Behavioural and Neural Correlates of Operant Conditioning in *Lymnaea stagnalis*: Role of Previous Experience During Development

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Abstract

The freshwater mollusc *Lymnaea stagnalis* was utilized in this study to further the understanding of how network properties change as a result of associative learning, and to determine whether or not this plasticity is dependent on previous experience during development. The respiratory and neural correlates of operant conditioning were first determined in normally reared *Lymnaea*. The same procedure was then applied to differentially reared *Lymnaea*, that is, animals that had never experienced aerial respiration during their development. The aim was to determine whether these animals would demonstrate the same responses to the training paradigm.

In normally reared animals, a behavioural reduction in aerial respiration was accompanied by numerous changes within the neural network. Specifically, I provide evidence of changes at the level of the respiratory central pattern generator and the motor output. In the differentially reared animals, there was little behavioural data to suggest learning and memory. There were, however, significant differences in the network parameters, similar to those observed in normally reared animals. This demonstrated an effect of operant conditioning on differentially reared animals. In this thesis, I have identified additional correlates of operant conditioning in normally reared animals and provide evidence of associative learning in differentially reared animals. I conclude plasticity is not dependent on previous experience, but is rather ontogenetically programmed within the neural network.

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In the name of God, Most Gracious, Most Merciful…

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Table of Contents **Pg**

List of Abbreviations

ANOVA	ANalysis Of VAriance
cAMP	Cyclic AMP
C/EBP	CCAAT/Enhancer Binding Protein
CNS	Central Nervous System
CPG	Central Pattern Generator
CREB	cAMP Response Element Binding protein
E	Experimental respiring
EJP	Excitatory Junction Potential
ENR	Experimental Non-Respiring
IP3	InPut 3 interneuron
MT	Memory Test
LTM	Long-Term Memory
N	Naïve respiring
NNR	Naïve Non-Respiring
TS	Training Session
RMP	Resting Membrane Potential
RPeD1	Right Pedal Dorsal 1
STNR	Straight-from-Tank Non-Respiring
VD4	Visceral Dorsal 4
VI	Visceral I
Y	Yoked respiring
YNR	Yoked Non-Respiring

List of Figures and Tables

INTRODUCTION

Animals demonstrate learning in a number of ways. Operant conditioning is a form of learning in which the animal forms an association between its behaviour and the consequence of that behaviour. Based on the experience, the animal is able to predict future outcomes and modifies its behaviour accordingly. A number of vertebrate (Wickens *et al.*, 2003) and invertebrate (e.g. Brembs, 2003) models have been used to study operant conditioning. The pond snail *Lymnaea stagnalis* has proven especially useful since its aerial respiratory behaviour can be operantly conditioned (Lukowiak *et al.*, 1996) and the underlying neural circuitry has been characterized (Syed and Winlow, 1991).

Lymnaea is a bimodal breather. When challenged with hypoxia, the animal supplements cutaneous respiration with aerial respiration, which involves migrating to the air-water interface and opening and closing its respiratory orifice, the pneumostome (Jones, 1961). Like most rhythmic behaviours, this aerial respiratory behaviour is controlled by a central pattern generator (CPG), the cellular components of which have been identified (Syed *et al.*, 1990). With operant conditioning, the animal learns to suppress hypoxia-induced ventilatory behaviour in response to a 'punishing' stimulus and, therefore, demonstrates a reduction in aerial respiratory behaviour (Lukowiak *et al.*, 1996).

With no apparent detriment to their health, *Lymnaea* can be reared from embryo to adulthood without ever performing aerial respiration. When allowed to do so, these animals spontaneously perform aerial respiration, demonstrating an ontogenetic development of the respiratory neural network and the resultant behaviour (Hermann and Bulloch, 1998). However, it is not yet known whether this behaviour is amenable to associative learning in these differentially reared animals. Can these animals modify a behaviour they have never experienced during their development? In other words, is plasticity activity- and/or experience-dependant?

In this study, the behavioural and neural correlates of operant conditioning were investigated in normally reared *Lymnaea*. The question of activity and experience-dependent plasticity was then addressed by performing the operant conditioning paradigm in the differentially reared animals. Finally, the mechanisms of associative learning are discussed with implications in development and adaptation to hypoxic stress.

BACKGROUND

Learning and Memory

Associative and Non-Associative Learning

While the phrase 'survival of the fittest' suggests the selection of beneficial anatomical and physiological characteristics over generations, learning is a survival mechanism that increases the probability of biological success within the life time of an organism. Indeed evolution has favored the development of learning and memory as a means of survival, in order for the organism to better adapt to its environment. Learning is defined as a change in behaviour as a consequence of experience, while memory is the retention and recollection of the learned behaviour.

In the taxonomy of learning, there are associative and non-associative forms of learning. Non-associative learning is considered the more primitive form in which the organism learns something about a repeatedly presented stimulus. Generally, an unexpected stimulus elicits an "orienting response", behavioural and physiological reactions meant to investigate the nature of the stimulus (Terry, 2003). Habituation is a decrease in the orienting response to a repeatedly presented stimulus while sensitization is an increase in the orienting response. Which form of learning is induced, generally depends on the type and intensity of the stimulus. For example, a person will habituate to monotonous office chatter but will become sensitized to all noise if they hear a scream.

If the stimulus happens to have significance to the animal, because it is followed by, or is temporally linked to another stimulus or event, then the orienting response may be replaced by conditioning. In associative learning two events, such as two stimuli or a stimulus and a response, become related such that one reinforces the occurrence of the other (Terry, 2003). Classical conditioning and operant conditioning are two such types of associative learning.

