Control of respiratory behaviour in *Lymnaea*

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The pond snail, *Lymnaea stagnalis* (L.), is an excellent system for studying integrative mechanisms underlying behaviour. For example, respiratory behaviour clearly consists of several locomotory reactions, shell movements, erection of the pneumostome and ventilation of the lung. Respiration is regulated by the oxygen content of the water, food, sex arousal, defensive behaviour and is seasonally modulated. The network controlling respiration modulates the neural ensembles underlying the component behaviours and vice versa. Our findings show that neurones controlling locomotion are connected to neurones of the cardiovascular and locomotory systems.

With respect to respiratory behaviour, it is known that three identified neurones make up the central respiratory generator (CRG) and their synaptic connections have been reconstructed in cell culture. A number of respiratory motor neurones have also been identified. During lung ventilation, both ciliary beating and muscular movements associated with locomotion are inhibited due to synaptic inputs from the CRG. Both direct and indirect connections from the CRG to heart motor neurones exist, but details of cardiorespiratory integration are as yet unclear. Thus neurones controlling the respiratory system lie within and interact with a complex neuronal network underlying several behaviours.

Neurotransmission and neuromodulation at rat carotid body chemoreceptors

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The mammalian carotid body (CB) is the principal arterial P_{O_2} detector that maintains blood homeostasis via the reflex control of ventilation. Thus in response to a fall in blood P_{O_2}, chemoreceptors (type 1 cells) located in the CB release neuroactive agents onto afferent nerve terminals, resulting in an increased neural discharge in the carotid sinus nerve (CSN). The cell bodies of the chemoafferent neurons reside in the petrosal ganglion and their central projections terminate in the nucleus tractus solitarii (NTS) in the brainstem. The neurotransmitter mechanisms that operate at synaptic sites between petrosal afferent terminals and CB type 1 cells have generated considerable interest and debate over the last ~60 years. Undoubtedly, much of the complexity arises from the broad diversity of neurotransmitters or neuromodulators expressed by type 1 cells, as well as from the fact that these neuroactive agents can potentially act on several receptor subtypes located either presynaptically on type 1 cells and/or postsynaptically on petrosal nerve endings. Among these agents are dopamine (DA), noradrenaline, acetylcholine (ACh), ATP, serotonin (5-HT), substance P and γ-aminobutyric acid (GABA). Several studies indicate that DA, for many years the leading transmitter candidate and best-studied indicator of the secretory status of type 1 cells, is not the main excitatory neurotransmitter, at least in the rat CB. There is evidence that DA may play a modulatory role in inhibiting release from type 1 cells via an autocrine or paracrine mechanism. I will review recent evidence, based mainly on the use of a co-culture model of rat type 1 cell clusters and petrosal neurons, for the idea that co-release of ATP and ACh onto petrosal endings is the principal mechanism leading to CB excitation by natural stimuli. I will also discuss possible mechanisms by which this principal pathway can be modulated by both inhibitory and excitatory 'presynaptic' influences. Firstly, I will discuss recent evidence that 5-HT release from type 1 cells during hypoxia may act in a positive feedback manner via autocrine or paracrine mechanisms to increase chemoreceptor gain. This appears to involve G protein-coupled 5-HT receptors (probably 5-HT_2 serotonergic receptors) and PKC-mediated inhibition of K^+ channels. Secondly, I will discuss an opposing negative feedback, PKA-mediated inhibitory pathway due to released GABA acting on presynaptic $GABA_B$ receptors on type 1 cells. The theme that is emerging is the presence of a slower presynaptic 'push-pull' integrated feedback system, superimposed on the faster postsynaptic events mediated by ionotropic receptors. While other pathways still remain to be elucidated, the reasons for this complexity remain speculative. Nevertheless, the system appears well suited for mediation of synaptic plasticity in the CB, and may well contribute to the well-known altered sensitivity of the organ during adaptation to chronic hypoxia.

Role of vagal afferents in maintenance of lung volume in the neonate

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Efferent vagal control of the heart in lower vertebrates

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Reflex, inhibitory control of heart rate, exerted by the parasympathetic vagus, is the predominant influence on cardiac output in vertebrates. However, vagal innervation of the heart is not homogeneous as some branches have greater or more complex influences on heart rate than others, and there seems to be particular heterogeneity in the extent to which different branches contribute to the generation of cardiorespiratory interactions (Taylor *et al.* 1999).

