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Hypoxia-induced modulation of the respiratory CPG

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1. ABSTRACT

Despite recent advances in our understanding of the neural control of breathing, the precise cellular, synaptic, and molecular mechanisms underlying the generation and modulation of respiratory rhythm remain largely unknown. This lack of fundamental knowledge in the field of neural control of respiration is likely due to the complexity of the mammalian brain where synaptic connectivity between central respiratory neurons, motor neurons and their peripheral counterparts cannot be mapped reliably. We have therefore developed an invertebrate model system wherein the essential elements of the central pattern generator (CPG), the motor neurons and the peripheral chemosensory cells involved in respiratory control have been worked out both *in vivo* and *in vitro*. We discuss our recent identification of peripheral, hypoxia sensitive chemoreceptor elements in a sensory organ of the pulmonate freshwater pond snail *Lymnaea stagnalis*, which provide an excitatory drive to the respiratory CPG neuron RPeD1 via direct chemical synaptic connections. Further studies using this unique invertebrate model system may reveal highly conserved principles of CPG neuromodulation that will remain relevant to more complex mammalian systems.

2. INTRODUCTION

Breathing is a fascinating rhythmic motor behavior that facilitates the exchange of oxygen (O_2) and carbon dioxide (CO_2) most commonly at the lungs or gills of animals. Like most rhythmic motor behaviors such as wing beating, walking and chewing, breathing activity is governed by a network of neurons that is typically referred to as a 'central pattern generator' (CPG). These CPGs generate patterned motor outflow to the relevant muscle groups, and in the case of breathing these are the respiratory pump muscles.

The respiratory CPG has been of particular interest in the study of rhythm generation for at least two principal reasons. First, the respiratory CPG governs a motor behavior which has a common homeostatic, physiological goal common to all animals: the extraction of O_2 from the ambient air or water, and the elimination of CO_2 into the surrounding environment. Second, as a homeostatic motor behavior, breathing is tightly regulated by inputs, which ensure that the level of gas exchange with the external environment is closely matched to tissue metabolism. This fine regulatory capacity enables a system wherein the influence of sensory inputs can be assessed and

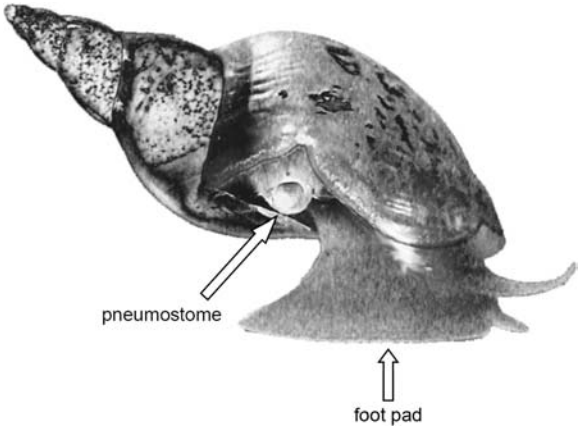

Figure 1. *Lymnaea stagnalis* pictured from above while breathing air at water surface. The animal's respiratory orifice, the pneumostome, can be seen open and allowing air to flow in or out of the rudimentary lung, the mantle cavity.

studied in the context of neuronal motor output required to match the behavioral demands of an animal in an ever-changing environment.

It has long been realized that while a CPG must by definition be sufficient to produce the patterned motor output which underlies a given behavior, afferent inputs or 'feedback' to the CPG is essential for this pattern of motor outflow to remain physiologically relevant to the environmental conditions surrounding the animal. The amplitude, shape of motor unit discharge, and cycle length of rhythmic activity can all be susceptible to modulation at all levels of neuronal organization – ranging from afferent input to the CPG itself. This is certainly the case with the respiratory CPG. Respiratory motor outflow is affected by a myriad of feedback mechanisms related to such things as ambient O_2 status, tissue metabolite levels, and body movement. At present however, little is known about the precise mechanisms through which peripheral inputs modify the CPG activity to either scale up or down the respiratory motor output.

Breathing behavior can be easily observed in a vast number of species, however the underlying CPG and the nature of its connectivity remains elusive. Indeed the respiratory CPG has proven to be incredibly intricate and adaptive network, making investigation at the neuronal and synaptic levels a challenging task. This is especially true for the mammalian CPG controlling breathing behavior, which has been the topic active research and great controversy (1, 2).

Invertebrate model systems have been fundamental in advancing our understanding of neurophysiology (3), and this is certainly true in the case in the study of central rhythm generation (4, 5). The central neurons of invertebrates exist within ganglia, and many are readily identifiable based upon their location and morphology. This feature of invertebrate nervous systems makes it possible to study a specific cell involved in a particular behavior across multiple animals of the same species. Therefore invertebrates are especially amenable to the study of rhythm generation, since networks can be mapped from individually identified cells, and subsequently studied at the system, cellular, synaptic and molecular levels.

In this review, we will discuss recent advances in our understanding of the cellular and synaptic mechanisms by which afferent signals from the periphery modulate respiratory CPG activity, made possible by *in vivo* and *in vitro* experiments performed in an invertebrate model system. We will further discuss how the use of this model system might help us to understand similar processes in the nervous systems of higher level organisms.