In classical conditioning a previously neutral stimulus is paired with a biologically relevant unconditioned stimulus, such that the response usually associated with the unconditioned stimulus is elicited by the presentation of the neutral stimulus alone. Behaviourally, the animal learns a new response to a stimulus. The most commonly discussed example is that of Pavlov's dog (Pavlov, 1927). One of the physiological responses to food is salivation. Pavlov presented the sounding of a bell immediately preceding the presentation of food and monitored salivation. Following a number of these bell-food trials, the dog began to salivate in response to the bell, even in the absence of food.

Edward Lee Thorndike (1898, 1911) is credited as the pioneer of instrumental conditioning, a form of associative learning very similar to operant conditioning. In his experiments, cats were placed in a wooden crate with a 'hidden' door-opening mechanism that would allow the cat to escape. Each time the cat tripped the mechanism and escaped the box, it was replaced back into the box to try again. In scientific terms, the discriminative stimulus (i.e. the box) would elicit an instrumental response (i.e. pulling a wire), which produced an outcome (i.e. escape) that reinforced the behaviour. Learning and memory was, thus, observed as an improvement in time required to escape the enclosure. Thorndike described this learning as trial-and-error based, whereby ineffective behaviours were gradually replaced with effective behaviours. In instrumental conditioning, the likelihood, form, and timings of these behaviours are modified by the consequences.

In 1938, B.F. Skinner described a more controlled experimental setup to condition animal behaviour. The Skinner box, as it is now called, allowed the precise control of the discriminative stimulus (e.g. presentation of a light), the operant response (e.g. pressing a lever), and the reinforcing stimuli (e.g. food pellet). Skinner coined the term 'operant' since the response *operated* on the *environment* to produce a consequence. Thus, in operant conditioning, the reinforcer strengthens the association between the discriminative stimulus and the operant response. It differs from instrumental conditioning in that the behaviour is continuous; when an animal subject is placed in the Skinner box, it is free to perform the response *ad libitum*. On the other hand, with instrumental conditioning, the subject is given separate occasions during which the behaviour and response can be elicited. None-the-less, instrumental conditioning and operant conditioning can be considered very similar and differ on the basis of who defined the training paradigms.

Operant conditioning is thought to be a higher form of learning than classical conditioning (Skinner, 1981). In classical conditioning, learning produces a reflexive response to unrelated stimuli. In operant conditioning, however, learning produces a change in motivational state such that the organism reflectively, and not reflexively, responds to the stimulus. It is influenced by a number of factors including the latency and intensity of the reinforcing stimulus, and its consistency and contingency. Also the reinforcement can be such that the occurrence of the operant response can increase or decrease. In punishment, the operant response is followed by the presentation of an aversive stimulus so that the animal learns to avoid the operant response. Thus, punishment produces a reduction in the occurrence of behaviour.

Molecular Memory

Having investigated the behavioural aspects, much of the recent research has focused on answering the question, "what are the molecular mechanisms of learning and memory?" What is memory? Where is it stored? And how is it stored?

In 1894, Santiago Ramon y Cajal proposed his theory of memory storage based on the observation that mature nerve cells do not replicate and divide. He suggested learning caused existing nerve cells to modify their connections to other nerve cells, so as to enhance communication between the two. Indeed a century of research has demonstrated that memory storage results from the changes in neurons that are components of the neural systems involved, and not neurogenesis or specialized 'memory cells'. Given the complexity of the intact organisms and the behaviour itself, neurobiologists initially adopted a reductionist approach to the study of memory including invertebrate preparations and mammalian isolated spinal cords and hippocampal slices (Milner *et al.*, 1998).

The cellular studies in the mollusc *Aplysia californica* by Kandel and the molecular genetic studies in the fruit fly *Drosophila melanogaster* by Benzer were fundamental to the understanding of molecular memory. Despite having only 20,000 and 300,000 neurons in the central nervous system (CNS), respectively, these invertebrate models systems demonstrate significant behavioural plasticity (Milner *et al.*, 1998). For example, *Aplysia* has been used to investigate the molecular correlates of many forms of learning including habituation (Bristol and Carew, 2005), sensitization (Khabour *et al.*, 2004), classical (Mozzachiodi *et al.*, 2003), and operant (Brembs *et al.*, 2004) conditioning. There are a number of reviews on the cellular mechanisms of learning and memory (Brembs, 2003; Carew and Sahley, 1986; Kandel, 2001; Milner *et al.*, 1998). Some of the cellular aspects of synaptic plasticity are discussed below, focusing on vertebrate and invertebrate models of operant conditioning.

A number of the molecular aspects of operant conditioning were deduced using *Drosophila* trained for odor avoidance and/or temperature preference (Drain *et al.*, 1991; Quinn *et al.*, 1974; Wustmann *et al.*, 1996). Initially, it was noted that some synaptic changes resulting from short-term behavioural modifications did not require protein synthesis and were mediated by second messenger systems (Glassman, 1969). Thus, cyclic AMP (cAMP)-mediated signaling in operant conditioning was hypothesized as a learning mechanism (Drain *et al.*, 1991; Dudai, *et al.*, 1976; Schwartz *et al.*, 1971). The signaling cascade results in the phosphorylation of the cAMP response element binding protein – 1 (CREB-1), a transcription factor that binds to response elements in the promoters of target genes that encode learning and memory (Brembs, 2003). Some of these target genes include activators of memory-enhancer genes (e.g. CREB-1),

repressors of memory-suppressor genes (e.g. CREB-2), and transcriptional regulators (e.g. the CCAAT/enhancer binding protein, C/EBP) (Kandel, 2001).

