Hypoxia-induced peripheral feedback is required for central respiratory rhythmogenesis in *Lymnaea*

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Aerial respiration in the fresh water mollusc *Lymnaea stagnalis* is controlled by an identified network of central pattern generating neurons (CPG). The CPG underlying central respiratory rhythmogenesis is comprised of three cells: namely RPeD1, VD4 and IP3I. IP3I and VD4 control expiration and inspiration, respectively, whereas RPeD1 initiates respiratory rhythmogenesis. Both *in vivo* and *in vitro,* RPeD1 stimulation is required to trigger respiratory episodes in IP3I and VD4; however, the intrinsic sources of this excitatory drive to RPeD1 *in vivo*, remain unknown. In this study, we demonstrate that the hypoxia-induced respiratory drive originates at the periphery and is conveyed to the CPG neurons via RPeD1. The peripheral chemoreceptor cells mediating this hypoxia sensitivity were subsequently identified and characterized and were found to resemble the mammalian carotid body chemoreceptors. Synapses between RPeD1 and the peripheral chemoreceptor cells (PCRC) were reconstructed in cell culture. We provide direct evidence that the efficacy of synaptic transmission between the PRCR and RPeD1 is highly modulated by both short- and long-term hypoxia and that this plasticity requires new gene transcription and *de novo* protein synthesis. Because both the peripheral (PCRC) and the central (CPC) components of respiratory rhythmogenesis in *Lymnaea* have features in common with mammals, the *Lymnaea* model thus provides us with an opportunity to elucidate mechanisms underlying neural control of breathing in a variety of animal species.

The snail feeding system: more fuzzy than we thought

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In motor pattern generating motor circuits, like that involved in molluscan feeding, we have classified neurons into specific types such as motoneurons, central pattern generator (CPG) neurons, modulatory interneurons and command neurons with the aim of ascribing individual functions to elements of the neural circuit (Benjamin & Elliott, 1989). Recent work on the pond snail *Lymnaea* suggests that these functional categories are far too simple to define the variety of roles carried out by identified neurons of the feeding circuit. For instance, motoneurons do not just mediate muscle contraction but play a key role in motor pattern generation. This is due to electrical coupling between CPG interneurons and motoneurons (Staras *et al.* 1998) and the modulation of their endogenous properties by serotonin (Straub & Benjamin, 2001). CPG interneurons initiate feeding as well being part of the oscillator mechanism (Kemenes *et al.* 2001). Command-like neurons can initiate feeding, but they also control detailed aspects of the motor pattern like the duration of a particular phase and the frequency of the feeding rhythm (Kemenes *et al.* 2001). These recent results suggest that the basic mechanisms involved in the control and generation of rhythmic motor behaviour are not the property of a single class of neurons, but are widely distributed across the neural network.

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Synaptic modulation of the pharyngeal muscle of *C. elegans*

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The nematode *C. elegans* feeds by rhythmic contraction of the pharyngeal muscle. The activity of the muscle is modified by environmental cues, e.g. the presence of *E. coli* increases the rate of pharyngeal activity. The neural circuit that mediates these effects has been completely mapped. However, the role of neurotransmitters in this pathway is not so clear. To address this we have made intracellular recordings from the pharyngeal muscle and characterised its resting properties (Franks *et al.* 2002). We have determined the response of the pharynx to putative neurotransmitters and neuromodulators (Rogers *et al.* 2001). By judicious use of mutant strains (Pemberton *et al.* 2001), or gene interference, physiologically important signalling pathways may be delineated.

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Inhibition and the control of motor responses in young *Xenopus* tadpoles

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The young *Xenopus* tadpole is a relatively simple vertebrate in which to explore the fundamental organisation of neuronal systems controlling motor behaviour. Immediately after hatching, these animals show distinct responses to particular stimuli, ranging from simple reflex bends or sustained rhythmic swimming when touched, to strong rhythmic struggling movements when grasped. Synaptic inhibition plays a key role in controlling all these responses. A small population of GABAergic reticulospinal neurons, which reliably terminate episodes of swimming when excited to fire by trigeminal afferents innervating the head skin, has now been characterised. These neurons may also play a role in control of long-term responsiveness to sensory stimulation. During self-sustained swimming, glycinergic inhibition from spinal interneurons has several distinct effects. Swimming frequency is very sensitive to changes in glycinergic inhibition. In contrast, rhythm generation and the left-right co-ordination mediated by characterised reciprocal inhibitory interneurons remain robust even after extensive surgical and pharmacological interference to inhibitory connections. These results have highlighted shortcomings with the use of the competitive antagonist strychnine in exploring the

role of glycinergic inhibition. The spinal interneurons that control the gating of sensory transmission from trunk skin Rohon-Beard neurons during swimming have also been characterised. In addition to their role in swimming, these interneurons are strongly recruited during the struggling motor pattern, which, unlike swimming, is readily disrupted by glycinergic block.

