Deciphering the molecular mechanisms underlying memory and learning: a functional genomics approach to studying the ageing and diseased brain

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Ageing of the brain may lead to a slow degradation of cognitive function, which ultimately leads to learning and memory deficits in the elderly. These processes are complex and their molecular mechanisms are still incompletely known. The hippocampus is a key region involved in several types of learning including spatial learning. Since both ageing and learning processes are known to involve changes in gene expression, we have studied gene expression changes during ageing in the event that these perturbations may be responsible for the learning deficits in the elderly and those suffering from neurodegenerative disease. To identify key genes involved in these processes, we measured gene expression in the dorsal hippocampus (dHPC) of 6-, 15- and 24-month-old mice following a hippocampus-dependent spatial learning protocol, the Repeated Acquisition and Performance Chamber (RAPC). Behavioural data showed a significant learning impairment in middle- and old-aged mice when compared with young mice, as assessed by an increased number of mistakes made by the subjects in the RAPC protocol. Gene expression changes in the dHPC were measured using high-density oligonucleotide microarrays. A number of statistical and supervised learning approaches have been employed to analyse the data set. One data analysis approach employs the Independent Consistent Expression Discriminator (ICED), which was designed to provide a more biologically relevant search criterion during predictor selection by embracing the inherent variability of gene expression in any biological state. The search criteria employed by ICED is designed to identify not only genes that are consistently expressed at one level in one class and at a consistently different level in another class but identify genes that are variable in one class and consistent in another. The result is a novel approach to accurately selecting biologically relevant predictors of differential biological states from a small number of microarray samples. Using both statistical and pattern recognition approaches, we found 175 genes significantly changed with ageing (59 % of which decreased), 305 with learning (58 % of which decreased) and 325 that showed interactive effects with both ageing and learning. In addition, we have generated class predictors from the analysis which can be used classify the memory and learning deficits as a function of ageing and disease. This study links gene expression to behaviour and shows the possibility of using microarray technology to profile gene expression in behavioural protocols. Our large-scale gene expression analysis after learning in young, middle and old aged animals provides for the first time an overview of genes that may be responsible for the spatial learning deficit in aged mice. Genes identified in this study and their proteins are potential candidates for therapeutic interventions that could revert or slow down age- and disease-dependent cognitive decline.

All procedures accord with current UK legislation.

The DRP2-dystroglycan complex in Schwann cells

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Mice that lack a functional Prx gene ensheath and myelinate peripheral nerve axons in an apparently normal manner, but this sheath later destabilizes and the mice develop a severe demyelinating neuropathy. Hence, periaxins play an essential role in the establishment of a stable Schwann cell-axon unit in the myelinated fibers of the vertebrate peripheral nervous system. In addition to a marked reduction in their peripheral nerve conduction velocities, periaxin-null mice display reflex behaviours associated with neuropathic pain. Damage to sensory nerves in human peripheral demyelinating disease is also known to be to linked to pain and excessive sensitivity to touch. Segmental demyelination in human disease can be associated with tactile allodynia, the perception of normally innocuous stimuli such as touching or brushing as painful, and hyperalgesia, a heightened response to painful stimuli; however, the mechanisms of neuropathic pain in demyelinating disease are poorly understood. Several families have now been identified with a variant of Charcot-Marie-Tooth disease which we call CMT4F in which the periaxin gene is mutated. Recent progress in this area has been marked by our identification of a new dystroglycan complex in the Schwann cell plasma membrane in which periaxin is a key component. It seems that disruption of this complex is at the heart of the derangement of myelination observed in the periaxin KO mouse, and probably in the human disease. We are presently seeking to identify the other components of the complex since these may represent candidate genes for other types of CMT disease.