Given that operant conditioning alters the probability of the occurrence of behaviour, biophysical properties that govern the probability of cellular activity are potential mechanisms of synaptic plasticity. Operant conditioning of the feeding behaviour in *Aplysia* has revealed such changes in biophysical properties *in vitro* and *in vivo* (Brembs *et al.*, 2002; Nargeot *et al.*, 1997; Nargeot *et al.*, 1999a,b,c). Briefly, activity in B51, a pre-motor neuron, is associated with ingestion-like pattern activity and is active during food rewarding behaviour. B51 burst threshold was lower and the input resistance higher in operantly conditioned animals compared to controls (Brembs *et al.* 2002; Nargeot *et al.*, 1999a). Physiologically, these changes increase the likelihood of B51 becoming active, so in the presence of food there is increased ability to generate ingestion-like pattern activity (Carew, 2002). Changes in biophysical properties have also been reported in vertebrate models of operant conditioning (Carp *et al.*, 2001; Wickens *et al.*, 2003; Wolpaw, 1987; Wolpaw and Tennissen, 2001). The H-reflex is an electrical model of the spinal stretch reflex, a tendon jerk. It is mediated by a two-neuron pathway consisting of an afferent neuron and a motoneuron, and is influenced by spinal activity (Wolpaw, 1987). It was found that the motoneuron firing threshold was shifted positively and the axonal conduction velocity was reduced in animals operantly conditioned to reduce the H-reflex (Wolpaw, 1997).

Neuronal morphological changes also accompany learning and long-term memory (LTM) (Bailey and Kandel, 1993; Carew and Sahley, 1986). The most common of these are functional changes in the pre-existing synapses and the number of synapses. For example, Shankaranarayana Rao *et al.* (1999) demonstrated, using a self-stimulation rewarding paradigm, increases in the number of spines in the dendrites of hippocampal and motor cortical pyramidal neurons of rats. Functionally, this was hypothesized to enhance the synaptic efficacy of these neurons. Similarly, Mahajan and Desiraju (1988) reported an increase in the number of branching points of the dendrites and spine density in learned groups compared to controls. Taken together, there are a number of molecular, biophysical, and ultrastructural correlates of operant conditioning.

Aerial Respiration in *Lymnaea stagnalis*

Recently, the freshwater pond snail *Lymnaea stagnalis* has become a popular model for investigating the neural correlates and cellular mechanisms of operant conditioning (Lukowiak *et al.*, 1996). There are two main reasons for this. First and foremost, *Lymnaea* displays an easily-

observable and quantifiable behaviour that is amenable to operant conditioning. Secondly, there are excellent candidate sites within the CNS where the changes associated with operant conditioning are likely to be found. Specifically, the aerial respiratory behaviour of *Lymnaea* can be operantly conditioned and resultant neural changes are hypothesized to manifest within the neural network, the respiratory central pattern generator (CPG), regulating this behaviour.

Respiration is an essential homeostatic behaviour. Thus, the underlying neural network controlling respiration must be able to regulate the rate of respiration to meet metabolic demands, as a function of environmental conditions. For example, hypoxia generally produces a hypoxic ventilatory response, in which the depth and/or rate of respiration increases. *Lymnaea* is fundamentally different from other molluscan model systems (like *Aplysia*) in that it is classified as a pulmonate mollusc (Harris, 2003). In pulmonate molluscs, respiration occurs partially across the somatic epidermis (cutaneous respiration) but also via a primitive lung (aerial respiration) (Syed *et al.* 1991). At the air-water interface, respiratory gases are expelled from the lung through the respiratory orifice, the pneumostome (Fig. 1), by forceful contraction of the mantle muscles. Inspiration follows and occurs by a passive process in which air is drawn into the lung cavity by negative pressure. A typical pneumostome opening can last anywhere from seconds to minutes and an animal can open and close its pneumostome a number of times before it re-submerges into the water (Jones, 1961).

The respiratory behaviour of *Lymnaea* has been extensively studied, initially to determine the function of its respiratory pigment, haemocyanin (Dawson and Wood, 1982, 1983; Jones, 1961). In 1961, Jones demonstrated that aerial respiratory behaviour in *Lymnaea* was inversely related to the level of dissolved oxygen in the water (partial pressure of oxygen, pO_2). It was also noted that oxygen consumption was relatively constant over a wide range of dissolved oxygen levels due to increased aerial respiration at low water pO_2 and reduced aerial respiration at high water pO_2. However, even in well-aerated pond conditions, *Lymnaea* will continue to perform aerial respiration, albeit with less frequency.

