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Light-induced pigment granule dispersion in *X. laevis* melanophores is mediated by an opsin photopigment

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Melanophores are a class of chromatophore found in the dermal layer of amphibian skin, which contribute to rapid and reversible changes in skin colouration. These cells contain thousands of melanosomes, a lysosome-related organelle, able to synthesise and store the brown-black pigment melanin. Melanosomes may be dispersed throughout the cell, or may become aggregated in the perinuclear region. The distribution of melanosomes within melanophores can be dramatically altered by various hormones, neurotransmitters and paracrine factors. Light can also trigger melanosome dispersion (Daniolos et al. 1990). This study examined the characteristics of light-induced melanosome dispersion in a clonal line of *X. laevis* melanophores. Melanophores (~4,000 cells/well) were seeded in black-walled, clear-bottomed 96-well plates 48 hr before use in 0.7x Leibovitz L-15/15% FCS medium. Pigment granule translocation was monitored by measuring the change in cell absorbance at 630 nm. Photodispersion on exposure to white light (100 $\mu W/cm^2$) could be detected after 5 min, reached a maximum after ~60 min, and was evident both in untreated, dark-adapted cells and in melanophores pre-aggregated with melatonin (100 pM, 1hr) in darkness. Measurement of the change in pigmented area using ImageJ (http://rsb.info.nih.gov/ij/) in individual melatonin-treated cells after light exposure revealed that ~95% of cells (67/70) showed significant photodispersion (>10% increase in pigmented area) with a mean increase in pigmented area of 117±9%. Dispersion increased with increasing light intensity and an irradiance-response curve to white light was generated and gave a mean IR_{50} (irradiance giving 50% dispersion) of $56 \pm 1 \mu W/cm^2$ (n=8). The spectral sensitivity of photodispersion was determined by using interference filters to generate irradiance-response curves at 7 wavelengths (456-578 nm). The IR_{50} at each wavelength was determined from which the relative sensitivity at each wavelength of light was calculated and an action spectrum (log relative sensitivity vs. wavelength) was constructed. The data were well-described ($r^2 = 0.8$) by the characteristic absorption spectrum of a vitamin A-based opsin photopigment with a wavelength of maximal sensitivity (λ_{max}) of 500 nm indicating that photodispersion is mediated by an opsin-like photopigment. A comparison of the action spectrum with the standard absorbance templates for the known *X. laevis* violet cone, green rod, red rod and red cone opsins demonstrates that these classical image-forming opsins do not mediate photodispersion, and suggest that a novel photoreceptor is responsible (Berson, 2003).

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Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

PC44

The impact of glutamate on the regulation of appetite

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Monosodium glutamate (MSG) is a widely used nutritional substance that potentially exhibits significant neuronal toxicity. The present study was undertaken to further investigate the links between obesity, voracity, and MSG toxicity, and to test the possibility of using a non competitive NMDA receptor blocker (memantine) to prevent this effect (1,2,3). The effects of orally administered MSG (250g/body weight) were investigated in adult female Wistar rats. The animals were divided into two groups (n=8 each) and served as their own controls. Animals were fed rat chow with either 0 or 2.5g MSG per day with or without memantine added (2mg/day), during alternate periods of 2 weeks. The sequence of food types was different in each group. Group 1 first received normal rat chow for 2 weeks (Control group), then chow with MSG (2.5g per day; MSG group) and finally chow with the same amount of MSG plus memantine (MSG-MEM group). Group 2 was submitted first to chow with MSG (MSG) for 2 weeks, then to chow with the same amount of MSG plus memantine (MSG-MEM) and finally to the standard food for 2 weeks (Control). Food intake of the animals was recorded in each period and blood samples of group 1 were taken from the jugular vein at the end of each diet period for hormonal levels determinations. All data were expressed as the mean ± SEM. To determine statistical differences, one-way ANOVA test for repeated measure was performed and differences among groups were subjected to contrast analysis test. Independently of the position in the sequence, during the periods of administration of MSG a significant increase in food consumption was seen: 8.1 ± 0.3 vs 5.6 ± 0.1 g/100 g BW; p= 0.006 and 9.7 ± 0.3 vs 8.1 ± 0.2 g/100 g BW; p=0.03 (MSG vs Control of Group 1 and Group 2, respectively). When MSG was given in the chow together with memantine, the food intake was significantly reduced reaching similar (Group 1) or lower (Group 2) levels than normal chow: 6.8 ± 0.7 vs $5,6 \pm 0.1$ g/100 g BW; p=0.06 (ns) and 6.7 ± 0.3 vs 8.1 ± 0.2 g/100 g BW; p=0.005 (MSG-MEM vs Control of Group 1 and Group 2, respectively). Similarly, significant increases in plasma adiponectin and CCK levels were observed in animals fed with MSG: 4.5 ± 0.3 vs 3.0 ± 0.2 µg/ml; p= 0.01 and 114.8 ± 3.5 vs 66.9 ± 10.0 pg/ml; p= 0.03 (MSG vs Control of Group 1 for adiponectin and CCK, respectively). Those effects were reversed also when memantine was added to the diet: 3.8 ± 0.3 vs 3.0 ± 0.2 µg/ml ; p= 0.1 (ns) for adiponectin and 58.18 ± 6.3 vs 66.84 ± 10.0 pg/ml; p=0.5 (ns) for CCK (MSG-MEM vs Control of Group 1). The effects observed in this experiment showed that MSG is able to increase voracity in adult animals, an effect reverted after the administration of memantine. These data actually confirm that by partially blocking the NMDA receptor, a reduction in voracity was obtained.