3. AN INVERTEBRATE MODEL SYSTEM FOR THE STUDY OF RESPIRATORY RHYTHM GENERATION

One invertebrate model system wherein the essential central, neuronal components of the respiratory CPG have been identified and studied, both *in vivo* and *in vitro*, is the pulmonate freshwater mollusk *Lymnaea stagnalis* (6). *Lymnaea* is an aquatic bimodal breather, meaning that they can exchange respiratory gasses across their skin with the aquatic environment, or alternatively breathe atmospheric air at the water surface. Aerial breathing activity in *Lymnaea* involves the exhalation and inhalation of air through their respiratory orifice, the pneumostome, and therefore requires a coordinated motor output to the muscles of the mantel cavity and pneumostome. As the animal surfaces with the respiratory orifice exposed to air (see Figure 1), contraction of muscles in the mantle cavity and pneumostome opening muscles allows 'stale' air to be expelled from the lung cavity, which is subsequently passively re-inflated by atmospheric air.

This breathing activity is governed by a three cell CPG consisting of the central-ring neurons: right pedal dorsal 1 (RPeD1), visceral dorsal 4 (VD4), and the input 3 interneuron (IP3I). Figure 2 shows the approximate locations of these cells in their respective central ring ganglia. This CPG network has been shown to be both necessary and sufficient to generate the basic rhythmic patterned activity underlying breathing behavior and controls the activity of respiratory motorneurons innervating the pneumostome opening and closing muscles. The rhythmic patterned activity of *Lymnaea* respiratory CPG has been studied using a reductionist approach - ranging from the semi-intact animal, to isolated cultured neurons *in vitro*. The connectivity between the cells has been fully characterized and the network reconstructed in cell culture. A schematic representation of the basic CPG and the nature of the connectivity within the three-cell network is shown in Figure 3.

4. HYPOXIA MODULATES RESPIRATORY BEHAVIOR IN LYMNAEA

4.1. Hypoxia provides a respiratory drive to breathe

Air breathing activity generated by the respiratory CPG in *Lymnaea* is a hypoxia-dependent

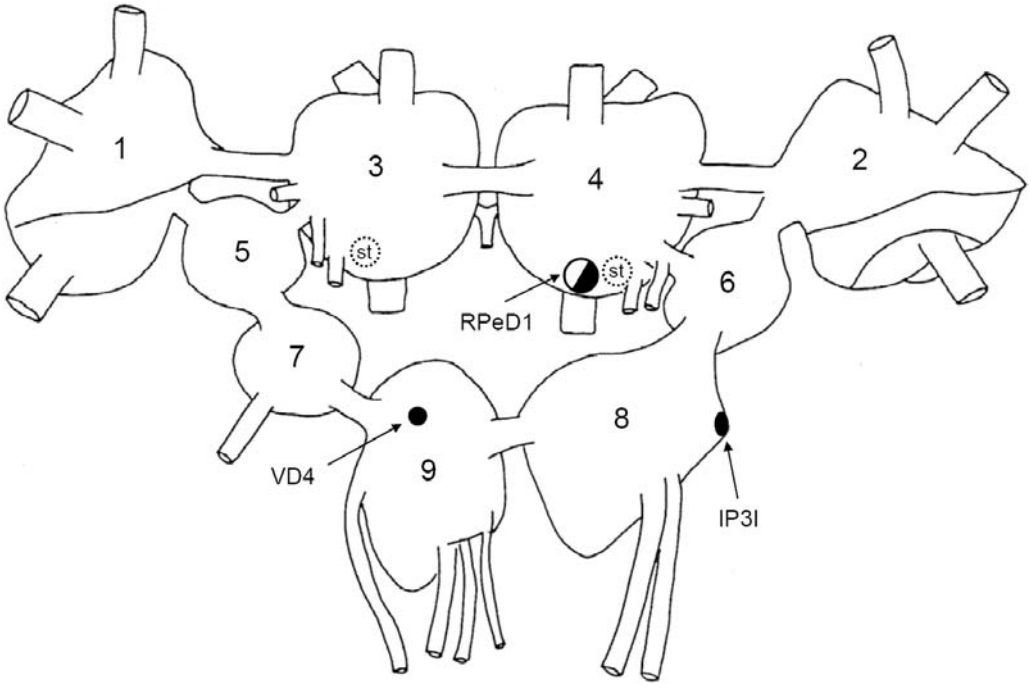

Figure 2. Dorsal View of the Central Ganglionic Ring of *Lymnaea stagnalis*. The ganglia are numbered as follows: 1, 2 left and right cerebral ganglia; 3, 4 left and right pedal ganglia; 5, 6 left and right pleural ganglia; 7, 8 left and right parietal ganglia; 9 visceral ganglion. The three identified cells involved in the central generation of respiratory rhythm are shown in their typical anatomical locations: RPeD1, right pedal dorsal one; VD4, visceral dorsal four; IP3I, input three interneuron. The degree of shading of the cell indicates coloration of the cell as it appears in the brain (filled indicates very white, open indicates pale orange). st, statocyst.

behavior (7, 8). When the aquatic environment becomes hypoxic, *Lymnaea* spend considerable time at the water surface and breathe more frequently in an effort to gain access to atmospheric oxygen (Figure 4). Indeed as an aquatic mollusk adapted to thrive in stagnant water, *Lymnaea* developed effective mechanisms through which it can sense ambient oxygen availability, and alter its air breathing behavior appropriately in order to cope with environmental conditions. The observation that oxygen availability changes breathing behavior underscores the importance of synaptic plasticity within the CPG neurons both of their sensory and motor counterparts. However, in order to directly study the cellular and synaptic mechanisms which underlie this plasticity, specific cells which function as oxygen sensors must first be identified in the animal.

4.2. The neural signal encoding oxygen status originates in the periphery

Inoue *et al* (9) demonstrated that the signal which stimulates breathing in an hypoxic environment originates in the periphery of the animal. In other words, the respiratory CPG neurons do not act as oxygen sensors and require other sensory input from the periphery (or 'body' of the animal) to sense oxygen availability and convey this information to the CPG via synaptic inputs. The study by Inoue *et al* involved the use of an inventive recording chamber wherein the bath surrounding the animal was separated into central and peripheral compartments. The 'brain' was bathed in a separate chamber from the 'body', but the nerves connecting the 'brain' to the 'body' remained intact. In this way, the CPG and the periphery could be independently exposed to hypoxia.