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Deletion of the gene for the NK1 receptor has multiple effects on neurochemistry and behaviour

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Polymorphisms within human monoamine transporters correlating with predisposition to behavioural disorders act as neuronal specific differential regulators of gene expression

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Clinical abnormalities in monoamine metabolism, in particular serotonin (5-HT) and dopamine have been implicated in the pathophysiology of many CNS-related disorders. Consistent with this, the use of drugs that block the effects of the serotonin

transporter (5-HTT) and the dopamine transporter (DAT1) have pointed to these being candidate genes involved in a variety of disorders including Parkinson's, Alzheimer's, schizophrenia and psychiatric disorders. Clinical studies have suggested a polymorphism within intron 2 of the 5-HTT gene and the 3′ untranslated region of the DAT1 gene are associated with the susceptibility to such disorders and can determine bioavailability of the DAT1 transporter *in vivo* (Battersby *et al.* 1997; Jacobsen *et al.* 2000). These polymorphisms are composed of a variable number of tandem repeats (VNTR) and the difference in copy number is correlated with predisposition to the disease. For example the 5-HTT transporter VNTR has 9, 10 or 12 copies of a 16 or 17 base pair element.

We have demonstrated that these VNTRs are functionally related, acting as transcriptional regulatory domains in reporter gene constructs both *in vitro* and *in vivo*. They support copy number dependent differential enhancer activity and additionally for the 5-HTT restricted neuronal regulation in the developing CNS in areas associated with endogenous 5-HTT expression (Fiskerstrand *et al.* 1999; MacKenzie & Quinn, 1999; Michelhaugh *et al.* 2001). Our data clearly indicate a potential mechanism by which such regulatory polymorphisms might influence physiology or susceptibility to disease by modulation of transporter gene expression.

We shall discuss both the variety of transcription factors that regulate these VNTR domains and how a base change within the tandem repeats can themselves change the transcriptional properties of the domain. The latter allows for a potentially greater degree of transcriptional plasticity directed from these domains than predicted for copy number alone. Thus clinical data correlating copy number alone of VNTRs with disease should be re-examined in light of these data to allow for such single base polymorphisms.

Related VNTRs, by primary sequence analysis, are found in several genes and we hypothesise that they are a novel class of regulatory domain that could determine both lineage expression and differential expression *in vivo*. Thus they act to regulate our normal physiology and their aberrant regulation could result in 'abnormal' behaviour.

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Function from behaviour: circadian control an exemplar

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The post-genomic era offers behavioural scientists unrivalled opportunities but also many pitfalls. The study of circadian biology is an example of where molecular and behavioural techniques have been effectively integrated to further our understanding of the underlying systems. Behavioural/lesion studies dating back 30 years first identified the suprachiasmatic nucleus (SCN) as the site of the principal mammalian 'clock'. The identification of this 'model' system in turn led to the identification of six genes that form the basis of the molecular 'clock' and an understanding of the biochemical mechanisms that allow them to interact to form a biological oscillator.

However, the nature of the control mechanisms that act upon the SCN is poorly understood. Recently, we have demonstrated that the neuropeptide receptor $VPAC_2$, when over-expressed in the mouse, leads to a distinctive behavioural phenotype. This was characterised by a rapid re-entrainment to shifts in the 12 h/12 h light/dark (LD) cycle and a short free running rhythm in constant darkness (DD). By contrast, $VPAC_2$ receptor knock-out ($Vipr_2^{-/-}$) mice exhibited an increase in the variance of activity onset under a 12 h/12 h LD cycle (Wt 0.116 ± 0.013, $Vipr_2^{-/-}$ 0.303 ± 0.059 h; $P = 0.002$) (data reported as means ± S.E.M. derived from ANOVA, $n = 10$ per group), were virtually arrhythmic in DD (Wt τ 23.9 ± 0.04 h, $Vipr_2^{-/-}$ undeterminable), and responded with a rapid onset of activity to short periods of darkness interpolated into the light phase unlike control animals (lag in activity onset: Wt 0.75 ± 0.09, $Vipr_2^{-/-}$ 0.28 ± 0.06 h, $P < 0.001$). Parallel studies assessing the molecular rhythmicity of the SCN confirmed that the 'clock' was silent.

It is also equally important to confirm which behaviours have been left unaffected following genetic modification. For example, we confirmed that $Vipr_2^{-/-}$ mice were able to learn a complex operant, visually guided task with which it was then possible to probe their visual competence. The performance of the knock-outs was found to be indistinguishable from wild-type littermates. Thus a general CNS depression and impairments in the primary visual system can be ruled out as explanations for the deficit in circadian control.

