSK3α(Ser21)/GSK3β(Ser9). Thus, activation of the ERK1/2 cascade leads to phosphorylation of GSK3α(Ser21)/GSK3β(Ser9), though this is probably mediated

by ERK1/2-activated protein kinases rather than ERK1/2 themselves. Somewhat unexpectedly, in view of its role in stimulating glycogen breakdown, activation of protein kinase A may also promote phosphorylation of GSK3α(Ser21)/GSK3β(Ser9). A role for GSK3 (the emphasis was largely directed towards GSK3β) in the development of cardiac/cardiac myocyte hypertrophy was recently proposed by Morisco *et al.* (2000) and Haq *et al.* (2000). As reviewed by Shevstov *et al.* (2006), one current proposal is that that GSK3 in its active state mediates phosphorylation of β-catenin promoting its proteasomal degradation. When GSK3 is inhibited, β-catenin is stabilised and enters the nucleus where it derepresses the Groucho-mediated inhibition of Lef/TCF transcription factors and promotes transcription of the genes they regulate. Alternatively or additionally, GSK3-mediated phosphorylation of NFATs (specifically NFATc1) results in their retention in the cytoplasm (Beals *et al.* 1997). By either pathway, PI3K - PKB/Akt-mediated inhibition of GSK3 by powerful hypertrophic agonists such as endothelin-1 (ET-1) or phenylephrine could promote transcription of suitably-susceptible genes either by stabilising β-catenin or by promoting NFAT dephosphorylation by protein Ser/Thr phosphatases such as calcineurin. Thus GSK3 in its catalytically-active state [GSK3α(Ser21)/GSK3β(Ser9)dephosphorylated] is potentially antihypertrophic. We have recently re-examined aspects of this scheme in cardiac myocytes from neonatal rat hearts in primary culture. In outline, our conclusions are as follows. 1. Both GSK3 isoforms are expressed in cardiac myocytes, thus GSK3α should receive as much attention as GSK3β. 2. Insulin, which strongly promotes activation of PKB/Akt but which does not activate the ERK1/2 cascade in cardiac myocytes, causes an LY294002-sensitive phosphorylation of GSK3α(Ser21)/GSK3β(Ser9). This coincides with inhibition of GSK3 activity as assessed using an FPLC-based assay. However, we have never been able to detect any pro-hypertrophic effects (e.g increased cell area and myofibrillogenesis) of insulin. 3. ET-1 (which is at best a weak activator of PKB/Akt but is a strong activator of the ERK1/2 cascade) causes a U0126-sensitive phosphorylation of GSK3α(Ser21)/GSK3β(Ser9), implying involvement of the ERK1/2 cascade. 4. In contrast to the results with insulin and with the caveat of lack of specificity, the GSK3 inhibitor 1-azakenpaullone promotes an increase in myocyte area with an increase in the myocyte length/width ratio. Overall, we conclude that whilst there may be a role for GSK3 activity in the inhibition of the cardiac myocyte hypertrophic response (and a role for GSK3 inhibition in the development and continuation of the response), the situation is not as clearly defined as with other signalling pathways.

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The authors' work was supported by the British Heart Foundation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA217

Differential activation of gp130 downstream signaling directs cardiac hypertrophy, collagen metabolism and angiogenesis after myocardial infarction

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The gp130 receptor is activated by interleukin-6 (IL-6) cytokines in response to inflammation, pressure overload, and ischemic injury and signals via JAK-STAT, SHP2-Ras-ERK and PI3/Akt pathways. In the heart, gp130 signaling has been implicated in cardiomyocyte hypertrophy and survival, ECM remodeling, and inflammation. Here we determined the role of individual downstream signaling pathways activated from the gp130 receptor in the mouse heart after myocardial infarction (MI).

Using the Cre-LoxP system, we generated mice harboring cardiomyocyte-restricted mutations in the gp130 receptor, e.g. a complete knock out of gp130 (CKO), a mutation in the gp130 receptor abrogating gp130 SHP2-Ras-ERK signaling (CKO-ERK) and a mutation in the gp130 receptor abrogating JAK-STAT signaling (CKO-STAT). All genotypes are viable and born with the expected Mendelian ratio. No specific cardiac phenotype was observed under the baseline condition. Intravenous injection with the IL-6 cytokine, leukemia inhibitory factor (LIF), induced a rapid activation of ERK and STAT3 in left ventricles (LVs) from WT mice while in CKO, LV activation of ERK and STAT3 was absent. In CKO-ERK LVs, LIF induced a normal activation of STAT3, but ERK1/2 activation was absent. In contrast, in CKO-STAT LVs, LIF-induced activation of STAT3 was absent and ERK1/2 was hyperactivated. No specific activation of Akt was observed upon LIF injection. Within the first 14 days post MI, mortality rate was higher, MI size was larger and cardiac function more deteriorated in all gp130 mutations compared with wildtype (WT) siblings. Long-term post MI mortality was highest in CKO-ERK (76%, $P<0.01$ vs WT) followed by CKO (61%, $P<0.01$ vs WT). No increased long-term MI mortality was observed in CKO-STAT compared with WT mice. CKO-ERK mice displayed attenuated cardiomyocyte hypertrophy and increased collagen degradation in the infarct border zone compared with WT mice. CKO-STAT mice showed elongation of cardiomyocytes and enhanced fibrosis compared with WT. CKO mice displayed elongation of cardiomyocytes and decreased capillary density compared with WT.