Fast inhibitory synapses: targets for neuromodulation of vertebrate motor behaviour

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Sensory modulation of mammalian locomotor pattern-generating networks

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Experiments with decerebrate cats during treadmill and fictive locomotion have provided much of what we know about the ways in which afferent feedback can regulate mammalian locomotion. Depending on the type of afferent and the timing of stimulus delivery during the step cycle, one can entrain the locomotor rhythm, cause a premature or delayed transition to the next step cycle phase, or enhance motoneurone activity in the on-going phase. Activation of ankle extensor group I muscle spindle or tendon organ afferents during extension simultaneously increases the duration and amount of extensor activity throughout the limb. These widespread reflex actions and the ability for prolonged afferent activation to disrupt step cycle timing and prevent the transition from stance to swing is evidence that locomotor-dependent reflexes can affect the locomotor pattern generating circuitry. As another example of how feedback from a limited subset of afferents can affect the CPG, activity in hip flexor muscle afferents plays a critical role in regulating the transition from swing to stance. Finally, activation of cutaneous afferents from the dorsum of the foot during both real and fictive locomotion evokes a corrective reflex that prevents stumbling. In this case a specialized reflex increases flexion at the hip, knee and ankle presumably by actions evoked in part through CPG circuitry without significantly affecting step cycle period. It is likely that identification of the interneurones mediating reflexes affecting pattern generating networks will provide insights into the organization of the CPG itself.

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Neural growth hormone: synaptic and extrasynaptic actions?

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It is now well established that growth hormone (GH) gene expression is not confined to the pituitary gland and occurs in many extrapituitary tissues, including the central and peripheral nervous systems. GH is present in neuronal and glial cells and in cerebrospinal fluid, and is present in the brain prior to the ontogenic differentiation of the pituitary gland. The colocalization of GH-receptors and GH-binding proteins in these sites suggests they are also sites of GH action. This possibility is supported by the presence of GH-responsive genes in neural tissues and by demonstrated actions of GH in neural development, behaviour and neurotransmission. GH may also have synaptic or extrasynaptic actions in the neural retina during the development of the eye.

GH immunoreactivity is abundantly present in cells of the neural retina in embryonic chicks, although this immunoreactivity is lost following hatch. This immunoreactivity is primarily associated with two proteins that are of smaller molecular size (15 and 17 kDa) than 'monomer' pituitary GH (24 kDa). The 15 kDa moiety (but not the 17 kDa variant) is present in the media of cultured retinal explants and is richly abundant in vitreous humour, suggesting that it is synthesized and secreted by the neural retina. The presence of GH mRNA in retinal tissue and the likely exclusion of circulating GH by the blood-retinal barrier supports this view. The presence of GH receptors in the retina also suggests autocrine/paracrine actions of this GH moiety in retinal neural tissue. However, as GH receptors are also present in the lens and cornea, there may also be non-neural sites of retinal GH action and the vitreous humour may provide a novel conduit for this retinal secretory product. GH may thus have synaptic or extrasynaptic actions within the eye.

Cerebrospinal fluid: a vital fluid in the developing rat brain

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The cerebrospinal fluid (CSF) has presented physiologists with an enigma for centuries. Reference to CSF in medical texts endows it with such functions as flotation of the brain, shock absorption, temperature regulation and waste disposal. However, all but flotation could arguably be ascribed to the immense vascular supply and capillary bed contained within the parenchyma of the brain. Significantly perhaps, the entire central nervous system begins as a fluid-filled neural tube and the fluid system is retained through development until, in some vertebrates, including humans, the tube is sealed in the spinal cord. CSF remains in the adult brain and is secreted by the choroid plexuses throughout life at a rate that produces four times the volume held in the fluid compartments per day. What then is the significance of this fluid?

Recent interest in CSF highlights the potential role of this fluid as a signal pathway (Nicholson, 1999; Johanson & Jones, 2001). Intense interest in brain development, and in particular development of the cerebral cortex, has exposed another potential role for CSF, a role co-ordinating the activity of germinal cells lining the cerebral ventricles and Cajal-Retzius

cells in the marginal zone (Marin-Padilla, 1998; Super *et al.* 1998; Meyer *et al.* 2000). In fetal-onset hydrocephalus there is retardation in the development of the cortex coincident with an obstruction of CSF flow. Cells of the germinal epithelium fail to proliferate and produce the number of neurones generated in normal cortex (Mashayekhi *et al.* 2001). However, neuronal progenitors from the hydrocephalic cortex proliferate as normal *in vitro* and CSF collected from lateral ventricles of the hydrocephalic cortex inhibits *in vitro* proliferation of neuronal progenitors (Draper *et al.* 2001). Analysis of normal and hydrocephalic CSF reveals protein differences that could underlie these effects.