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Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

PC45

Role of cyclooxygenase-1 (COX-1) and -2 (COX-2) in the decrease of growth hormone and IGF-I in arthritic rats

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Experimental arthritis is an animal model of chronic inflammation that is associated with a decrease in body weight gain, growth hormone (GH) and insulin-like growth factor-I (IGF-I). Cyclooxygenase enzymes (COX) are responsible for prostaglandin (PG) production. COX-1 is responsible for the physiological production of PGs and COX-2 for the elevated production of PGs that occurs in sites of disease and inflammation. The aim of this work was to analyze the role of COX-1 and COX-2 in arthritis-induced decrease in GH and IGF-I. For this purpose, arthritis was induced in male Wistar rats by an intradermal injection of Freund's adjuvant (1mg) in the sole of the right paw. Fifteen days after adjuvant injection control and arthritic rats were divided in three groups. One group was daily injected for 8 days with saline; another one was injected with 4mg/kg s.c. of indomethacin (unspecific inhibitor of COX) and a third group of rats was injected with 1 mg/kg s.c. of meloxicam, a specific inhibitor of COX-2. Arthritis induced a decrease in body weight gain (12.2 ± 3 vs 39.4 ± 1.9 g ($\pm$ SEM), $P<0.01$, n=10-12), pituitary GH gene expression (43 ± 6 vs 100 ± 22, % over control values, $P<0.01$, n=7-9) and IGF-I serum levels (730 ± 38 vs 109 ± 32 ng/ml, $P<0.01$, n=10-12), whereas it increased the serum levels of PGE2 (6.94 ± 0.3 vs 6.558 ± 1.6 ng/ml, $P<0.05$, n=10-12). In arthritic rats, indomethacin and meloxicam treatments markedly decreased the arthritis score and the swelling of the paws ($P<0.01$), and totally prevented the decrease in body weigh gain ($P<0.01$). Meloxicam injection did not have any effect in control rats but indomethacin induced a decrease in IGF-I serum levels in control rats. In arthritic rats, both COX inhibitors prevented the decrease in IGF-I serum levels as well as in pituitary GH gene expression. All these data suggest that, during chronic inflammation, prostaglandins have an inhibitory effect on body weight, GH and IGF-I, since treatment with COX inhibitors reverses these harmful effects in arthritic rats.

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Melanopsin activates photodispersion in *X. laevis* melanophores

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Translocation of pigment granules in *X. laevis* melanophores activated by light (photodispersion) is mediated by a vitamin A-based opsin photoreceptor. This study examined the expression of mRNAs encoding classical rod and cone opsins, and the novel non-image forming opsin, melanopsin, by quantitative real-time PCR (qPCR). In addition, the functional role of melanopsin was examined by inhibiting melanopsin expression using siRNA and examining the effect on photodispersion of pigment. Total RNA was isolated from melanophores and *X. laevis* retina, reverse transcribed with MMLV-RT and random 10-mers and qPCR done using primers specific for red rod, green rod, red cone, violet cone opsins and melanopsin. Melanopsin specific siRNAs were designed and prepared with the Silencer™ construction kit (Ambion) and transfected by electroporation. Pigment granule dispersion was measured in melanophores (6-8000/well) in 96-well plates by measuring the change in absorbance at 630 nm, or the change in pigmented area using ImageJ (http://rsb.info.nih.gov/ij/) in individual cells. Specific, sensitive qPCR assays readily measured all opsins in *X. laevis* retina cDNA, but could detect only melanopsin mRNA in melanophore cDNA (4.9×10^3 copies/10^8 copies 28S rRNA) indicating that melanopsin is the opsin mediating photodispersion. To examine its role further, a pool of four melanopsin-specific siRNAs were transfected into melanophores. Melanopsin mRNA expression was dramatically reduced (~70%) 3 days after transfection, yet expression of mRNAs encoding other GPCRs known to trigger pigment dispersion (5-HT_7 receptor) and aggregation (melatonin_{1c} receptor) was unaltered, and STAT1 (indicative of activation of the interferon response pathway) was not up-regulated. Transfection of melanophores with a negative control siRNA did not reduce melanopsin mRNA. Western blotting using a specific melanopsin antibody showed that melanopsin mRNA knock-down was accompanied by a reduction in melanopsin protein expression. Irradiance-response curves to white light were compared in negative control transfected and melanopsin siRNA transfected melanophores. Melanopsin knock-down reduced maximal photodispersion by $50 \pm 4\%$ (n=6), while the irradiance-response curve after transfection of a negative control siRNA was identical to untransfected cells. The inhibition of dispersion in siRNA transfected melanophores was specific to the light stimulus, as treatment with α-melanocyte stimulating hormone (1 nM) induced substantial additional dispersion ($82 \pm 2\%$ of maximum, n=6). Measurement of pigmented area on individual siRNA transfected melanophores confirmed that photodispersion was reduced. These experiments show that light-induced dispersion of pigment granules in *X. laevis* melanophores is mediated by melanopsin, an opsin found in the mammalian retinal ganglion cells that mediate non-image forming functions such as the pupil light reflex, circadian photoentrainment and light inhibition of the synthesis of the pineal gland hormone, melatonin.