By simultaneously recording from the respiratory CPG neuron RPeD1, and the pneumostome opener motor neuron visceral J cell, respiratory activity was observed to increase in response to hypoxia only when the compartment housing the periphery tissue was exposed to hypoxia (see Figure 5). Exposure of the central compartment containing the CPG neurons to hypoxia challenge did not however, trigger respiratory activity. These data suggested that the respiratory activity in *Lymnaea* originates at the periphery. The authors also found that the hypoxia driven stimulatory drive from the periphery was abolished when the peripheral compartment was bathed with Ca^{2+}-free saline. The neural signal driving respiration in hypoxia also appeared to be relayed via nerves projecting to the mantle cavity and pneumostome regions of the animal. It was therefore concluded that a chemosensory element in the periphery, likely residing in the mantle or pneumostome tissues, relays an excitatory drive to the respiratory CPG via chemical synaptic connections located at the periphery of the animal. However, neither the precise nature of these hypoxia sensitive elements nor the underlying mechanisms were elucidated.

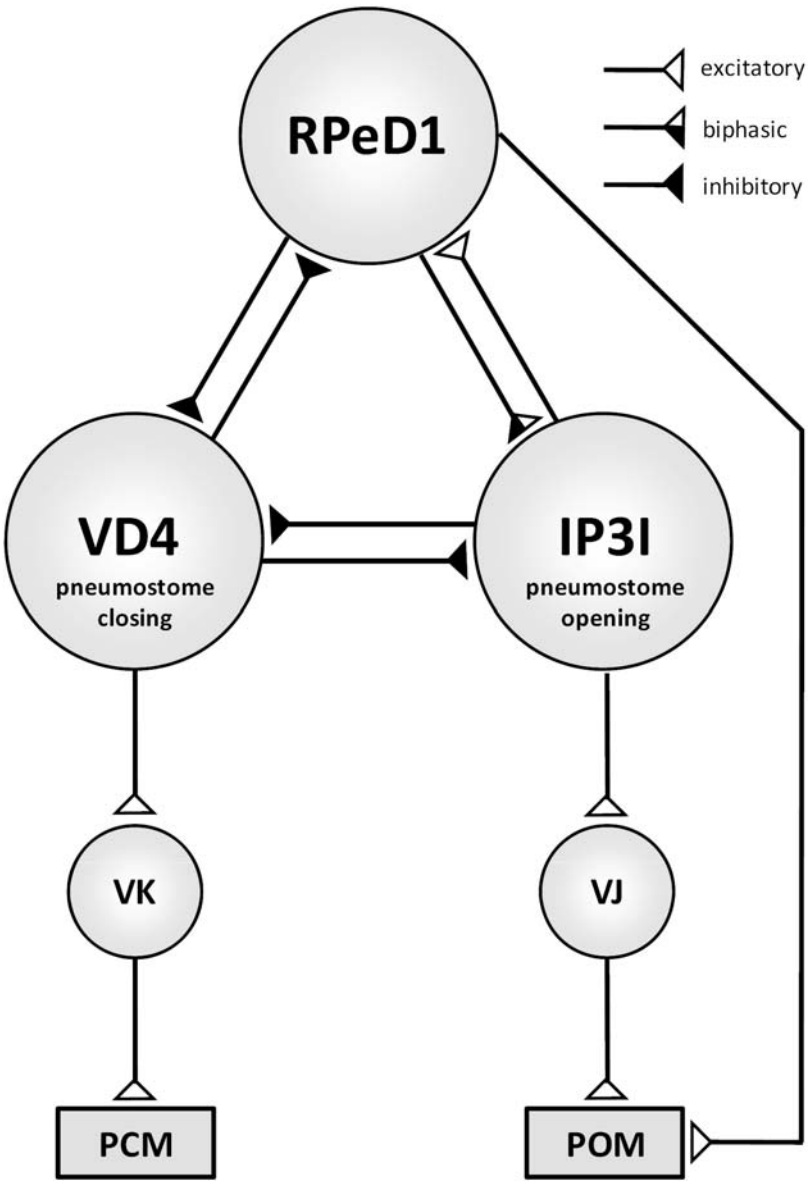

Figure 3. A schematic representation of the respiratory CPG and the motor pathways controlling the pneumostome muscles of *Lymnaea*. Breathing behavior is controlled by a CPG consisting of at least three identified interneurons. RPeD1 activates respiratory interneuron IP3I via dual excitatory / inhibitory connections and triggers respiratory patterned activity in the other respiratory neuron visceral dorsal 4 (VD4). The respiratory rhythm is then conveyed to the pneumostome opening muscles (POM) and closing muscles (PCM) via the motor neurons visceral J (VJ) and visceral K (VK), respectively. All of these connections are chemical and monosynaptic, and have been reconstructed in culture. Activation of RPeD1 can initiate rhythmogenersis, and receives excitatory, chemosensory and mechanosensory input from the periphery which modulates the activity of the CPG.