Within the series studies conducted with the over-expressing and knock-out mice, a number of issues arose. These illustrate some of the potential pitfalls in application of molecular techniques to the study of biological function. In this case the knock-out studies were performed using 129P2 stem cells as the basis for the construct. As should be the case the circadian phenotype of the 129P2 strain was also determined. This was found to be abnormal. Although the salient features of the behavioural phenotype were observed in F2 hybrids on a mixed 129P2/OlaHsd × C57BL/6J background and were not seen in littermate controls, or in 129P2/OlaHsd mice, the KO strain was taken to isogenicity prior to final phenotyping.

These data illustrate that with careful hypothesis-driven experimental design, subtle phenotypes can be used to further elucidate the underlying mechanisms in particular functions. In this case peptidergic intercellular signalling, probably by VIP through the $VPAC_2$ receptor, appears to be implicitly involved in normal circadian control.

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Probing the function of a family of neuronal calcium sensors

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The neuronal calcium sensor family of Ca^{2+}-binding proteins are expressed predominantly or only in neurons (Burgoyne & Wiess, 2001). They possess EF-hand motifs and are almost all are N-terminally myristoylated. The different members of the family show varying affinities for free Ca^{2+}, suggesting that they may have similar or distinct physiological functions that are determined by the level of free Ca^{2+}. It is likely that they play important roles in the regulation of neuronal function. Some significant roles have been identified. For example, NCS-1 is involved in the control of neurotransmitter release (McFerran *et al.* 1998), Ca^{2+} channel function (Weiss *et al.* 2000) and learning and memory (Gomez *et al.* 2001). The KChIP proteins regulate A-type potassium channels (An *et al.* 2000). In general, however, much is still to be learnt about their cellular functions and in particular the molecular basis for their actions. We have developed a dominant negative mutant of NCS-1 that has allowed us to probe the role of NCS-1 in Ca^{2+} channel regulation (Weiss *et al.* 2000; Weiss & Burgoyne, 2001). The use of the N-terminal myristoylation for reversible or permanent membrane association of the NCS proteins has been probed in live cells using fluorescent tagged forms of NCS-1 and hippocalcin (O'Callaghan *et al.* 2002) and also KChIP1. To further address their function, we have developed a method for the isolation of proteins that interact with the NCS proteins in a calcium-dependent manner and tested the feasibility of the approach for one family member known as neurocalcin δ (Ivings *et al.* 2002). Binding proteins were identified by gel electrophoresis and MALDI-TOF analysis and included clathrin heavy chain, actin, and tubulin (Ivings *et al.* 2002). These interactions were confirmed using independent assays.

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Targeted regulation of CGRP: implications for migraine

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The calcitonin gene-related peptide (CGRP) and P/Q voltage-gated calcium channel genes have been implicated in the underlying pathophysiology of migraine headaches. CGRP levels are elevated during migraine, then are restored to normal coincident with headache relief following treatment with the serotonergic drug sumatriptan. Patients with the rare familial hemiplegic migraine disorder have mutations in a subunit of the P/Q-type calcium channel. However, whether there is a connection between calcium and CGRP expression in the sensory neurons that are activated during migraine is not known. The approach that we have taken is to study the regulation of CGRP gene expression in primary cultures of rat trigeminal neurons. We have previously demonstrated that CGRP secretion from these cultures is increased by depolarization and inflammatory signals and decreased by treatment with 5-HT_1 agonists, including sumatriptan. We now report that the CGRP promoter is activated by depolarization and inflammatory signals. These signals cause a transient increase in calcium and activation of MAP kinase pathways. The stimulation requires a helix-loop-helix regulatory element within a cell-specific 18 bp enhancer that is sufficient to target expression of a reporter gene preferentially to neurons. The MAP kinase-stimulated CGRP promoter activity was markedly reduced by treatment with sumatriptan. Sumatriptan regulation of CGRP gene expression did not couple to a G_i/G_o pathway but rather caused a prolonged increase in intracellular calcium. The sustained elevation in calcium was shown to be sufficient to repress MAP kinase stimulation of the CGRP promoter. Finally, we provide evidence that the inhibitory effects of sumatriptan are probably mediated via induction of serine/threonine protein phosphatases. This raises the possibility of using the cell-specific CGRP enhancer for targeted expression of a therapeutic phosphatase. Based on our results, we propose that the CGRP promoter is sensitive to the amplitude and duration of a calcium signal, such that transient calcium signals increase CGRP gene expression during migraine while prolonged calcium signals induced by serotonergic migraine drugs could reduce CGRP levels. While the role of the P/Q calcium channels in this model remains completely speculative, we suggest that channel mutations may alter the calcium dynamics in the trigeminal neurons so as to result in increased levels of CGRP.