For the first time we have dissected gp130-mediated signaling pathways according to their specific role on cardiac remodeling after MI. In this regard, we could show that gp130 mediated ERK1/2 activation controls cardiomyocyte hypertrophy and limits collagen degradation in the MI border zone, while gp130 mediated STAT activation protects from cardiomyocyte elongation and extensive fibrosis.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA219

Autocrine/paracrine actions of natriuretic peptides in myocardial infarction

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The natriuretic peptides ANP, BNP and CNP are cardiac autacoids exerting local actions in the heart and, in the case of ANP and BNP, endocrine actions on the peripheral vasculature and kidneys. The major actions of the peptides are brought about by binding to membrane-bound receptors (NPR-1 and NPR-2), leading to activation of particulate guanylate cyclase and elevation of intracellular cGMP (Baxter, 2004). Accumulating evidence suggests that the natriuretic peptide/cGMP pathway may be an important autocrine/paracrine mechanism that attenuates both the extent of acute injury during infarction and the long-term proliferative responses relevant to the development of post-infarct cardiomyopathy (D'Souza et al., 2004).

During acute myocardial ischaemia, tissue release of BNP is observed to be marked and rapid, consistent with an endogenous cardioprotectant role of the peptide. Recent evidence suggests that exogenously administered ANP and BNP are cardioprotective in a variety of ischaemia-reperfusion models. For example, ANP is anti-arrhythmic in coronary artery occlusion models and both ANP and BNP limit infarct size when administered prior to coronary occlusion. These protective effects are clearly related to elevation of cGMP concentration but the distal effectors of the survival signalling cascade are unclear. We have proposed that cGMP-dependent protein kinase (PKG) is a salvage kinase at reperfusion and demonstrated that pharmacological inhibition of PKG abrogates the infarct-limiting effect of postconditioning. More recently we have observed that BNP (10 nM) administered during the early reperfusion period limits infarct size, serving as a pharmacological mimetic of ischaemic postconditioning. Further work has revealed that the protective action of BNP at reperfusion is dependent on the opening of KATP channels since pharmacological inhibition with glibenclamide, 5-hydroxydecanoate or HMR1098 abrogates the protective action of BNP. Whether or not endogenous natriuretic peptides play a role in ischaemic postconditioning remains to be determined. Nevertheless, evidence such as that outlined above suggests that therapeutic activation of the natriuretic peptide/cGMP pathway may have potential to limit infarct size by salvaging myocardium from the injurious consequences of reperfusion.

Following infarction, during the proliferative remodelling phase, the steady elevations of circulating ANP and BNP correlate with the extent of left ventricular dysfunction. The induction of natriuretic peptide production and release as a feature of the neurohormonal response is now well appreciated, and it seems likely that the peptides play local autocrine/paracrine roles that counter-regulate the proliferative signalling activated by angiotensin-II, catecholamines and endothelin. Although upregulation of ANP and BNP gene expression is a feature of cardiac myocyte hypertrophy, the peptides exert a marked anti-hypertrophic action in myocytes in vitro. There is also experimental evidence that cardiac fibroblasts may be a major source of ANP and BNP in remodelling, and that via a cGMP-dependent mechanism natriuretic peptides inhibit fibroblast proliferation and collagen synthesis. The effects of ANP and BNP on cardiac myocyte apoptosis are less clearly defined but these actions might also favourably influence remodelling. Recent work identifying the heart as a source of CNP during the evolution of heart failure raises questions about the possible autocrine/paracrine roles of this peptide and how these relate to the actions of ANP and BNP. Ultimately, the balance of proliferative and anti-proliferative signals will determine the extent of adverse remodelling and the natriuretic peptide family is likely to be very important in providing physiological antagonism of proliferative neurohormones. The reasons why there is apparent overwhelming of the beneficial effects of natriuretic peptide signalling in progressive hypertrophy and decompensation are unknown and represent a challenge for harnessing of the peptides' therapeutic potential.

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The author acknowledges the generosity and support of the British Heart Foundation, Heart Research UK and The Wellcome Trust.

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C92

TNFα-induced IL-1 and IL-6 expression in human cardiac fibroblasts: mechanisms and modulation by thiazolidinediones

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The proinflammatory cytokine TNFα (tumor necrosis factor α) plays an important role in the remodelling of the heart that occurs following myocardial infarction. In addition to exerting direct effects on cardiac cell function, TNFα contributes to adverse myocardial remodelling by increasing production of other proinflammatory cytokines, such as interleukin (IL)-1 and IL-6. Thiazolidinediones (TZDs) are insulin-sensitizing agents primarily used for treating Type 2 diabetes. However, in addition to improving insulin resistance, TZDs also exert beneficial pleiotropic anti-inflammatory effects and can reduce adverse myocardial remodelling. The aim of the present study was to determine whether TNFα could stimulate expression of other proinflammatory cytokines in cultured human cardiac fibroblasts, to examine the underlying intracellular mechanisms, and to investigate the modulatory effects of TZDs.

Human cardiac fibroblasts were cultured from biopsies of right atrial appendage. Cytokine mRNA expression and secretion was measured using quantitative real-time RT-PCR and ELISA. Activation of signalling pathways was determined by immunoblotting with phospho-specific antibodies.

TNFα (0.1-10 ng/ml) stimulated IL-6, IL-1α and IL-1β mRNA expression in human cardiac fibroblasts in a concentration-dependent manner. The use of pharmacological signalling pathway inhibitors (PD98059, SB203580, LY294002 and IMD-0354) and receptor neutralising antibodies established that both

TNFα-induced IL-6 and IL-1β expression were mediated via the TNF-RI receptor and p38 MAP kinase, PI3K/Akt and NF-κB pathways. In contrast, TNFα-induced IL-1α expression was mediated via both TNFRI and TNFRII subtypes and stimulated via the p38 MAP kinase and PI3K/Akt pathways, but negatively regulated by the NF-κB pathway. Despite TNFα increasing mRNA levels of all three proinflammatory cytokines, only IL-6 was detected in conditioned media following TNFα treatment. The ability of three different TZDs (ciglitazone, rosiglitazone and troglitazone) to modulate cytokine expression was then investigated. All three TZDs increased TNFα-induced IL-1α or IL-1β mRNA expression, and ciglitazone and troglitazone (but not rosiglitazone) significantly increased IL-6 mRNA and secretion.