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Melanopsin activates photodispersion in *X. laevis* melanophores via a G_s-cyclic AMP-PKA-dependent pathway

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Melanopsin is a novel opsin expressed in a small sub-set (~1%) of directly photosensitive retinal ganglion cells which project to brain areas involved in various non-image forming functions of the eye, including the pupil light reflex, circadian photoentrainment and light-suppression of the synthesis of the pineal gland hormone, melatonin. The mechanisms by which melanopsin transduces photic information remain poorly understood (Peirson & Foster, 2006). In the present study a clonal *X. laevis* melanophore cell line which endogenously expresses melanopsin was used to investigate the phototransduction cascade activated by light. Exposure of melanophores to white light (100 μW/cm^2) triggered a dramatic dispersion of pigment granules throughout the cell, but did not alter intracellular cyclic GMP concentration. Also photodispersion of pigment was not blocked by a cyclic GMP analog (8Br-cGMP, 100 μM) or a cyclic GMP phosphodiesterase inhibitor (zaprinast, 100 μM) suggesting that the classical rod and cone opsin phototransduction cascade is not activated by melanopsin. Furthermore, in melanophores, RT-PCR did not detect mRNA for transducin α-subunit or arrestin, key components of this pathway. As melanopsin has high homology with invertebrate opsins, the G_q-phospholipase C-protein kinase C (PKC) phototransduction pathway utilised by invertebrate rhabdomeric photoreceptors was examined. A PKC inhibitor (Ro31-8220, 100 μM) did not alter photodispersion but did effectively block dispersion in response to a PKC activator (PDBu, 100 nM), indicating that this pathway is not involved in the response to light. In contrast, photodispersion was significantly inhibited in melanophores transfected with a $G_{s\alpha}$ dominant negative mutant, and light exposure produced a rapid, large (~2-fold), sustained (≥ 60 min) and highly significant ($p<0.001$) increase in melanophore cyclic AMP concentration. A role for protein kinase A (PKA) was indicated as microinjection of PKA inhibitors (PKI 6-22 amide or Rp-8Br-cAMPS) completely blocked photodispersion: increase in pigmented area in control melanophores, 124 ± 46%; PKI 6-22 amide treated melanophores, -6 ± 8% (n=4-6, $p<0.05$, Student's t test). These experiments show that the signalling mechanism activated by melanopsin in melanophores is neither of the previously characterised mechanisms of phototransduction found in vertebrates or invertebrates. Rather, in these cells light activates G_s, which increases adenylate cyclase elevating intracellular cyclic AMP leading to activation of PKA. As PKA is known to be physically associated with pigment granules in a complex with the molecular motors which drive translocation (Kashina et al. 2004), this may provide the basis for light regulation of pigment distribution in melanophores.

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PC48

Real-time detection of vasopressin release from the neurohypophysis of transgenic rats *in vivo*

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The temporal resolution for measuring of vasopressin (VP) release from neurohypophysial nerve terminals in response to various challenges is relatively poor. By combining advances in both fluorescent protein (FP) and transgenic rat technology, we describe a fibre optic approach for monitoring vasopressin (VP) release from the neurohypophysis in response to hyperosmotic stimulation and baroreceptor reflex unloading in vivo. Enhanced green fluorescent protein (eGFP) fluorescence is highly pH dependent (Kneen et al. 1998), being low in acidic conditions, but increasing markedly towards neutral pH. We used this property of eGFP to directly monitor eGFP release from the acidic conditions of the secretory granule to the neutral conditions of the extra-cellular space.