In the elasmobranch fish, *Scyliorhinus canicula*, a relatively primitive vertebrate, the sympathetic nervous system does not extend forwards to innervate the head and pharynx, so that the heart receives solely parasympathetic innervation via the vagus nerve. However, there is a dual innervation to the heart from branchial cardiac and visceral cardiac branches (Taylor *et al.* 1977). These may have separate roles, with efferent control exerted chiefly by activity in the branchial cardiac branches (Short *et al.* 1977). The activity in the branchial cardiac branches can be discriminated into two discrete types: rhythmical bursting units, which show respiration related activity, and sporadic or regular but not rhythmical units. These two types of activity have been shown to have separate origins in the brainstem (Barrett & Taylor, 1985). Electrical stimulation of the peripheral cut end of a branchial cardiac branch with continuous trains of impulses

(2–4 ms pulses at 50 Hz) caused cardiac slowing or arrest. However, stimulation with short bursts (5–10 impulses) at rates between 19 and 43 bursts min^{-1} caused the heart to beat at the imposed rate, even when this was faster than the intrinsic rate. This implies that the respiration-related bursting units recorded from the intact branchial cardiac branch could recruit the heart, providing a mechanism for the generation of cardiorespiratory synchrony (Taylor *et al.* 1999).

While peripheral stimulation of visceral cardiac branches had very little inhibitory action on the heart, central stimulation caused a much greater degree of cardiac slowing than central stimulation of a branchial cardiac branch. Thus it appears that the visceral cardiac branches have a predominantly afferent role in the reflex control of heart rate, indicating that they may innervate receptors in the heart resembling the atrial receptors of mammals.

The vagus nerve in reptiles runs to the heart, trachea, lungs and pulmonary and coronary vasculature. In snakes, peripheral electrical stimulation of the vagus results both in a bradycardia and an increase in pulmonary vascular resistance, because the vagus innervates a sphincter on the pulmonary artery as well as having an inhibitory influence on heart rate (Lillywhite & Donald, 1994). The vagus nerve in the rattlesnake, *Crotalus durissus*, is asymmetrical in its effects. Peripheral stimulation of the left cervical branch markedly slows the heart, while transection causes cardioacceleration. The right branch is relatively without effect on cardiac chronotropy. The functional and neuroanatomical bases of this marked asymmetry are presently under investigation.

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Cellular mechanisms of respiratory rhythm generation in vertebrates

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The generation of rhythmic activity dominates animal behaviour. Rhythmic activity plays important roles in the control of locomotion, breathing, feeding and circadian activity, and also in the control of various forms of cortical activity. Despite these diverse roles, common cellular principles underlie rhythm generation in invertebrate and vertebrate neuronal networks.

It can be demonstrated that rhythmic activity relies on a combination of intrinsic membrane properties, such as the activation of voltage-dependent calcium and sodium channels, and synaptic properties involving chemical and electrical transmission. These cellular properties are targets to neuromodulation, thus providing neural networks with the flexibility to respond to changes in the behavioural and environmental state of the animal.

This lecture will use the neural network that controls breathing in mammals as a model system to discuss general principles of rhythm generation. Our data indicate that the rhythm generating mechanisms in the respiratory network are state dependent. Under normoxic conditions, respiratory rhythm generation relies primarily on synaptic mechanisms. In contrast, during anoxia, the respiratory rhythm is primarily driven by pacemaker properties.

The transition from a primarily network-driven to primarily pacemaker-driven activity reflects two distinct forms of breathing: normal respiratory (eupnoeic) activity and gasping, respectively.

In vivo gene transfer to dissect neuronal mechanisms regulating cardiorespiratory function

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We have recently adopted adenoviral vectors to express specific proteins confined to restricted areas of the brainstem to understand nervous control of cardiorespiratory function (Kasparov & Paton, 2000; Paton *et al.* 2001). Virally mediated gene transfer can be used to enhance the levels of expression of a transmitter/modulator as well as antagonise intracellular pathways through the expression of dominant negative proteins. This approach allows (i) gene manipulation confined to a specific central nervous region of interest, (ii) assessment of the chronic effects of gene expression, (iii) avoidance of problems relating to non-selectivity of drugs or their absence, (iv) control data to be acquired prior to gene intervention and (v) limited time for compensation. All told, viral vectors provide an alternative to transgenic animals.

Normal levels of blood pressure are regulated by arterial baroreceptors that project to neurones residing in the nucleus of the solitary tract or NTS. This structure is essential for cardiorespiratory afferent integration. We have applied adenoviral-mediated gene transfer to the NTS. Specifically we have assessed the mechanisms by which angiotensin II (ANGII) acting within the NTS depresses the baroreceptor reflex, which ultimately allows arterial pressure to rise. Indeed, in hypertension, the gain of the baroreceptor reflex is reduced but blockade of central ANGII type 1 (AT1) receptors helps restore arterial pressure in hypertensive rats (e.g. Gyurko *et al.* 1993; Phillips *et al.* 1977).