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Hormonally derived pheromones in fish: exogenous communication from gonad to brain

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Living in a medium that can limit visual information but readily exposes the olfactory organ to hormonal compounds released by conspecifics, fish throughout their long evolutionary history have had both clear cause and ample opportunity to evolve olfactory responsiveness to these potentially important chemical cues (hormonal pheromones). Indeed, steroids, prostaglandins and their metabolites are reported to be hormonal pheromones in major fish taxa including carps (goldfish), catfishes, salmon and gobies. Best understood are goldfish, where periovulatory females sequentially release a preovulatory steroid pheromone and a postovulatory prostaglandin pheromone that dramatically affect male behaviour and physiology. Three major components of the preovulatory pheromone are the oocyte maturation-inducing steroid 17β,20β-dihydroxy-progesterone, a sulphated metabolite, and androstenedione, which together induce endocrine responses that increase the quantity and quality of releasable sperm and behavioural responses that enhance male spawning success. At ovulation, femles decrease release of the steroid pheromone, and increase synthesis of prostaglandin $F_{2\alpha}$ that acts internally as a hormone to induce female sex behaviour and externally as a pheromone triggering both endocrine and courtship responses in males. Hormonal pheromones not only challenge the classical concept that sex hormone actions are limited to reproductive synchrony within the individual but also provide examples where endocrine response to a hormonal pheromone could provide the basis for a pheromonally mediated endocrine feedback system involving at least two individuals and potentially many more. Given the conserved chemistry of fish endocrine systems and the evidence for similar hormonal pheromones among related fish, it is as yet unclear if or how hormonal pheromones are species specific.

The role of prolactin in the photoperiodic control of avian gonadotrophin secretion

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In most birds living at subtropical and temperate latitudes, seasonal changes in photoperiod play a key role in determining the timing and duration of the breeding season. Most photoperiodic birds breed on long days but some, such as the emu, are short day breeders. A unifying hypothesis has been developed to account for long or short day breeding in birds (Blache *et al.* 2001). Central to this hypothesis is that long or short day avian breeding is the product of an interaction between two independently controlled, asymmetrical cycles of photoperiodically dependent prolactin and luteinizing hormone (LH) secretion. Co-incidence between increased prolactin and LH secretion results in a suppression of LH secretion. Prolactin acts at the level of the anterior pituitary gland to suppress LH β gene expression, and at the hypothalamic level to depress gonadotrophin releasing hormone (GnRH) gene expression. Studies on mammalian GnRH cell lines suggest that prolactin may act directly on GnRH neurones through the prolactin receptor. Support for this possibility in birds comes from neuroanatomical studies showing the presence of prolactin receptors in the vicinity of GnRH neurones. Photoperiodically dependent prolactin release may also control reproductive function at the level of the gonads, which are also a site of prolactin receptor gene expression. Prolactin may have several functions at the gonadal level, including the inhibition of LH-dependent steroidogenesis.

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Bi-directional communication between pineal gland and immune system

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The pineal gland is a vertebrate neuroendocrine organ able to transduce the information on external lighting condition into the biochemical message. Rhythmical synthesis and release of its principal hormone, melatonin (MEL), bring about an essential message on both the diurnal and seasonal changes in the external environment. This message may be, in turn, involved in the regulatory network responsible for keeping homeostasis within the vertebrate's body. It comprises the nervous, endocrine and immune systems, able also to communicate reciprocally and to co-operate, using the same message and receptor molecules. MEL involvement in this network has been recently accepted as its receptors were discovered not only within central nervous system but also in several peripheral tissues. Using different experimental approaches both membrane-bound and nuclear MEL receptors were demonstrated within lymphoid glands and circulating immune cells. In lymphocytes a negative correlation between MEL-regulated cAMP content, via specific G protein-coupled receptors, and lymphocyte proliferation has been shown.