Respiratory Central Pattern Generator

Aerial respiration in *Lymnaea* is controlled by a well-defined respiratory CPG (Fig. 2a) (Syed *et al.*, 1990; Syed and Winlow, 1991). CPGs are neural networks that can produce rhythmic, patterned outputs in the absence of extrinsic inputs (Syed and Winlow, 1991). CPGs control a number of rhythmic behaviours such as respiration, locomotion, swimming, and feeding, in invertebrates and vertebrates (Marder and Rehm, 2005). This particular respiratory

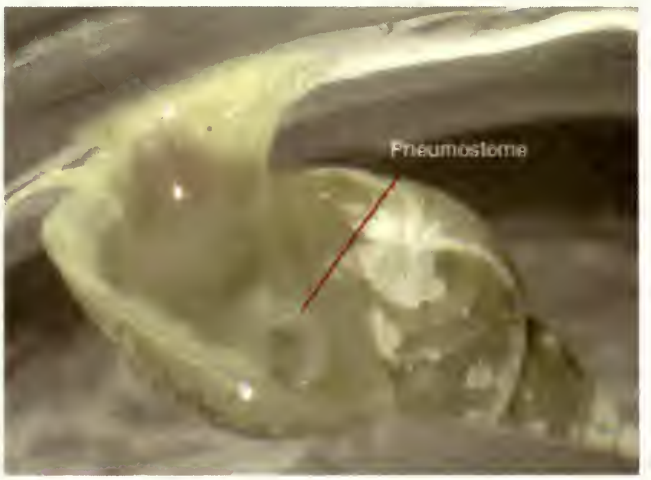
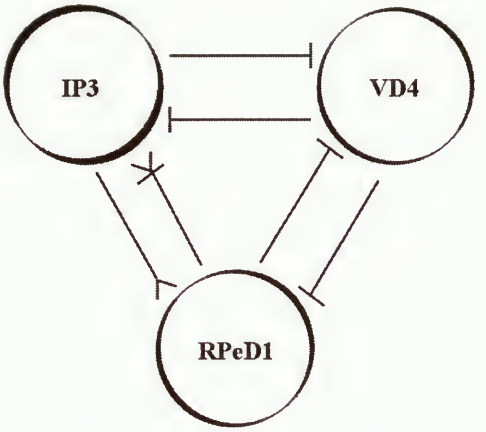


Figure 1: *Lymnaea* performing aerial respiration. At the air-water interface, *Lymnaea* opens and closes its respiratory orifice, the pneumostome, to exchange respiratory gases.

A)



B)

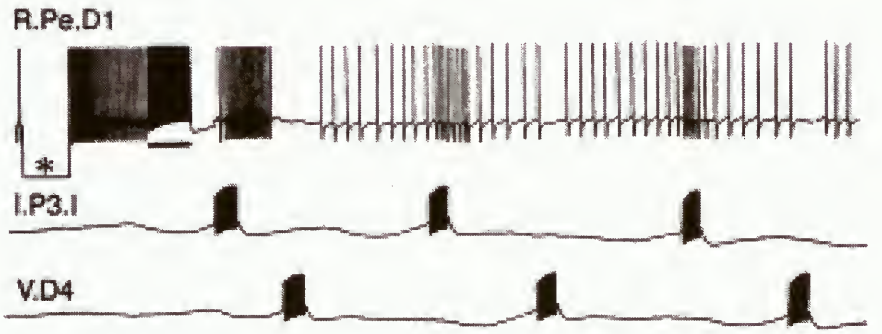


Figure 2: Respiratory CPG of *Lymnaea stagnalis*. **(A)** Diagram of respiratory CPG. Activity in RPeD1 initiates the respiratory rhythm. IP3 activity results in pneumostome openings while activity in VD4 results in pneumostome closing. Excitatory connections are represented as open triangles and inhibitory connections are represented as short bars. **(B)** Sample electrophysiological recordings of CPG neurons in cell culture (Syed *et al.* 1990). The cyclical respiratory rhythm is maintained by the reciprocal inhibitory activity of IP3 and VD4.

CPG consists of three monosynaptically connected interneurons located within the central ganglionic ring. The three cells are: Right Pedal Dorsal 1 (RPeD1), Input 3 (IP3) interneuron, and Visceral Dorsal 4 (VD4). The sufficiency and necessity of this three-cell CPG in generating the coordinated patterned firing activity has been demonstrated in isolated brain preparations and also in cell culture (Fig. 2b) (Syed *et al.*, 1990, 1991; Syed and Winlow, 1991).

RPeD1 is a large (100-120 μm diameter) neuron that lies on the posterior, dorsal surface of the right pedal ganglion, medial to the statocyst. Chemosensory information concerning pO_2 is thought to be relayed to RPeD1 and there are two lines of evidence for this. Peripheral projections from RPeD1 ramify the blood vessels supplying the anterior part of the lung roof (Syed and Winlow, 1991). Thus, it is hypothesized that RPeD1 may be sensitive to changes in the haemolymph pO_2. Also, RPeD1 firing frequency increases when the pneumostome and osphradial area, the molluscan olfactory organ, are made hypoxic (Inoue et al., 2001; Wedemeyer and Schild, 1995). However, this increased firing activity is not observed when the CNS alone is made hypoxic (Inoue *et al.*, 2001). Taken together, low blood and/or water pO_2 induce patterned impulse activity in RPeD1.

IP3 and VD4 are the other two respiratory CPG neurons. IP3 activity produces a pneumostome opening. It is located deep in the lateral, ventral surface of the parietal ganglia and forms monosynaptic, excitatory connections with the pneumostome opener motor neurons, the visceral I and J (VI/J) cells. Action potentials in these motor neurons evoke 1:1 excitatory junction potentials (EJPs) in the pneumostome opener muscles (Syed *et al.*, 1991). VD4, on the other hand, produces a pneumostome closing. It is located on the anterior, dorsal surface of the visceral ganglion and forms excitatory, monosynaptic connections with the pneumostome closer motor neurons, the visceral K (VK) cells. Impulse activity in the VK cells produces EJPs in the muscle fibres controlling pneumostome closing (Syed *et al.*, 1991).