Transgenic rats expressing a VP-eGFP fusion gene (Ueta et al. 2005) were anaesthetised with ketamine:medetomidine (60:250 mg kg^{-1} i.m.). A femoral artery and vein was catherterised for blood pressure measurement and intravenous administration, respectively. Using our recently developed technology (Bradley et al. 2003), a dual 'optrode' consisting of two optic fibres was constructed; one fibre coupled to a 488nm Ar laser was used for excitation and the other fibre attached to a photomultiplier tube was used for signal detection. The optrode was stereotaxically placed immediately dorsal to the neurohypophysis during bolus intravenous injections of hypertonic saline (HS; 0.1ml, 3M NaCl) and sodium nitroprusside infusion (SNP; 100μM; 100μl/min for 5 min) to unload baroreceptors.

HS induced a rapid increase in fluorescence (40.2±3.2%; n=5, $P<0.05$) signal lasting approximately 40 s. This produced a significant increase in plasma omolality from 296±4 to 336±6 mosmol kg^{-1}. The evoked change in fluorescence was not related temporally to the evoked changes in arterial blood pressure. However, infusion of SNP to induce a sustained fall in arterial pressure was associated with a stimulus-locked increase in fluorescence

(19.5±1.5%; n=3). Neither bolus injections nor infusions of saline (0.9%) caused a change in fluorescence. Post-hoc histological analysis confirmed that in those animals where the optrode was placed immediately dorsal to the neurohypophysis was a change in fluorescent signal was detected to HS (n=5) and SNP (n=3) but no fluorescence signal was detected when optrode placements fell distant to the neurohypophysis (n=7).

We conclude that optrode recording permits detection of putative vasopressin release from the neurohypophysis *in vivo*. This approach can be used for real-time monitoring of fluorescent reporter proteins during homeostatic cardiovascular reflexes evoked by physiological stimuli.

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PC49

Glucose produces different and characteristic metabolic patterns in alpha-, beta- and delta-cells in mouse islets of Langerhans

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The main function of the islet of Langerhans is the control of glucose homeostasis. Several types of endocrine cells, mainly alpha-, beta- and delta-cells, respond to changes in extracellular glucose concentrations with the release of glucagon, insulin and somatostatin, respectively. In the pancreatic beta-cell, the model for the stimulus-secretion coupling is currently accepted, and implies a key role for mitochondrial function. Extracellular glucose elevations trigger mitochondrial metabolism, activating the tricarboxylic acid cycle, and thereby increasing the concentration of redox electron carrier molecules, which stimulate oxidative phosphorylation and ATP synthesis. The increase in ATP/ADP ratio inhibits ATP-dependent K^+ channels, leading to a depolarization-induced Ca^{2+} signal that triggers insulin release. In contrast, little is known in alpha- and delta-cells about the metabolic responses involved in the signalling pathways that lead to secretion.

This lack of information is mainly due to the scarcity of non-beta-cells in the islet and certain limitations of conventional methods. In the present research, we monitored the fluorescence of either flavoproteins or NAD/NADH by redox confocal microscopy to analyse the mitochondrial metabolic responses to glucose of individual alpha-, beta- and delta-cells in fresh intact islets (Quesada et al. 2006). Activation of mitochondrial metabolism leads to a decrease in the fluorescence of flavoproteins. The percentage of decrease was calculated as the change in fluorescence from the signal obtained with 0.5 mM glucose. Data were expressed as means ± SEM. Statistical significance was analysed using a Student's t test ($p<0.05$). All cases were found significant. After recording autofluorescence in single cells within the islet, we performed immunochemical protocols to identify the different cell types in the same optical slice (Quesada et al. 2006).

Pancreatic beta-cells responded with a homogeneous dose-dependent metabolic pattern from 0.5 to 25 mM glucose. At 25 mM glucose, the fluorescence decreased 34.11 ± 2.16% (n=45 cells). Delta-cells exhibited a similar pattern although the response was about 3-fold lower (10.41 ± 2.54% at 25 mM glucose, n=13 cells) than beta-cells. However, glucose produced minor or no effect in the fluorescence of alpha-cells (2.88 ± 0.69% at 25 mM glucose, n=36 cells), despite their metabolism being sensitive to drugs acting on mitochondrial function. After autofluorescence records, these cells were able to develop Ca^{2+} signals, indicating their normal function. Similar results were obtained in cultures of dispersed cells.

These observations indicate important metabolic differences in glucose utilisation among alpha-, beta- and delta-cells, and further indicate divergences in the signalling pathways that lead to hormone release in each cell type.

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