5. IDENTIFICATION OF PERIPHERAL HYPOXIA-SENSITIVE CELLS WHICH DRIVE RESPIRATION

5.1. Osphradial denervation compromises the respiratory response to hypoxia

The osphradium in *Lymnaea* is a known peripheral sensory structure which has been hypothesized to be involved in respiratory and feeding behaviors as well as osmoregulation (10-12). Structurally, the osphradium has been described as a Y -shaped epithelial canal which leads inward from the surface of the mantle. At least 3 morphologically distinct neuronal cell subtypes have been identified in the osphradium (13). There are large (80-100 μm dia.) white 'ganglionic' cells located adjacent to the origin of the osphradial nerve. Smaller cells (20-30 μm dia.) that are light yellow in color and much more numerous are located near the ganglionic cells, along the epithelial canal, and along the dorsal and ventral surfaces of the structure. The third type of cell is much smaller (3-5 μm dia.), and is located near the centre branching of the

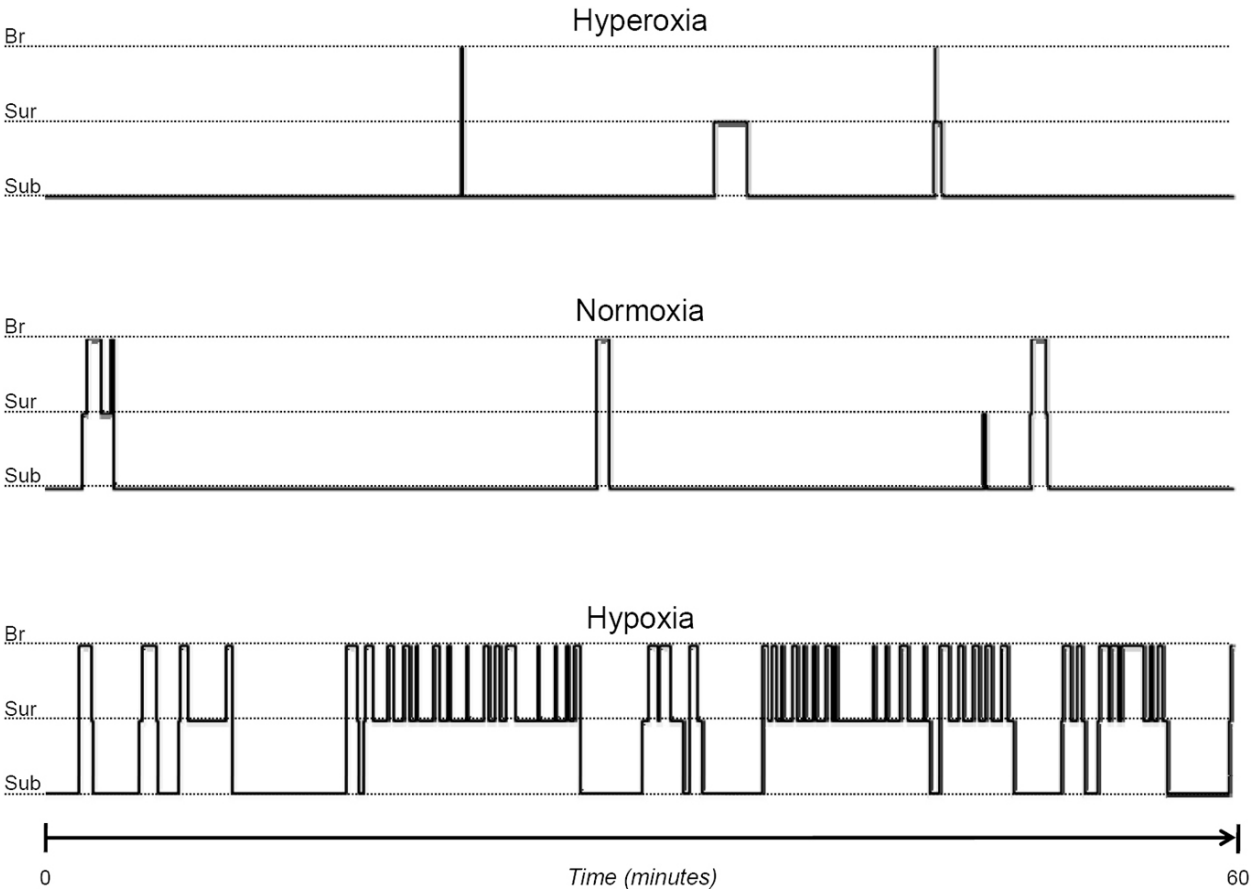

Figure 4. Breathing in *Lymnaea* is a hypoxia-driven behavior. A breathing behavior histogram for one snail monitored over 60 minutes on separate occasions in three ambient oxygen conditions: hyperoxia (PO_2 >> 300 mmHg), normoxia (PO_2 ~ 140 mmHg), and hypoxia (PO_2 ~ 55 mmHg). The water was gently aerated with hyperoxic, normoxic or hypoxic gas mixtures so that the water and the air at the water surface were in equilibrium. Snails were then recorded as being in one of 3 behavioral states in these backgrounds: Sub – 'submerged' under water; Sur – 'surfaced' with pneumostone closed; Br – surfaced with pneumostome open, 'breathing'. Notice that breathing behavior is heavily influenced by amibient oxygen status. In a hyperoxic background, animals remain submerged much of the time, and rarely if ever open their pneumostome at the water surface. In a normoxic background, animals occasionally visit the water surface to engage in aerial breathing. In a hypoxic background, animals spend most of their time at the water surface and repeatedly open and close their pneumostome, performing regular breathing movements. This powerful effect of hypoxia on breathing behavior clearly illustrates the modulatory influence of oxygen over respiratory rhythm generation in the freely behaving animal.

epithelial canal. Anatomically, the osphradium is located superficially in the mantle tissue, in close proximity to the pneumostome. Indeed, the structure and the nerve branches leading to it can be readily identified through the skin of intact animals using a stereo microscope (8).