The respiratory CPG cycle is initiated by hypoxia-induced patterned activity in RPeD1 (Inoue *et al.*, 2001). RPeD1 activity excites IP3 and simultaneously inhibits VD4 (Syed et al., 1990). IP3 activity in turn excites RPeD1 while inhibiting VD4, producing a pneumostome opening (Syed and Winlow, 1991). Upon release from inhibition, VD4 activity inhibits both RPeD1 and IP3, and produces a pneumostome closing (Syed and Winlow, 1991). The cyclical CPG rhythm is maintained by the reciprocal inhibitory activity of IP3 and VD4 (Fig. 2b).

Operant Conditioning of Lymnaea's Aerial Respiratory Behaviour

Lymnaea's aerial (i.e. pulmonary) respiratory behaviour can be operantly conditioned to demonstrate long-term memory (LTM) (Lukowiak *et al.*, 1996, 1998). As mentioned previously,

a punishment paradigm can be used to reduce the occurrence of an operant response. Operant conditioning of the aerial respiratory of *Lymnaea* is a punishment-based training paradigm. The animals form an association between aerial respiration and an aversive stimulus, which acts to decrease aerial respiration.

The original training paradigm employed by Lukowiak (1996) was designed to punish aerial respiration, the operant response, when the animals were challenged with hypoxia. The experiment consisted of five sessions with three phases each: a pre-test, a training period, and a post-test. Both the pre- and post-test were free observation periods, meant to quantify respiratory parameters. During the training period the snails received a tactile stimulus to the pneumostome area with every attempt to perform aerial respiration. This resulted in the immediate closure of the pneumostome. The amount of force required to cause pneumostome closure is non-damaging to the musculature. Also, Jones (1961) has previously shown cutaneous respiration is sufficient for maintaining blood pO_2. Thus, animals do not incur any injury by the use of this procedure.

Two control groups were used in the training paradigm. Naïve animals were allowed to freely perform aerial respiration during the training sessions and received no tactile stimulation. This was done to control for the effects of intermittent hypoxia on metabolism and respiration. Yoked animals were also allowed to perform aerial respiration but received tactile stimulation, not contingent to pneumostome openings, to control for the physiological response to the tactile stimulus itself. Learning and memory was quantified using a number of respiratory parameters. Learned animals demonstrated a significant reduction in the number of attempted pneumostome openings in the training sessions as conditioning progressed. These animals also demonstrated a significant reduction in both the number of pneumostome openings and total breathing time in the post-test phase compared to the pre-test phase. In other words, the hypoxic ventilatory response was reduced in these animals compared to naïve and yoked controls following training. Hence, operant conditioning of *Lymnaea* results in behavioural suppression of aerial respiration.

Molecular and Neural Correlates of Operant Conditioning in Lymnaea

The transcriptional regulators CREB-1 and CREB-2 have recently been identified in *Lymnaea* (Sadamoto *et al.*, 2004a,b). The same group has also identified the *Lymnaea* C/EBP homolog, which is known to activate transcription of downstream genes that give rise to the growth of new synaptic connections (Hatakeyama *et al.*, 2004a,b; Kandel, 2001). Thus, mechanisms of memory storage are likely conserved in *Lymnaea*. However, the roles of CREB and C/EBP as molecular substrates of memory have not been demonstrated using the operant conditioning paradigm. Gene expression and protein synthesis is none-the-less important in LTM

formation and has been investigated in *Lymnaea*. Animals injected with transcription and translation blockers did not reduce their aerial respiratory behaviour as a consequence of operant conditioning (Sangha *et al.*, 2003). Specifically, expression of new genes from the soma of RPeD1 is required for LTM formation (Scheibenstock *et al.*, 2002). Also, Lowe (2004) and Lowe and Spencer (2006) have recently demonstrated that LTM formation was augmented by silencing RPeD1 activity in between training sessions, presumably due to altered gene expression induced by experimental hyperpolarization.

Spencer *et al.* (1999; 2002) demonstrated significant changes in the respiratory CPG in animals previously trained for LTM. In the isolated CNS, RPeD1 was quiescent more often in ganglia derived from trained animals compared to yoked controls (Spencer *et al.*, 1999). Also, there was a reduction in spontaneous IP3 activity, which was reflective of the reduction in pneumostome openings in the intact conditioned animals. Finally, the authors provided evidence of a change in synaptic plasticity between RPeD1 and IP3. In the semi-intact preparation, retention of memory was evident as a reduction in pneumostome activity (Spencer *et al.*, 2002). Furthermore, RPeD1 activity was reduced following the application of the reinforcing stimulus in conditioned animals compared to controls. However, as the authors pointed out, not all experimental animals demonstrated these neural changes. Thus, other network parameters are likely to be affected by learning and memory. It also remains to be established whether or not other experimental conditions, such as development and rearing environment, can produce similar changes in behaviour and neural activity, revealing conserved mechanisms of plasticity.

Nature versus Nurture

The Role of Experience in Development

The balance between genetic disposition and phenotypic flexibility is important in the development of an organism. This is especially true in the development of the vertebrate and the invertebrate nervous system. While the architecture is determined by genetic factors, activity plays an important role in the refinement of neural networks (Harris-Warrick and Marder, 1991; Lnenicka and Murphey, 1989).