Our data provide important insights into the regulation of proinflammatory cytokine expression in human cardiac fibroblasts, and suggest that the beneficial effects of TZDs on the myocardial remodelling process are not due to inhibition of TNFα-induced IL-1 or IL-6 expression by cardiac fibroblasts. Rather, the TZDs appear to exert "pro-inflammatory" effects on these cells, which may raise questions regarding the use of this class of drugs in patients with cardiovascular disease.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA220

Insulin resistance and endothelium dysfunction

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Insulin resistance is now well established as an independent risk factor for the development of type 2 diabetes and cardiovascular atherosclerosis. Consistent with this humans with insulin resistance such as obese, type 2 diabetic or subjects of South Asian origin are at substantially increased risk of cardiovascular atherosclerosis and its complications. Most studies have examined atherogenesis in models of severe insulin resistance or diabetes. However, by the time type 2 diabetes is diagnosed, these patients already demonstrate a significant atheroma burden. Furthermore, recent studies suggest that even in adolescence, relative insulin resistance may increase cardiovascular risk. Future therapies must therefore target progression of vascular dysfunction in mildly insulin resistant states prior to the development of diabetes. In the current work we studied the impact of very mild insulin resistance on endothelial function during ageing from adolescence through to adulthood. Endothelium dysfunction is a hallmark of insulin resistant conditions and is a key step in the initiation and progression of atherosclerosis. The term endothelium dysfunction embraces a number of abnormalities amongst which reduction in bioavailability of the antiatherosclerotic signalling molecule nitric oxide (NO)is of particular importance. The present series of studies used a murine model of insulin resistance at a global level to explore the temporal changes in NO bioavailability as mice moved from adolescence to adulthood.

We used mice with haploinsufficiency for the insulin receptor (IRKO) a model of mild insulin resistance with preserved glycaemic control. We previously demonstrated that 2 month-old (Young) IRKO mice have preserved vasorelaxation responses to acetylcholine (Ach). We have now studied the effect of ageing on endothelium function in male IRKO mice compared to wildtype littermate controls. Despite no significant deterioration in glucose homeostasis, 6 month-old (Adult) IRKO mice had marked blunting of Ach-mediated vasorelaxation (IRKO Emax 66+/-5% versus wildtype 87+/-4%, $p<0.01$). This was restored by the superoxide dismutase mimetic MnTMPyP (IRKO Emax to Ach with MnTMPyP 85+/-5%). Dihydroethidium fluorescence of aortae and isolated coronary microvascular endothelial cells demonstrated a substantial increase in endothelium-derived reactive oxygen species in IRKO mice. These data demonstrate that mild insulin resistance is a potent substrate for accelerated endothelium dysfunction during ageing, and also suggests that reduction of endothelial superoxide production may preserve endothelium function over time.

This work was supported by The British Heart Foundation

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SA221

The adventitial fibroblast NAD(P)H oxidase as a paracrine mediator of vascular hypertrophy: Signal for vascular disease?

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Reactive oxygen species (ROS) have been implicated in tissue remodeling in a variety of cardiovascular diseases including hypertension, stroke, myocardial infarction, congestive heart failure and renal microvascular disease. Undoubtedly, the mechanisms involved are complex; however there is now compelling evidence that an increase in ROS during hypertension plays a pivotal role in this process. Studies suggest that in hypertension and other vascular disease, there is an imbalance in oxidant-generating vs. oxidant-catalyzing systems, leading to a build-up of endogenous superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). We and others have shown that the adventitia is a major site of vascular ROS production and have proposed that adventitial fibroblast NAD(P)H oxidase-derived ROS is the sensor and messenger for the early development of systemic vascular disease. This presentation will focus on the paracrine contribution of adventitial fibroblasts, their NAD(P)H oxidase(s) and the role of attendant ROS in neointimal growth and medial hypertrophy in systemic arteries. Until recently the contribution of the adventitia to vascular function has largely been ignored except for reference to its role in the structural support for the blood vessel and as a scaffold for sympathetic nerve endings and the vasa vasorum. As such, the adventitia has been viewed with much the same skepticism as once was reserved for the endothelium, largely being considered an inert physical barrier separating the medial smooth muscle from other tissues. This "outsider" status of the adventitia is clearly giving way to a rapidly growing interest in this relatively new frontier in vascular biology. Multiple findings

support a seminal and complex role for adventitial ROS in the recruitment of inflammatory cells, production of extracellular matrix and general remodeling of the vessel wall in cardiovascular disease.

NIH Grant #HL-55425 and the American Heart Association (AHA). PJP is an Established Investigator of the AHA.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C93

Cranberry juice consumption ameliorates endothelial dysfunction in ovariectomized rats

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Consuming vegetables and fruits can reduce the risk for cardiovascular diseases. Nutraceutical products have been increasingly used to retard the onset of cardiovascular complications and to treat postmenopausal symptoms. Cranberries are rich in antioxidant polyphenols, such as flavanoids and related phenolic acids. In this study, we aimed to examine whether consumption of cranberry juice could ameliorate endothelial dysfunction in an animal model of estrogen deficiency.