While the exact sensory functions of the different neuronal subtypes in the osphradium are not fully understood, studies using direct nerve recordings have indicated that the osphradium does respond with an increased nerve discharge in response to low partial pressure of oxygen (PO2) (14, 15). Based upon this information, it seemed reasonable for us to propose that the osphradium may be the peripheral source of the afferent respiratory drive related to the status of ambient oxygen availability.

This possibility led us to perform experiments to directly investigate the role of the osphradium in the control of breathing behavior (8, 16). As a first step, we surgically denervated the osphradium by cutting the branch of the internal right parietal nerve that innervates it, so as to prevent any afferent signal originating in the osphradium from reaching the respiratory CPG in the central ring ganglia, and observed the effects of this denervation on respiratory behavior. We found that animals in which the osphradial nerve was lesioned demonstrated normal respiratory movements, suggesting that motor control of the pneumostome musculature remained intact. However, in the absence of sensory input from the osphradium, animals breathed less often and spent more time away from the water surface, regardless of ambient oxygen

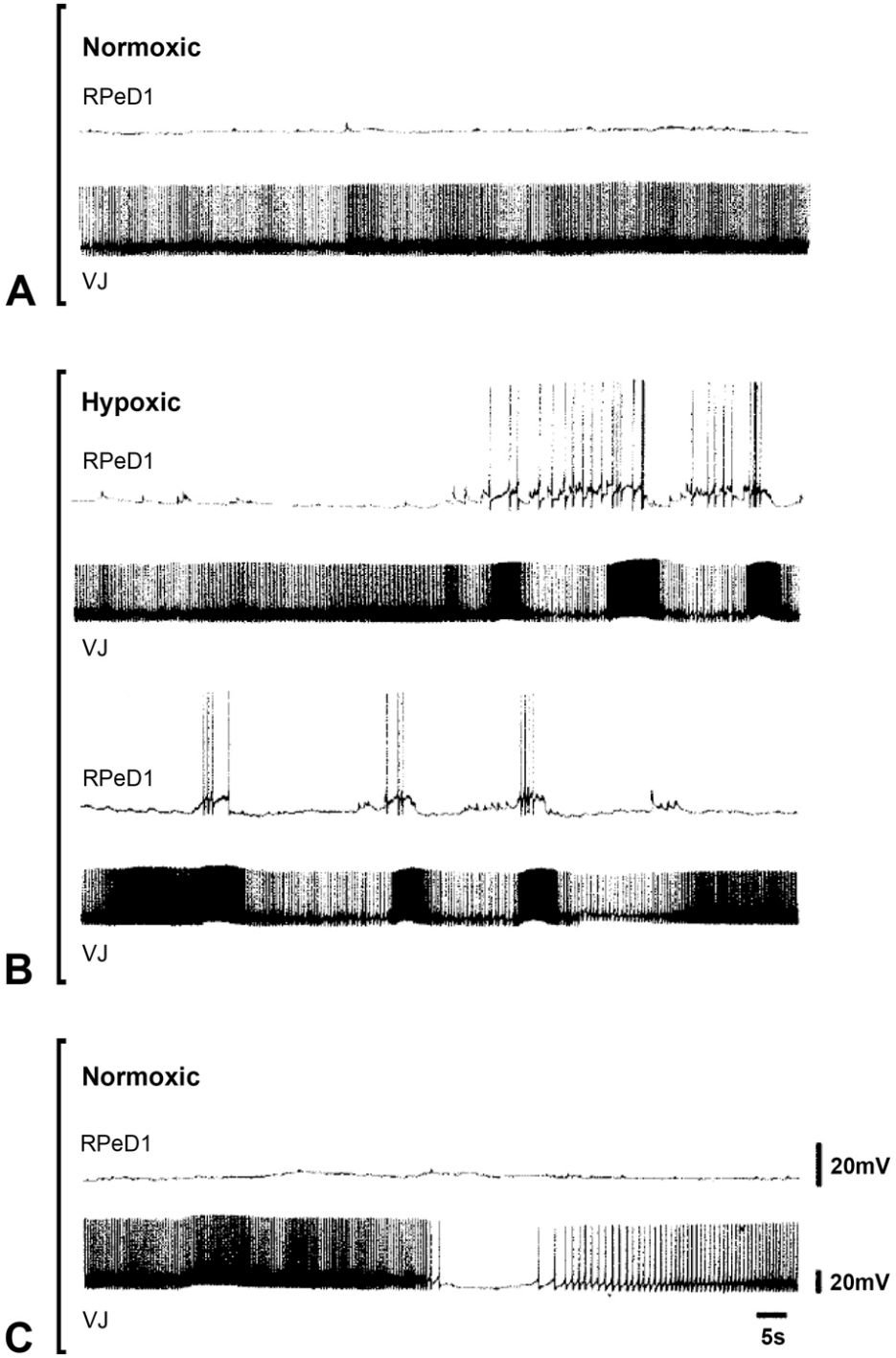

Figure 5. Hypoxia-induced respiratory drive in *Lymnaea* originates at the periphery. To test for the origin of chemosensory driven, respiratory activity in *Lymnaea*, right pedal dorsal neuron 1 (RPeD1), and the pneumostome opener motor neuron visceral J (VJ) cell were recorded simultaneously in a semi-intact preparation. The compartment containing the central ring ganglia was physically separated from the periphery (pneumostome, mantle, lung, kidney, and heart) and sealed. A: Under normoxic conditions, the CPG neuron RPeD1 and a VJ cell did not show spontaneous respiratory discharges (top panel), nor were pneumostome opening and closing movements observed (not shown). B: Within minutes of perfusing hypoxic saline (2nd panel), characteristic rhythmic respiratory discharges appeared in both RPeDl and VJ cell (2nd and 3rd panels). C: spontaneous respiratory activity returned to baseline levels after wash out with normoxic saline. All traces represent continuous recordings. Reproduced with permission from (9).

availability. As such, while surgical denervation of the osphradium did not completely abolish breathing, the respiratory drive and the ability to increase ventilation in response to hypoxia was significantly compromised.