Searching for new genes required for embryonic nervous system development in *Drosophila* using RNAi

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Although the sequences of the ~13 500 genes of *Drosophila melanogaster* have been described, the functions of most of the encoded proteins are unknown. The classical genetic approach of starting with a mutation of interest followed by the subsequent molecular analysis of the corresponding gene have ascribed functions to only a small subset of the total number of genes. Large scale transposon mutagenesis screens and the isolation of a collection of lethal loss of function mutants has led to the assignment of vital functions for a larger fraction of the genes. However, many of the remaining genes appear to be infrequent targets of transposon insertions. Reverse genetic approaches such as gene targeting provide an alternative approach to generating loss of function mutations in a single gene, but are currently not practical for genome wide application. We are using RNA interference (RNAi) as a screening tool to identify new genes that are required for the development of the embryonic nervous system. Double stranded RNAs corresponding to ~1700 genes were synthesized *in vitro* and injected into preblastoderm embryos. After allowing the embryos to develop, the nervous system was visualized using mononclonal antibodies that stain subsets of neurons or axon tracts. Seventeen dsRNAs were identified whose injection resulted in a range of developmental abnormalities affecting the central and/or peripheral nervous system. Mutant phenotypes have not been described for most of the genes identified which included diverse classes of proteins including transcription factors, cell signalling components and enzymes. Mutants were also found in genes previously identified

by classical genetic methods including members of the *hedgehog* signalling cascade, the anti-apoptotic gene, *thread* and the dynein light chain encoded by *cut-up*, and the transcription factor *lola*.

Functional regulation of AMPA receptors by proteins interacting with the c-terminus of the GluR2 subunit

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The AMPA type glutamate receptors are heteromultimeric proteins consisting of 4 or 5 subunits (GluR1–4). We, and others, using yeast two-hybrid and biochemical analyses have identified a number of proteins that interact with specific motifs on the intracellular c-terminus of the GluR2 AMPA receptor subunit. These proteins, some of which are novel, are thought to be involved in the targeting to and regulation of AMPARs at excitatory synapses in neurons. However, the mechanisms by which these interactors achieve this are only starting to be investigated.

To study the functional roles of these interacting proteins we have developed approaches to investigate these interactions in hippocampal neurons in acute brain slices. To block the interactions of specific proteins we used short peptides corresponding to binding motifs for these proteins and introduced them into hippocampal neurons during whole-cell patch-clamp recordings. The effects of the peptides on AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) during basal transmission and during long-term synaptic plasticity were investigated. These acute studies show that interaction proteins such as NSF, PICK1 and GRIP regulate AMPA receptors over a time scale of minutes at synapses, and that these interactions are required for the expression of long-term synaptic plasticity.

To investigate the more long-term effects of these interactions, we used Sindbis virus to express blocking peptides, full length interacting proteins, or mutant interacting proteins in acute hippocampal slices maintained overnight in culture. These studies show that the interactors can regulate surface expression and subunit composition of synaptic AMPA receptors but that distinct interactors have different roles in this regulation. Using this approach to study mutant forms of the interactors, this approach has also allowed us to investigate the downstream signalling mechanisms of these proteins.

These novel approaches have allowed us to directly investigate the functions of these poorly characterised proteins in neurons in acute brain slices. When combined with other techniques such as biochemical analyses and immunocytochemistry, these studies have enabled much progress to be made in understanding the role of protein–protein interactions in regulating AMPA receptors at synapses in neurons.

All procedures accord with current local guidelines.