Female Sprague-Dawley rats, weighted 200-230 g, were anaesthetized using sodium pentobarbital (40 mg kg^{-1} body weight, i.p.) under aseptic conditions and then ovariectomized via the abdominal route. Sham-operated rats were used as controls. The rats were divided into three experimental groups: sham-operated, ovariectomized, and ovariectomized rats receiving cranberry juice. Changes in vascular reactivity (constriction and dilatation) in aortas, carotid arteries, and renal arteries from all three groups were studied in organ bath or on myograph. The expression level of endothelial nitric oxide synthase (eNOS), Akt, angiotensin 1 receptor and nitrotyrosine were detected by Western blot. The endothelium-dependent relaxation to acetylcholine was significantly reduced in the three types of isolated arteries from ovariectomized rats and the impaired endothelial function can be partially prevented by acute treatment with angiotensin 1 receptor blocker. Chronic consumption of cranberry juice significantly ameliorated endothelial dysfunction due to estrogen deficiency in ovariectomized rats. The vascular tension developed by the addition of phenylephrine was higher in ovariectomized rats than in control rats. This increased contraction was attenuated in rats receiving cranberry juice. Last, there is also an increased oxidative stress, as indicated by nitrotyrosine level, which was restored by cranberry consumption. Chronic consumption of cranberry juice (i) increased phosphorylation of eNOS at ser1177 without affecting the total aortic protein contents of eNOS and Akt, and (ii) decreased protein expression for angiotensin 1 receptor in aortas. The results of the present study suggested that chronic oral administration of cranberry juice to ovariectomized rats clearly augments the bioavailability and function of endothelial nitric oxide in systemic blood vessels via stimulating eNOS phosphorylation and inhibiting expression and/or function of angiotensin 1 receptor-mediated vascular dysfunction. The active ingredients in cranberry juice responsible for restoring endothelial function remain to be further elucidated.

Supported by RGC grant, CUHK Li Ka Shing Institute of Health Sciences and Focused Investment Scheme of Hong Kong.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C94

Upregulation of aldo-keto reductases AKR1B and AKR1C in aorta: an adaptive response to reactive aldehydes

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Reactive oxygen species (ROS) are known to be produced during the development of atherosclerosis, and are thought to play a detrimental role in the progression of the disease, for example through the oxidation of LDL (1). Reactive aldehydes generated through the peroxidation of lipids have also been implicated as agents in the cytotoxic processes that are initiated by ROS. In particular, the reactive aldehydes 4-HNE and acrolein are known to trigger apoptosis, and at high doses necrosis (2). Both acrolein and 4-HNE have been shown to reach high concentrations in and around atherosclerotic plaques, and may be more destructive than the initial production of ROS (3).

Several enzyme families are able to detoxify reactive aldehydes, including the aldo-keto reductases (AKR), a superfamily consisting of over 40 NADPH-dependent enzymes that are capable of reducing aldehydes and ketones to alcohols (4). The reduction of 4-HNE and acrolein to the corresponding alcohols is a detoxication step, and may play a role in reducing the damage caused by lipid peroxidation (5).

In this study, we have examined the aortic expression of two aldo-keto reductases, AKR1B and AKR1C, in a mouse model of atherosclerosis in order to examine their role in protection against reactive aldehdyes. Apolipoprotein E is essential for maintaining normal cholesterol levels, and ApoE -/- mice that lack this protein are frequently used as a model as they develop early atherosclerotic coronary artery disease. Immunohistochemistry of aortic segments showed increased expression of both AKR enzymes in ApoE -/- mice compared to control C57BL/6 mice. Quantitative Western blot analysis of aortic homogenates indicated over 5-fold increase in expression of AKR1B and AKR1C protein levels. Aortic homogenates from ApoE -/- mice were also shown to have increased ability to metabolise acrolein and 4-HNE compared to control. In an attempt to understand the mechanism responsible for increased expression, cultured C57BL/6 aortic smooth muscle cells were exposed to a sub-lethal concentration of 4-HNE. Western blot analysis revealed increased expression of AKR1B in exposed cells. In addition, pretreated cells expressing increased levels of AKR1B were more resistant to the deleterious effects of 4-HNE. These results indicate that

aortic smooth muscle cells display an adaptive response to toxic aldehydes, and suggest that this can provide cytoprotection from toxic insults such as 4-HNE.

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RL is a recipient of a Bell College Research Fund studentship.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA222

Disruption of methylarginine metabolism impairs vascular homeostasis

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Asymmmetric dimethylarginine (ADMA) and monomethyl arginine (L-NMMA) are endogenously produced amino acids that inhibit all three isoforms of nitric oxide synthase (NOS) [1]. ADMA accumulates in various disease states including renal failure, diabetes and pulmonary hypertension, and its concentration in plasma is strongly predictive of premature cardiovascular disease and death [2-4]. Both L-NMMA and ADMA are eliminated largely through active metabolism by dimethylarginine dimethylaminohydrolase (DDAH) [5] and thus DDAH dysfunction may be a crucial unifying feature of increased cardiovascular risk. However, despite considerable interest in this pathway and the role of ADMA as a novel risk factor, there is little evidence to support a causal role in pathophysiology. Here we reveal the structure of human DDAH-1 and probe the function of DDAH-1 both by deleting the DDAH-1 gene in mice and by use of designed specific inhibitors which we demonstrate by crystallography bind to the active site of human DDAH. We show that loss of DDAH activity leads to accumulation of ADMA and reduction in NO signalling. This in turn causes vascular pathophysiology including endothelial dysfunction, increased systemic vascular resistance and elevated systemic and pulmonary blood pressure.