5.2. The osphradium contains oxygen-sensing peripheral chemoreceptor cells

Based upon our observation that denervation of the osphradium reduced the breathing activity of animals, we went on to perform electrophysiological studies to determine whether cells in the osphradium itself can respond to hypoxia as a stimulus. We tested whether osphradial neurons exhibit oxygen sensitivity by performing sharp electrode recordings from them using a semi-intact preparation (8). We found that osphradial cells were generally quiescent when bathed in well-oxygenated saline, but within seconds of exposure to hypoxic saline they produced burst-like periods of activity (Figure 6, Panel A). This demonstrated that hypoxia provided an excitatory stimulus for osphradial cells. To test whether osphradial cells possess an inherent sensitivity to background oxygen levels, these cells were isolated *in vitro* and studied in cell culture. In this way, any potential inputs or connectivity which might have masqueraded as inherent oxygen sensitivity in the semi intact preparation would be eliminated (8). Even when isolated osphradial neurons were exposed to a hypoxic background, their membranes exhibited depolarization and an induction of spikes. Osphradial neurons therefore exhibit properties which are characteristic of respiratory 'peripheral chemoreceptor cells' in mammals and we therefore designated them as PCRCs.

5.3. Osphradial PCRCs provide direct excitatory input to the respiratory CPG

RPeD1 is the only respiratory CPG neuron with projections to the periphery (17). Moreover, this projection via the right internal parietal nerve and the osphradial nerve has a branch which terminates in the osphradium. These observations supported our contention that RPeD1 may receive chemosensory information directly from the osphradium (8). Using simultaneous direct intracellular recordings from osphradial neurons and RPeD1 in the semi-intact preparation, we confirmed that the stimulation of osphradial neurons using current injection triggered action potentials in a normally quiescent RPeD1 (Figure 6, Panel B). We then performed these same simultaneous recordings from the preparation while bathed in a 'HiDi' saline. *Hi*gh *di*valent cation saline solutions contain higher than normal concentrations of extracellular cations, specifically Mg^{2+} and Ca^{2+}. HiDi solutions have commonly been used as a means of testing for evidence of monosynaptic connectivity between identified neurons *in vivo* (18, 19), and while their mechanism of action is complex, they are believed to increase the activation threshold for voltage dependent currents in the positive direction. In this way, the likelihood of activating any putative interneurons is greatly diminished. We observed that when osphradial neurons were directly activated by intracellular current injection, that action potentials continued to be observed in the post synaptic cell, RPeD1, despite the background presence of a HiDi solution. This observation strongly suggests that the respiratory CPG neuron RPeD1 receives monosynaptic excitatory input from osphradial PCRCs in the semi-intact preparation.

5.4. An excitatory synapse between the PCRC and RPeD1 studied *in vitro*

We further examined the nature of synaptic connections between the osphradial PCRCs and the respiratory CPG neuron RPeD1 using *in vitro* cell culturing techniques (20). Specifically, PCRC and RPeD1 soma were isolated *in vitro* and paired in cell culture in a soma–soma configuration (8, 21). In culture, the specific synapses between osphradial neurons and RPeD1 re-established in a manner similar to that seen *in vivo*. Action potential induced in PCRCs via direct current injection resulted in one-for-one EPSPs in the paired RPeD1 neuron. In an effort to identify the specific chemical transmitter(s) involved at this synapse, we applied a variety of pharmacological interventions. Most importantly, the cholinergic antagonist mecamylamine completely and reversibly blocked the synaptic transmission between the cells. Dopaminergic, serotonergic and P2X purinoceptor antagonists had no significant effects in altering the generation of EPSPs in the post-synaptic RPeD1. These data thus suggested that the hypoxia drive in *Lymnaea* originates at the periphery among the osphradial cells and is conveyed to RPeD1 via direct synaptic connections.

We also tested the effect of hypoxia on the PCRC-RPeD1 synapse isolated in culture. Exposing the PCRC-RPeD1 cell pair to hypoxia elicited spiking activity in the PCRCs which in turn generated 1:1 Excitatory postsynaptic potentials (EPSPs) in the post synaptic RPeD1. Interestingly, we also found that in the presence of hypoxia, the amplitude of these EPSP was potentiated. In other words, the PCRC-induced EPSPs in RPeD1 were significantly larger when the cells were exposed to hypoxia, compared to normoxic conditions (Figure 7). Hypoxic exposure did not affect action potential amplitude or duration in the PCRCs, and neither the resting membrane potential nor the reversal potential of RPeD1 appeared to be significantly affected by exposure to hypoxia. These observations suggest that the potentiation that we observed in EPSP amplitude at the PCRC-RPeD1 synapse during hypoxia exposure, probably occurred via changes in synaptic physiology. While the precise mechanisms resulting in the hypoxia-induced modulation of EPSP amplitude at this synapse remain to be determined, we speculate that they may be similar to those observed at synapses in the afferent arm of the chemosensory reflex pathway in vertebrate systems, and involve either an inhibition of K^+ channels or the activation of L-type Ca^{2+} channels (22).