Communication between immune and neuroendocrine systems (including the pineal gland) may be considered on two levels: an antigen-independent strategic one and an antigen-stimulated emergency circuit. The strategic level means that the normal development and function of both parts of this network are reciprocally dependent. In chicken embryo, pinealectomised very early during incubation, a retarded development of the primary lymphoid glands and a decreased immune response accompanied by the significant changes in the biogenic amines concentration in the spleen and brain were demonstrated. Thus development of the immune system seems to remain under regulatory influence of the pineal gland, exerted either directly on the lymphoid organs, and/or indirectly, via the neuroendocrine network. Recently, a parallel pattern of the diurnal rhythms of MEL and thymic hormones was demonstrated in rats and humans. On the other hand, an avian primary lymphoid gland, bursa of Fabricius, influences normal development of the pineal gland function, as the embryonic bursectomy evoked an alleviation of the circadian rhythm of MEL synthesis and this effect was reversed by the treatment with very low doses of bursin, a bursal tripeptide hormone. In laboratory rodents, pinealectomy performed during postnatal life caused an involution of the thymus, associated with a depression of several immune parameters, restored by the evening administration of MEL. Additional support for the strategic level of relationships between MEL and the immune system comes from the demonstration that this hormone was not only present, but also synthesized within lymphoid cells and haemopoietic tissue.

The influence of MEL on immune response (emergency level) was examined in various experimental approaches, but the results are still controversial. The effects observed strongly depended on the experimental model, including species, sex and age of the animal examined, method and extent of immune system activation, immune parameter examined, MEL dose and duration of treatment, as well as on factors not frequently taken into consideration, such as the circadian rhythm (both the pineal gland and immune system function), season, stressing conditions, etc. MEL has been shown to modulate several immune functions, such as antibody production, lymphocyte proliferation, ADCC activity, NK cell cytotoxicity, cytokine synthesis and release, inflammatory reaction, etc. Taking into consideration a versatility of MEL function, its facility to penetrate cells as well as the binding sites present in the cell membrane and the nucleus, one can assume numerous mechanisms involved in its regulatory activity. Among them, a mediation of the endogenous opioids and/or various cytokines as well as free radical scavenging were postulated.

To close a regulatory loop between the immune system and the pineal gland it is absolutely necessary that the messages sent by the activated immune system are understood by the pineal gland, i.e. the activation of the immune system influenced the pineal gland activity and, therefore, peripheral MEL level. The effects exerted by the immune system activated in different way (cytokine injection, immunisation with non-pathogenic antigen, inflammatory reaction) clearly indicated that both circadian rhythm of the pineal gland activity and serum MEL concentration varied in these conditions. Therefore, one can assume that the biochemical message sent by the pineal gland may be adjusted not only to the external lighting conditions but also to the actual performance of the immune system.

Comparative aspects of neurosteroids: production and function

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The actions of peripheral steroid hormones upon the brain, crossing the blood–brain barrier, are well known. Furthermore, the expression of specific receptors for such hormones have been localised in specific and discrete areas of the CNS in numerous species. Recently, evidence indicates that the brain itself, in addition to being a target tissue for steroids, may also have the ability to synthesize steroids *de novo* from cholesterol. Such steroids have been termed 'neurosteroids'. The existence of such neurosteroids was first established in mammals (Baulieu, 1997) and the majority of related studies have been carried out using the rat as an animal model. Only limited studies have been performed so far on non-mammalian vertebrates. However, evidence is now accumulating that neurosteroid production by the brain is common throughout the vertebrate kingdom. Furthermore, although little is still understood regarding their physiological significance in these other vertebrates, some evidence now exists to suggest a number of possible associations with both early development and reproduction. For instance, in the amphibian brain, the amount of pregnenolone and pregnenolone sulphate together with steroidogenic enzymes such as cytochrome P450 side-chain cleavage, express significant changes over the year which may be related to the breeding cycle. Furthermore, in species such as the *Xenopus*, the concentration of pregnenolone in the brain is significantly higher than that in either the gonads or plasma. The existence of P450scc in these lower vertebrates is thus indicative of an early and conserved property of vertebrate brains. The existence of neurosteroids has also recently been demonstrated in birds. In the quail, biochemical analysis of progesterone metabolism indicates the brain may be involved in the active production of 5β-dihydro-progesterone from progesterone only during embryogenesis, perhaps indicating a role for this neurosteroid in neuronal development. In contrast, in the ring dove, a species in which both sexes share in the incubation of eggs and the care of the young, the *in vitro* incubation of sections of diencephalon with tritiated pregnenolone, in order to determine the rate of biosynthesis of progesterone, demonstrated a greater synthesis of progesterone in the brains of brooding male birds compared with non-breeding males ($P < 0.05$). Consistent with this is the observation that although circulating plasma progesterone levels are low during the brooding phase, progesterone concentrations in the brain of brooding male doves are higher compared with non-breeding birds ($P < 0.05$). The expression of parental behaviour in this species can be induced by progesterone and so these studies possibly provide a further functional significance for synthesis of progesterone in the dove brain.

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