Pioneering experiments by Wiesel and Hubel (1963a,b,c) demonstrated the importance of visual experience in the development of the vertebrate visual system. Experiments in rats and kittens have revealed several changes in the visual system following monocular and binocular deprivation during development, including differences in the number and morphology of receptive cells, the cortical receptive field size, visual acuity, and response latency to visual

stimuli (Pizzorusso *et al.*, 2000). Zebra finch males also demonstrate activity-dependent plasticity in the development of song. During early development, auditory experience is necessary for juvenile males to imitate the song of an adult male. Experimentally deaf finches are not capable of controlling vocal behaviour since it is dependent on auditory feedback from specialized structures within the CNS like the anterior neostriatum. Song errors are detected by the anterior neostriatum and results in synaptic remodeling until the song matches the adult finches (Bottjer, 2002). In the *Drosophila* invertebrate model, dark rearing affects the size of the optic lobes but has no effect on the higher vertical system of motion sensing neurons of the visual system (Barth *et al.*, 1997; Scott *et al.*, 2003). Electrophysiology of the photoreceptors indicates a greater membrane resistance in dark reared flies so that, behaviourally, the dark reared *Drosophila* respond to changes in light contrast slower than light reared *Drosophila* (Wolfram and Juusola, 2004). Also affected is the preference for visual patterns and colors (Hirsch and Tompkins, 1994).

Mechanistically, factors involved in intracellular (e.g. kinase signaling) and synaptic (e.g. N-methyl-D-aspartate receptor, NMDAR) signaling are implicated in experience-dependent neural refinement of the underlying networks (Berardi *et al.*, 2003; Bottjer, 2002). The changes in physiology and the resultant behaviour are permanent and the incurred adaptations are irreversible if the animals are not exposed to sensory input within a critical period. In the visual system during monocular deprivation, cortical representation is permanently lost in the deprived eye (Pizzorusso *et al.*, 2000). Furthermore, if the deprived eye is allowed to open following the critical period, binocular vision remains permanently affected and vision in the 'good' eye deteriorates to poor levels. Similarly in the finch, song cannot be further refined and new syllables cannot be learned if they are restricted from auditory input beyond a critical period, which ends at around the age of 90 days (Bottjer, 2002). In the *Drosophila* model, even short-term exposure to light during the critical period can reverse many of the physiological and behavioural changes associated with sensory deprivation (Wolfram and Juusola, 2004). Thus, sensory deprivation and differential rearing can permanently affect activity and experience-dependent plasticity.

Developmental Plasticity of Respiratory Behaviour in Lymnaea

Hermann and Bulloch (1998) have previously shown that *Lymnaea* can be raised from eggs to adulthood without ever experiencing aerial respiration. Aside from being slightly smaller than normally reared snails, these differentially reared snails appear normal regarding locomotion, reproduction, feeding, and other physiological processes. When allowed to do so,

these differentially reared *Lymnaea* will surface and perform aerial respiration in a manner similar to normally reared *Lymnaea*. Thus, regarding respiration, cutaneous respiration is sufficient to meet the metabolic demands of these animals and maintain homeostasis in normoxic pond water conditions. More interestingly, the aerial respiratory behaviour of *Lymnaea* is genetically programmed and is activity and experience-independent.

There are, however, qualitative and quantitative differences in aerial respiration between differentially reared and normally reared snails. Differentially reared snails perform significantly less aerial respiration in normoxic water conditions compared to normally reared snails. Differences were reported in both the number of pneumostome openings and total breathing time during the observation sessions (Hermann and Bulloch, 1998). In hypoxic water conditions, the quantitative differences in respiration were exaggerated since normally reared snails demonstrated the hypoxic ventilatory response and the differentially reared snails did not (Hermann and Bulloch, 1998). Although there are adaptive processes regulating respiration, the authors concluded that most of these differences were mainly due to experience-independent development of the underlying neural network.

Thesis Objectives

Overall Objectives

With no known detriment to their health, *Lymnaea* can be raised in an environment in which they are prevented from rising to the water's surface to perform aerial respiration. Hermann and Bulloch (1998) have previously demonstrated that aerial respiratory behaviour in *Lymnaea* develops independent of experience. However, it has not yet been determined whether the ability of the CPG to change is independent of previous behaviour. The aim of this study was to investigate the plasticity of the respiratory CPG in differentially reared animals. I sought to determine if higher-order plasticity (i.e. associative learning) is dependent on experience during development. I hypothesized there would exist limitations in the adaptive properties of the respiratory CPG in non-respiring animals due to inexperience, as is observed in other vertebrate and invertebrate models of sensory deprivation and differential rearing.

To my knowledge, *Lymnaea* is the only example of differential rearing in which an important homeostatic behaviour can be eliminated from the development of the animal. This offers a unique model to investigate the effects of activity and experience on the development and function of the neural network controlling respiration, the plasticity of the network, and the resultant plasticity in respiratory behaviour. The work can be extended to vertebrate models and

has implications in the physiologic effects of environmental hypoxia on development and the ventilatory response. The advantage of this invertebrate preparation, however, is the ability to investigate at the level of single identified cells, the molecular and cellular properties underlying neuronal plasticity. Experiments can be performed on the intact animal, the semi-intact preparation, and also in the isolated brain. Whole animal experiments provide information on the behavioural aspects of operant conditioning while the semi-intact preparation permits the correlation of behavioural and cellular activity from defined neurons. In the isolated brain, the same cellular activity can be examined in the absence of peripheral sensory input from the pneumostome and osphradial area to further investigate the CPG network properties. Such studies are not yet possible in vertebrates.