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This work was funded by grants from the British Heart Foundation (PG20007 and PG/02/165/14797), Wellcome Trust (065612/Z/01) and MRC (G0000002). PV, JL and MN are part of the European Vascular Genomics Network funded by the EU (Contract No. LSHM-CT-2003-503254). This study received financial support from the European Commission under the 6th Framework Programme (Contract No: LSHM-CT-2005-018725, PULMOTENSION).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA223

Endothelial dysfunction and atherothrombosis

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The initiation, modulation and resolution of thrombus associated with eroded or unstable coronary plaques are critical determinants of acute coronary events. This itself is dependent on the cellular function of the surrounding endothelium and vascular wall. In particular, the regulation of vascular tone and the acute release of tissue plasminogen activator by the endothelium make important contributions to the defence against intravascular thrombosis. These aspects of endothelial function may provide major new insights into the pathophysiology of cardiovascular disease, and to shape future therapeutic interventions.

Professor Newby is supported by the British Heart Foundation

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA224

PKC control of cell division

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The duplication of cellular contents and their precise, equal division between daughter cells is a characteristic of dividing cell populations. Successful achievement of this complex process is effected by a substantial repertoire of cellular machinery and associated controls. The latter ensures the appropriate completion of individual steps prior to the commitment to the next. Thus, for example, the integrity and completion of DNA replication is ensured prior to exit from S-phase. The final, irretrievable step in this cascade of cell division events is the separation of daughter cells, i.e. cytokinesis. As with other elements of the cell cycle, cytokinesis is a highly orchestrated event and recent studies have uncovered a critical role for Protein Kinase

C (PKC) in this process. The relevant PKC-dependent properties, the redundancy of PKC action and the regulatory inputs via PKC will be described.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA225

Functional PKC *in vivo* analysis using deficient mouse models

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The aim of our group is to identify Protein kinase C in vivo function by analysing individual PKC knock outs we have generated over the past few years. The general approach we are using to identify target tissues and/or defined cell populations within the mouse for further investigation is a detailed expression analysis of individual PKC isoforms. For these purposes we have established several specific tools in the past which allows us to follow up isoform specific PKC expression on a very precise level. Doing so we have started to investigate PKC expression profiles under various tumour conditions in mice. As predicted we were able to identify various PKC isoforms to be either up or down regulated during the development and progression of certain tumours implying that these isoforms are substantially linked to the biology of these tumours. In order to proof this hypothesis we then cross relevant PKC knock out lines on the appropiate tumour background and analyse tumour growth and progression under PKC deficient conditions. Examplary for this approach recent data of PKCalpha deficient APCmin mice will be presented.
Along the line of isoform specific PKC expression profiles we also identified various tissues and cell population with overlapping PKC expression without showing a phenotyp in the single knock out raising the question whether there is redundancy within the PKC family. To address this question we have started to generate PKC double knock outs and in fact some combinations clearly show phenotyps not present in the single knock out indicating that compensation takes place among PKC isoforms. Recent data will be presented.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C95

Aldosterone rapidly activates protein kinase D via a mineralocorticoid receptor/epidermal growth factor receptor trans-activation pathway in kidney cortical collecting duct cells

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Aldosterone promotes Na^+ absorption and K^+ secretion in target tissues such as the epithelia of the distal colon and distal nephron. The osmotic movement of water concurrent with Na^+ absorption means that the net effect of aldosterone release on the whole body is to increase extracellular fluid volume and consequently raise blood pressure [1]. The cytoplasmic mineralocorticoid receptor(MR) acts as a ligand-dependent transcription factor [2]. However, the earliest investigation of rapid physiological responses to aldosterone describes the hormone's effects on Na^+ and K^+ excretion into the urine within 5 min following its intra-arterial administration [3]. Rapid signaling responses have been observed following aldosterone treatment, including the activation of protein kinase signaling cascades and a rise in $[Ca^{2+}]_I$ [4,5]. The rapid autophosphorylation and activation of the novel Serine/Threonine protein kinase Protein kinase D1 (PKD1) in the murine M1 renal cortical collecting duct (M1-CCD) cell line within 5 min of aldosterone (10 nM) treatment (n=6) was demonstrated. M1-CCD cells were routinely propagated in the presence of dexamethasone (5 μM). It was established that dexamethasone withdrawal resulted in a reversible suppression of MR expression by these cells (n=4) that blocked the autophosphorylation of PKD1 at Sre916 in response to aldosterone (n=6). It was demonstrated that the epidermal growth factor receptor (EGFR) inhibitor Tyrphostin AG1478 (30 nM) inhibited the autophosphorylation of PKD1 (n=4). It was further established that EGFR became phosphorylated at Ser845 in response to aldosterone and that both this phosphorylation event and the autophosphorylation of PKD1 were sensitive to the c-Src kinase family inhibitor PP2 (100 nM) (n=4). Aldosterone treatment promotes the association of c-Src with Heat shock protein 84 (Hsp84) the murine homologue of human Hsp90. It was demonstrated that the Hsp90 antagonist 17-allylaminogeldanamycin (17-AAG) (5 μM) inhibitor both the Src tyrosine kinase-dependent phosphorylation of EGFR and also the autophosphorylation of PKD1 in response to aldosterone (n=4). An M1-CCD derived cell line that was stably suppressed in its PKD1 expression failed to translocate green fluorescent protein tagged epithelial sodium channel subunits to its apical surface following aldosterone treatment. Consequently the aldosterone-induced activation of PKD1 has a significant role in priming the physiological responses stimulated by this hormone.