7. SUMMARY AND PERSPECTIVES

In this review, we have summarized recent research from our laboratory wherein we have developed a simple invertebrate model system in which the fundamental

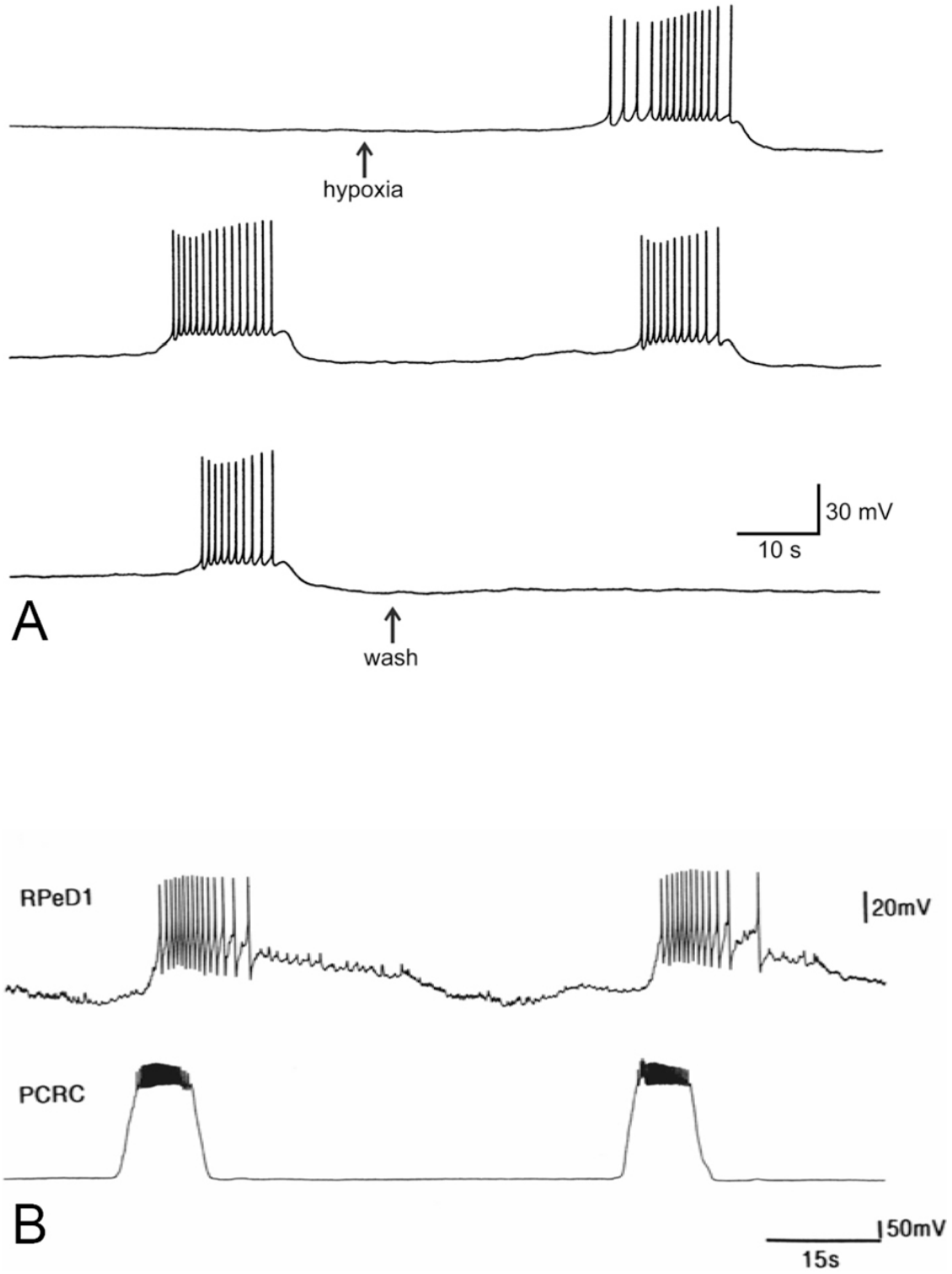

Figure 6. Osphradial neurons are hypoxia-sensitive peripheral chemoreceptors which drive the respiratory CPG. Panel A: Burst-like activity in an osphradial chemosensory neuron elicited by the transition from normoxia (PO_2 ~ 140 mmHg) to hypoxia (PO_2 ~ 65 mmHg; n = 23 cells from 11 preparations). RMP pre-hypoxia = − 46 mV. Panel B: Activity in a PCRC, induced via sustained direct depolarizing current injection, causes spiking activity in the respiratory CPG neuron RPeD1 (RMP = −57 mV, held to −70 mV). Panels A and B reproduced with permission from (16).