We found that exogenous ANGII in the NTS depressed the baroreceptor reflex-mediated bradycardia: ANGII suppressed both the reflex activation of cardiac vagal activity and withdrawal of inferior cardiac sympathetic nerve activity during hypertension (Boscan *et al.* 2001). The depressant effect of ANGII in the NTS on the baroreceptor reflex was mediated via release of nitric oxide (NO). In the absence of selective antagonists for the various isoforms of nitric oxide synthase (NOS), we employed an adenovirus (Ad-TeNOS; see Kantor *et al.* 1996) to express a dominant negative form of endothelial (e) NOS in the NTS. In these transfected rats, disabling eNOS prevented the depressant effect of ANGII on the baroreceptor reflex (Paton *et al.* 2001). In addition, in naïve rats, various NO donors all depressed the reflex. Further, using conventional pharmacological tools we found that ANGII-mediated eNOS activation was calcium–calmodulin dependent and occurred through the activation of phospholipase C (PLC): the ANGII-mediated inhibition of the baroreceptor reflex bradycardia was abolished in the presence of a PLC inhibitor U73122 and a calmodulin antagonist – W7. The coupling of AT1 receptors to PLC is likely to be mediated by Gq protein. Thus using a different adenoviral vector that expressed a dominant negative form of the α-subunit of the Gq protein, Ad.GaqDN, we blocked Gq-

mediated signalling in the NTS. In these transfected animals, exogenous ANGII in the NTS only marginally reduced baroreceptor reflex gain, suggesting that Gq protein is involved in the ANGII-mediated attenuation of the baroreceptor reflex.

Our next quest was to determine the cellular compartment harbouring eNOS. Scanning confocal microscopy revealed that ANGII (200 nM) increased the intracellular calcium concentration of NTS endothelial cells but not neurones. Because intracellular calcium elevation is prerequisite for the ANGII-induced inhibition of the baroreceptor reflex, these data indicate that the endothelium is the primary source of ANGII-triggered release of NO. Moreover, we demonstrate that inhibition of soluble guanylyl cyclase blocked the effect of ANGII. It is therefore likely that NO enhances the efficacy of inhibitory synaptic transmission (GABA) in the NTS, as revealed in earlier studies (Paton & Kasparov, 2000).

Because arterial hypertension is a chronic disease, the long-term effects of gene manipulation in the NTS should be considered also. Using radio telemetry, we measured cardiovascular variables in conscious, freely moving rats in which the NTS was transfected with Ad-TeNOS to disable eNOS. After 2 weeks the baroreceptor reflex gain was enhanced significantly. These data reveal that endogenous eNOS activity within the NTS plays a major role in setting the sensitivity of the baroreceptor reflex and support our previous findings in acute experiments (see above).

We propose a hypothesis of vascular-neuronal signalling in the NTS whereby circulating ANGII releases NO from endothelial cells of the blood vessels that diffuses into the NTS to potentiate the release of GABA, which depresses neurones mediating the baroreceptor reflex. Further, our data demonstrate unequivocally that endogenous eNOS activity in the NTS is a major factor determining baroreceptor reflex function in conscious rats. Whether over-activity of eNOS in the NTS, due to heightened levels of ANGII, results in hypertension awaits investigation.

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A malleable 'hard-wired' central pattern generator mediating aerial respiration in *Lymnaea*

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Aerial respiration is mediated by a 3-neuron CPG whose sufficiency and necessity has been demonstrated. However, the output of this 'hard-wired' CPG is 'plastic'. For example, aerial respiratory behaviour can be operantly conditioned, a form of associative learning, and it exhibits long-term memory. With the passage of time the memory weakens. We hypothesize that this forgetting, or memory transience, is also a form of learning.

To test this hypothesis we have performed a number of different experimental procedures following the establishment of memory. (1) Cold shock (intact snails are maintained at 4 °C) blocks new protein synthesis, and snails subjected to cold shock have their memories significantly extended. (2) If we remove the soma of RPeD1, one of the CPG neurons, after memory has been formed, forgetting does not occur. We remove the soma in such a way as to not damage the primary neurite (where all synaptic activity occurs) and the primary neurite remains functional for up to 3 weeks. However, since there is no longer any nucleus, altered gene activity, which we hypothesize to be necessary for forgetting, cannot occur. (3) If a new association is prevented (snails are not allowed to perform aerial respiration and thus the association of opening the pneumostome and no reinforcing stimulus) memory is also extended. All the results are consistent with the hypothesis that altered gene activity and new protein synthesis have to occur in order to forget. Forgetting of a learned behaviour thus appears to be a memory of a new learned behaviour that resembles the naïve state.