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This work was supported by the Higher Education Authority of Ireland under the Programme for Research in Third Level Institutions Cycle 3 and by the Wellcome Trust.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C96

Mitochondrial import of PKC epsilon is mediated by the HSP90-TOM pathway: role in cardioprotective signaling

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Myocardial ischemia-reperfusion (IR) injury occurs due to acute myocardial infarction (AMI) and triggers a variety of pathological processes that culminate in necrotic cell death. The protein kinase C (PKC) kinase family plays an important role in mediating the endogenous cardioprotective response to IR. Previously, our lab developed rationally designed PKC isozyme-selective regulatory compounds which were used to elucidate the role of specific PKC isozymes in mediating the protective response to myocardial IR (1). These studies found that selective activation of εPKC before ischemia (preconditioning) or immediately at the onset of reperfusion (postconditioning) can protect the heart from IR injury. The cardioprotective target of εPKC is thought to reside within mitochondria, as εPKC directly regulates opening of the mitochondrial permeability transition pore (mPTP) and mitoKATP channels (2). In order to access intramitochondrial targets, εPKC must first transverse the mitochondrial outer membrane, however, the mechanism for mitochondrial import of εPKC remains to be described. HSP90 is a member of the heat-shock-protein family of molecular chaperones and is activated by cellular stresses including IR injury. Recent evidence suggests that HSP90 also plays a role in mediating the mitochondrial import of cytosolic proteins, in tandem with TOM (translocase of the outer membrane) (3). The aim of the current study was to determine whether the HSP90-TOM import pathway plays a role in the mitochondrial import of εPKC. We employed an ex vivo rat heart model of myocardial IR in combination with electron microscopy (EM) and western blotting and found that IR induced significant mitochondrial import of εPKC. EM analysis showed that mitochondrial εPKC was predominantly localised on the inner mitochondrial membrane. To determine whether IR-induced mitochondrial import of εPKC required the HSP90-TOM import pathway, we used the HSP90-specific antagonist geladanamycin (GA). Infusion with 5μM GA at reperfusion prevented mitochondrial import of εPKC and dramatically worsened IR injury when compared to hearts exposed to IR alone. Biochemical analysis of mitochondrial εPKC revealed that εPKC forms a complex with both HSP90 and TOM20 at the mitochondria following exposure to IR but not under normoxic conditions. Co-precipitation of mitochondrial εPKC with HSP90 or TOM20 was prevented by GA at reperfusion. These findings demonstrate that cardiac IR induces the mitochondrial import of εPKC, which is mediated by the HSP90-TOM import pathway and is a required step in the endogenous cardioprotective response to IR. This is the first description of the mitochondrial import of εPKC and provides a novel mechanistic insight into εPKC-mediated cardioprotection.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA226

Structure-based optimization of PKC-theta antagonists

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Protein kinase C-theta (PKC-theta) is a central signaling molecule in the T cell receptor activation pathway and is a target for treatment of a number of diseases. Several PKC inhibitors are in the drug-discovery pharmaceutical programs today for the treatment of cancer, diabetes and arthritis. CD4+ T lymphocytes also play a critical role in the initiation and progression of allergic airway inflammation. Our goal is the development of PKC-theta antagonists as a means to control asthma and autoimmune diseases (1), with the strategy based on developing small molecule agents that would block the enzyme's catalytic activity. Implementation of this strategy led to the discovery of two lead chemical series, which are being synthesized and characterized in enzymatic and cell assays. To help guide the optimization of these compounds, structural and modeling approaches, including site-directed mutation and a structure-surrogate strategy, have been developed. We first obtained structural information using X-ray crystallography, revealing the association between staurosporine and the PKC-theta ATP-binding site (2, 3), and this complex was used as a surrogate structure for subsequent high-throughput docking studies with inhibitors under development. Our attempts of solving crystal structures of advanced hits with wild type PKC-theta, as well as with the designed PKC-theta mutants (site-directed surface mutants), have repeatedly led to a crystal form with poor diffraction (~ 6 A resolution at best). Through an iterative process of chemical synthesis, molecular modeling and biochemical testing we have found highly potent sub-nanomolar inhibitors, but they suffered from lack of selectivity over Src kinases. To address this issue, we determined the crystal structures of several lead compounds with a Src kinase. These co-structures revealed interaction of headpiece with Glu 428, which we never observed in binding mode predictions with PKC-theta due to small difference in protein conformation. Consequently, the SAR around the headpiece become well explained with this "hybrid" PKC-theta model, where parts being modeled based on Src kinase co-structures. The results of this structural effort helped produce compounds with significant selectivity improvements over the initial lead molecules. To further enhance structure-guided lead optimization we are also pursuing a PKA-based surrogate approach. Production, characterization and use of different PKA-PKCtheta chimeras as a structural mimic are discussed and details presented.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA227

Orphan GPCRs and the regulation of food intake

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The superfamily of the GPCRs can be classified into the somatosensory GPCRs (500-700 members) and "transmitter" (about 360 members) GPCRs. These last GPCRs bind all the known neurotransmitters, neuropeptides and peptide hormones and are the omnipresent modulators of brain function. Of the 360 transmitter GPCRs, 260 bind known ligands, while the others bind ligands that have not been thus far described. These are the orphan GPCRs, which carry the promise to lead us to the discovery of novel physiological responses. However, orphan GPCR research is hampered by the lack of these receptors natural ligands. To be studied, orphan GPCRs need to be deorphanized, i.e. their natural ligand need to be characterized.