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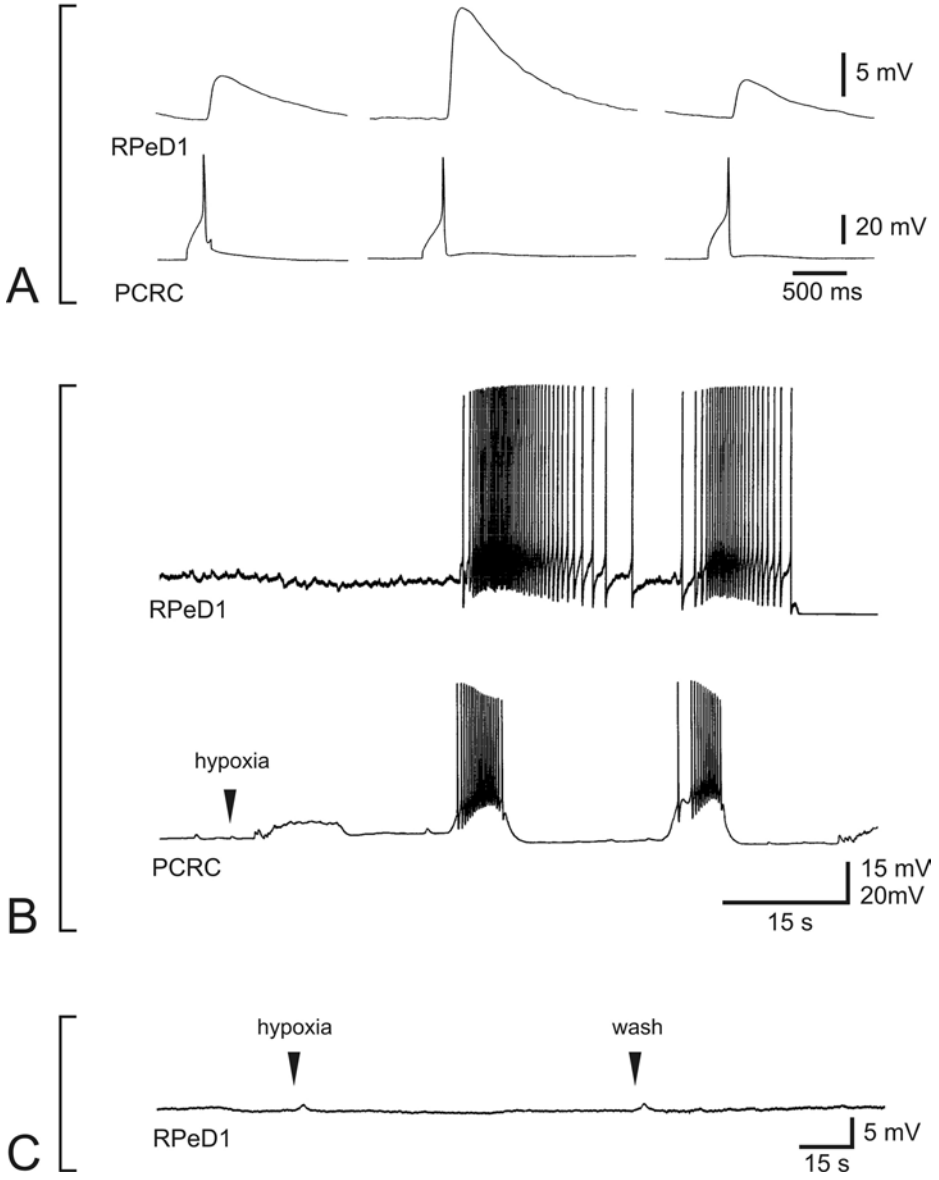

Figure 7. Panel A: To test whether osphradial peripheral chemoreceptor cells (PCRCs) re-establish specific synapses with right pedal dorsal 1 (RPeD1) *in vitro*, the cells were isolated and paired overnight in a soma–soma configuration. After 16–24 h of cell culture, simultaneous intracellular recordings revealed an excitatory synapse between the paired cells. Shown are recordings from one PCRC–RPeD1 synapse, where the RMP in RPeD1 was -59 mV. Baseline membrane potential in RPeD1 was adjusted to -120 mV at rest using bridge current injection controls to maintain consistency across experiments for post-synaptic potential measurements. Induced action potentials in a PCRC triggered 1:1 excitatory post-synaptic potentials (EPSPs) in RPeD1. The amplitude of the PCRC-induced EPSPs was significantly potentiated in the presence of hypoxic (PO_2 65 mmHg) saline and this synaptic transmission returned to its baseline upon washout with normal (PO_2 140 mmHg) saline. Panel B: Hypoxia drives the PCRC–RPeD1 synapse when cells are isolated in culture. Upon exposure of the PCRC–RPeD1 synapse in co-culture to hypoxic saline (PO_2 35 mmHg), periodic changes in membrane depolarization in the PCRC lead to bursting activity that excites RPeD1. Shown is a simultaneous intracellular recording from a PCRC–RPeD1 soma–soma synapse where the cells (RMP -55 and -59 mV, respectively) were co-cultured for 24 h. Panel C: isolated RPeD1 cells do not respond during exposure to this same hypoxic background (RMP -57 mV). Reproduced with permission from (8).

cellular and synaptic mechanisms underlying respiratory rhythm generation and modulation can be directly investigated. The essential elements of the respiratory CPG of the fresh water pond snail *Lymnaea stagnalis* have been identified and studied both *in vivo* and *in vitro*. Moreover, we have located peripheral chemosensory elements which provide an afferent modulating signal to the respiratory CPG, which is related to the status of ambient oxygen availability. Oxygen sensitive PCRCs located within the osphradium exhibit hypoxia-induced activity which provides direct excitatory input to the respiratory CPG neuron RPeD1 via cholinergic synaptic transmission. This model system therefore provides an ideal opportunity in which to study the underlying basis of afferent modulation of respiratory rhythm at varying levels of reduction; from whole animal experiments, to *in vitro* cell culture studies. Detailed studies using this invertebrate model system approach have the potential to provide an unprecedented level of understanding of the cellular, synaptic and molecular basis of respiratory rhythm generation and neuromodulation.

9. ACKNOWLEDGEMENTS

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Abbreviations: CPG: central pattern generator; PO_2: partial pressure of oxygen; PCRC: peripheral chemoreceptor cell; RPeD1: the neuron right pedal dorsal 1; VD4: the neuron visceral dorsal 4; IP3I: the input 3 interneuron

Key Words: Hypoxia, Oxygen, Respiration, Control of Breathing, Respiratory Rhythm, Modulation, Peripheral Chemoreceptor, Osphradium, Central Pattern Generator, CPG, Afferent Signal, *Lymnaea Stagnalis*, *In vivo*, *In vitro*, Review

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