Specific Objectives

Aerial respiration can be operantly conditioned in normally reared *Lymnaea* and this plasticity in behaviour is a result of plasticity in the underlying neural network (Lukowiak *et al.* 1996; Spencer *et al.*, 1999, 2002). To ascertain the role of activity and experience in behavioural and neural plasticity, the same operant conditioning paradigm will be applied to non-respiring, differentially reared *Lymnaea.* In this thesis, I compare aerial respiratory behaviour and CPG activity in normally reared, respiring *Lymnaea* and differentially reared, non-respiring *Lymnaea.* I will also determine (in respiring *Lymnaea*) where neuronal changes that underlie operant conditioning occur in the respiratory CPG. Finally, I will ascertain the behavioural and neural plasticity of non-respiring *Lymnaea* using an operant conditioning paradigm.

MATERIALS AND METHODS

Specimens

Normally Reared Lymnaea

Normally reared animals will also be termed 'respiring' throughout the thesis, indicating the ability to perform aerial respiration during development. Specimens of *Lymnaea stagnalis*, originally derived from the stocks of the Vrije University in Amsterdam, were laboratory bred and maintained in well-aerated, artificial pond water (Instant Ocean; Aquarium Systems, Ohio, USA). The breeding containers were open to the atmosphere and *Lymnaea* were freely able to perform aerial respiration. The snails were kept on a light:dark cycle of 12:12 hr and a diet consisting of Spirulina algae flake food (Nutrafin Max; Rolf C. Hagen Inc., Quebec, Canada), lettuce, and carrots. All snails used for training and electrophysiology were between 20 and 25 mm in shell length, corresponding to an age of 3-6 months.

Differentially Reared Lymnaea

Differentially reared animals will also be termed 'non-respiring' throughout the thesis, indicating the inability to perform aerial respiration during development. Clear, plastic breeding containers with fine mesh walls were submerged in well-aerated, artificial pond water aquariums (Fig. 3). Special care was taken to ensure no air bubbles were trapped in the enclosures. A single egg sack was hatched under each enclosure and snails were raised to adulthood without ever experiencing aerial respiration. The snails were kept on the same light:dark cycle and the same diet as normally reared *Lymnaea*. These snails were age-matched to normally reared *Lymnaea* but as reported previously, were slightly smaller in size (Hermann and Bulloch, 1998). All animals used for training and electrophysiology were between 18 and 25 mm in shell length.

Procedures

Operant Conditioning

Snails were selected and randomly assigned to one of three groups: naïve, yoked, or experimental. During the training sessions and the memory test, naïve *Lymnaea* were allowed to freely perform aerial respiration. The experimental, operant conditioning group received a punishing tactile stimulus that was contingent on the animal opening its pneumostome at the air-water interface to perform aerial respiration (Fig. 4a). The stimulus was of sufficient intensity to

Figure 3: Enclosure used to differentially rear *Lymnaea* to adulthood. Special care was taken to ensure no air bubbles were trapped in the submerged enclosures. There is no known detriment to the animals' health in preventing aerial respiration; the aquarium was kept well-aerated and the walls of the container consisted of mesh to permit water flow and oxygen diffusion.

A)

B)

Figure 4: Operant conditioning of *Lymnaea's* aerial respiratory behaviour. **(A)** In this paradigm, aerial respiration was punished by the application of an aversive stimulus to the open pneumostome. As training progressed, the animal demonstrated fewer attempts to perform aerial respiration. **(B)** Schematic of training paradigm. Prior to each session, 100% nitrogen gas was bubbled in the beaker for ten minutes to increase the animals' aerial respiratory drive.
(Pre-Obs = pre-observation session, TS = training session, MT = memory test, Post-Obs = post-observation session)

cause immediate pneumostome closure. Yoked animals also received the tactile stimuli but this was not contingent on their own pneumostome opening. Rather, it was contingent on the experimental animal to which it was yoked. Table 1 summarizes the various groups and the abbreviations used to describe them.

Snails were individually identified by a series of markings applied to their shells and placed in an 800 mL beaker with well-aerated artificial pond water. The snails were given ten minutes to habituate and explore the new environment. The beaker was then capped with a perforated barrier and topped off with artificial pond water, thereby preventing subsequent aerial respiration. All animals were subjected to hypoxic stress by bubbling 100% nitrogen gas in the water for ten minutes prior to and for the duration of each session. Generally, there was a 10-fold reduction in oxygen content after the ten minute period, as recorded using a Thermo Orion dissolved oxygen meter (Model 835A; Thermo Electron Corporation, Massachusetts, USA). This was done in order to increase the animals' drive to perform aerial respiration. 200-300 mL of water were siphoned at which point the snails entered the 0.5-hour pre-observation session, in which all animals were allowed to freely perform aerial respiration to determine the number of pneumostome openings and the total breathing time. After the pre-observation session, air was bubbled into the water and the beaker was capped and filled with water. After one hour, nitrogen was again bubbled into the water for ten minutes, the water level was lowered, and the snails entered the first of four 0.5-hour training sessions. The number of attempted pneumostome openings was recorded for the experimental *Lymnaea* while the number of pneumostome openings and total breathing time was determined for both naïve and yoked control groups. Each training session was separated by one hour to allow consolidation of memory (Lukowiak et al., 2000). The beaker was capped and filled with water between each training session. 18 hours after the final training session, the snails entered the memory test, which was procedurally similar to the training sessions. One hour following the memory test, the snails entered the post-observation period in which all of the snails were again allowed to freely perform aerial respiration. A schematic of the training paradigm is illustrated in Figure 4b. The number of pneumostome openings and total breathing time was determined for all animals.