We have devised a strategy to identify the natural ligands of orphan GPCRs. We use expressed orphan GPCRs as a targets and tissue extracts as source of their natural ligands. Since 1995, this strategy has led to the discovery of 10 novel transmitter families. The novel transmitters have been studied for their pharmacological characteristics, for the localizations of their sites of expression and sites of action in the CNS and for their functional roles. This has led to a broadening of our understanding of particular physiological functions. For example, the first transmitter to be discovered via the orphan receptor strategy, Orphanin FQ/Nociceptin, has shown to be of great interest to neurosciences. It is foreseen that most of the other novel transmitters will follow this path.

Interestingly, several of the novel neuropeptides discovered as ligands of orphan GPCRs exhibit regulatory roles on food intake (1). The behavioral response to food intake rests on a balance between orexigenic and anorexigenic signals. The regulation of this balance starts in the periphery, reaches the arcuate nucleus and ultimately converges onto the lateral hypothalamus. The regulatory role of the lateral hypothalamus is thought to be carried out by two ligands of orphan GPCRs, the neuropeptides orexins and melanin-concentrating hormone (MCH). To understand the functional significance of the MCH system, we have isolated a synthetic MCH receptor antagonist. This antagonist was identified through the screening of synthetic combinatorial libraries. The antagonist inhibits MCH-induced Ca2+ mobilization with an IC50 value of 6 nM for and is specific for the MCH1R. When administered i.c.v., it blocks spontaneous food intake in a dose dependent manner. However, it has only a weak and transient inhibitory effect on food intake. On the other hand, when administered peripherally, it reduces nocturnal food intake and induces significant metabolic changes. Using this selective antagonist we thus can differentiate the central and peripheral effects of the MCH system.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA228

Nicotinic acid receptor - pharmacological and physiological functions

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Low plasma concentrations of high density lipoprotein (HDL) cholesterol are an independent risk factor for atherosclerotic cardiovascular diseases. Raising HDL cholesterol levels is therefore one of the therapeutic strategies for the prevention of cardiovascular diseases. Besides the development of new HDL cholesterol-elevating pharmacological procedures, the oldest lipid-modifying drug, nicotinic acid (niacin), has recently attracted new interest. It has by far the strongest HDL cholesterol-elevating effect among the drugs currently approved for the treatment of lipid disorders. The recently identified receptor for nicotinic acid, GPR109A, is expressed in adipocytes and various immune cells. It has the expected pharmacological properties, and studies in mice lacking GPR109A have shown that it mediates the acute pharmacological effects of nicotinic acid. Recent progress in the understanding of the physiological and pharmacological role of the nicotinic acid receptor will be summarized and its potential use as a new target for the development of anti-dyslipidemic drugs to prevent and treat cardiovascular diseases will be discussed.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C97

Involvement of α-ketoglutarate receptor in diabetic nephropathy

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α-Ketoglutarate was recently identified as the endogenous ligand of GPR80, a member of orphan G protein-coupled receptors (oGPCR). However, the physiological and pathological meanings of α-ketoglutarate receptor still remain unclear. Because kidney is the tissue where α-ketoglutarate receptor mRNA was highly expressed, we have conducted in vivo and in vitro experiments to explore its correlation with diabetic nephropathy (DN). A model of rats with DN were induced by once intraperitoneal

injection of streptozotocin (STZ) at a dose of 65mg/Kg, and α-ketoglutarate receptor protein expression was examined with immunohistochemical method at 6, 10, 12, 16 and 32 weeks after STZ injection. It was found that the protein expression of α-ketoglutarate receptor in the medullary loop of the DN rat kidney was increased from 6 weeks on. To mimic the situation of DN, the cultured rat renal tubular epithelial cells(RTEC)were exposed to a high concentration of glucose (30mmol/L). By using MTT colorimetric method and RT-PCR, effects of the high concentration of glucose with or without α-ketoglutarate sodium on the RTEC proliferation and TGF-β,α-SMA and MCP-1 mRNA expressions were detected. It was indicated that high concentration of glucose-treated RTEC was higher in its proliferation and TGF-β,α-SMA and MCP-1 mRNA expressions, but α-ketoglutarate sodium could inhibit the above effects of glucose. In conclusion, relevance between α-ketoglutarate receptor and DN implied that α-ketoglutarate receptor might be involved in the development of DN.

Key words: α-Ketoglutarate receptor; GPR80; Diabetic nephropathy; Rat

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This project was supported by Beijing Foundation of Natural Sciences.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C98

Novel function of the inflammatory chemokine MIP1-β, a ligand for the HIV co-receptor CCR5, as a vasoconstrictor of human blood vessels: antagonism by maraviroc

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HIV-infected individuals are at increased risk of cardiovascular events, possibly owing to accelerated atherosclerosis. The mechanisms involved are unclear. The chemokine receptor CCR5 is a major co-receptor for the HIV virus. Interestingly, CCR5 deficiency results in a reduction in diet-induced atherosclerosis in mice (Braunersreuther et al., 2007) and CCR5 and its ligand MIP1-β are expressed in human vascular smooth muscle (Schecter et al., 2000). A direct effect on vascular tone of chemokines has not been reported. Human saphenous vein (SV) is a widely used graft in patients with coronary artery disease however, grafts, particularly SV, exhibit a form of accelerated atherosclerosis making this blood vessel a useful model of disease. In initial experiments we have determined whether MIP1-β has direct constrictor/dilator action in SV and confirmed that this is a CCR5 mediated action using the selective antagonist maraviroc (UK-427,857; Dorr et al., 2005).

Rings (4mm) of human SV were denuded of their endothelium and mounted in organ baths, containing Krebs' solution at 37°C, for isometric tension recording. Following a normalisation procedure to optimise basal tension, endothelium removal was confirmed by the absence of a vasodilator response to ACh in phenylephrine constricted veins. Cumulative concentration-response curves (CRC) were then constructed to MIP1-β (10^{-11}-$1.1x10^{-7}$M). In adjacent rings of SV from some patients, 300nM maraviroc or vehicle (DMSO 0.01%) were added to the bathing medium and cumulative CRC to MIP1-β constructed 30 minutes later. In additional experiments, CRC to MIP1-β were constructed in SV that had been preconstricted with endothelin-1 (10nM) and experiments terminated by addition of 30μM SNAP. Vasoconstrictor responses were expressed as a %phenylephrine and vasodilator responses as %reversal of ET-1 constriction. Data were analysed using FigSys (Biosoft, Cambridge, UK) to determine values of pD_2 and maximum response. n-Values are the number of patients from whom tissue was obtained.

In ET-1 constricted SV, MIP1-β did not elicit direct vascular smooth muscle relaxation over the concentration range tested. In contrast MIP1-β produced a concentration-dependent contraction of SV with pD_2 of 7.73±0.17 (n=12). In the presence of 300nM maraviroc (n=4), the constrictor response to MIP1-β was abolished (Fig 1). These data reveal a previously unidentified role for the chemokine MIP1-β as a potent constrictor of human SV, an effect mediated through the CCR5 receptor present on vascular smooth muscle.

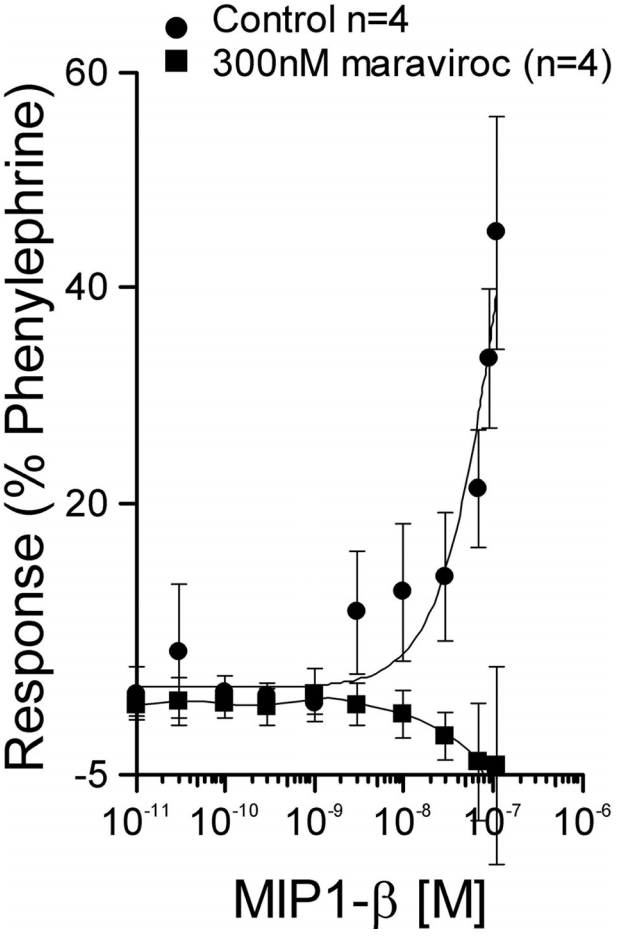

Fig. 1 Antagonism of MIP1-β by maraviroc in SV

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SA229

GPR119: a receptor for oleoylethanolamide, and its potential role in diabesity

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In search of new treatments for obesity and type 2 diabetes ("diabesity"), we have de-orphanized GPR119, a $G_{\alpha S}$ protein-coupled receptor whose expression is localised predominantly in the pancreas (β-cells) and gastrointestinal tract (L-cells). We recently reported oleoylethanolamide (OEA) to be the most active endogenous ligand thus far identified[1]. OEA has been described as a peripherally-acting agent which reduces food intake and body weight gain in rodent models, leading us to hypothesise that GPR119 may represent a novel and attractive drug discovery target. We have therefore developed several biological tools, including a yeast reporter system as a high-throughput assay, and have identified novel, selective, orally-available GPR119 agonists. Such agents could provide attractive new oral therapies for type 2 diabetes and obesity, offering significant improvements in metabolic parameters such as glucose tolerance, as well as body weight loss. The results of our studies with representative synthetic small-molecule agonists in various cell systems and in rodent models of diabetes and obesity will be presented.

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We should like to thank the many colleagues at (OSI)Prosidion who have participated in our GPR119 project, and also to acknowledge colleagues at Renasci Consultancy Limited and Rheoscience A/S who performed many of the pharmacology studies on a fee-for-service basis.

Prosidion Limited is a subsidiary of OSI Pharmaceuticals Inc.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

SA230

A new receptor for cannabinoid ligands

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Endocannabinoids and extracts of the cannabis plant C. sativa affect a plethora of biological processes via CB1 and CB2 receptors. However, much evidence has emerged to suggest that additional receptors exist that mediate certain cannabinoid functions. Here we show that mammalian and plant derived cannabinoid ligands bind to and affect the activity of the orphan G-protein coupled receptor (GPCR) GPR55. In turn this results in activation of the intracellular signaling mediators RhoA, Rac1 and cdc42 via activation of G13 G-proteins. RT-PCR expression analysis, immunohistochemistry and in situ hybridisation have been used to reveal the tissue distribution of GPR55. In vivo, a GPR55 selective agonist, O1602, induces a reduction in blood pressure in spontaneously hypertensive rats. This effect is significantly enhanced by concomitant selective CB1 receptor blockade, and is sensitive to a GPR55 selective antagonist. These data suggest that GPR55 is a novel cannabinoid receptor that may play a role in blood pressure regulation.

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