Dissection of Semi-Intact Preparations

A similar approach to that of Lowe (2004) and Lowe and Spencer (2006) was used to dissect the semi-intact preparations (Fig. 5a). Briefly, snails were anaesthetized in a *Lymnaea* saline solution [composition in mM: 51.3 NaCl, 1.7 KCl, 4.1 $CaCl_2 \cdot 2\ H_2O$; 1.5 $MgCl_2 \cdot 6\ H_2O$,

Group	Abbreviation	Development	Behaviour During Training
Naïve respiring	N	Normally Reared	freely allowed to perform aerial respiration
Yoked respiring	Y	Normally Reared	receive tactile stimulus but not contingent on pneumostome opening
Experimental respiring	E	Normal Reared	receive tactile stimulus contingent on pneumostome opening
Naïve Non-Respiring	NNR	Differentially Reared	freely allowed to perform aerial respiration
Straight-from-Tank Non-Respiring	STNR	Differentially Reared	N/A; dissected straight from rearing tanks into semi-intact preparation without exposure to air
Yoked Non-Respiring	YNR	Differentially Reared	receive tactile stimulus but not contingent on pneumostome opening
Experimental Non-Respiring	ENR	Differentially Reared	receive tactile stimulus contingent on pneumostome opening

Table 1: Terminology given to animals used in this study. The term 'respiring' refers to the normally reared animals that were allowed to perform aerial respiration during development. The term 'non-respiring' refers to the differentially reared animals that were restricted from performing aerial respiration during development. The terms do not imply anything about the behavioural performance of the intact animals or semi-intact preparations during operant conditioning.

A)

B)

Figure 5: Semi-intact and isolated brain preparation of *Lymnaea stagnalis*. (**A**) Compared to previous studies, the semi-intact preparations used in these experiments maintain peripheral input from the anterior and posterior of the body, as well as the pneumostome/osphradial area. **(B)** Central ring ganglia showing RPeD1 and VI. The two cells are morphologically distinct and can be impaled accurately and reliably for electrophysiological recordings.

5 mM HEPES buffer, pH to 7.9 with NaOH] containing 30% Listerine (Pfizer Canada Inc., Toronto, Canada) for three minutes. The anaesthetic agent in Listerine is menthol and its use does not affect learning or memory (Spencer *et al.*, 2002). The outer shell was removed and the body was pinned dorsal side-up. The pneumostome was propped on a small piece of sylgard to visualize pneumostome openings. A medial incision from the base of the mantle to the head was made to expose the inner cavity. The esophagus and the reproductive organs were excised and sylgard was positioned under the CNS. The commissure linking the left and right pedal ganglia was severed and the CNS was pinned to the sylgard. The preparations were given 20-30 minutes to recover from surgery prior to electrophysiological and behavioural analysis.

Dissection of Isolated Central Nervous System Preparation

Following the semi-intact recordings, the electrodes were removed from the cells and all remaining nerve bundles to the head and body were severed. The electrodes were repositioned to record RPeD1 and VI activity. The preparation was given five minutes to recover from damage-induced firing activity prior to further electrophysiological analysis.

Electrophysiology

Intracellular recordings were simultaneously obtained from RPeD1 and the VI cell (Fig. 5b) using standard electrophysiological techniques (Spencer *et al.*, 1999, 2002). RPeD1 initiates the CPG rhythm and VI innervates the pneumostome opener muscles. IP3 activity can be monitored indirectly as distinct bursts in the VI cell. Cell penetration was aided by proteolytic enzymatic treatment (Protease, Type IX; Sigma-Aldrich Co., Missouri, USA) over the surface of the right pedal ganglion and the visceral ganglion. Glass microelectrodes with a resistance of 20-60 MΩ were pulled on a Kopf electrode puller (Model 730; David Kopf Instruments, California, USA) and back filled with saturated K_2SO_4. Signals were obtained using a Neuro Data IR283A amplifier (Cygnus Technology, Inc., Pennsylvania, USA) connected to a PowerLab/4SP digital acquisition system (ADInstrumnets, Inc., Colorado, USA) and Chart recording software (v4.2; ADInstrumnets, Inc., Colorado, USA).

Data and Statistical Analysis

In the intact animal, the respiratory parameters were quantified across all sessions. A two-way repeated measures analysis of variance (RM-ANOVA) was carried out to test for a possible interaction effect between the two variables (i.e. the pre-/post-observation sessions and treatment group), followed by a Bonferroni's corrected *post hoc* analysis to reveal the differences

between and within the groups. Within group differences were also identified using a paired t-test or a one-way RM-ANOVA followed by a Bonferroni *post hoc* analysis.

In the semi-intact preparation, all parameters were quantified over the five-minute pre-test and post-test sessions. Data are presented as the pre-test values for any given parameter as well as the change in parameter value from pre-test to post-test.

Change in parameter from pre-test to post-test

= (post-test value) – (pre-test value)

In the isolated brain preparation, the resting membrane potential was determined immediately prior to removing the electrodes from RPeD1 and VI. All remaining parameters were determined for the final five minutes of a ten-minute recording. Significance was established using a Bonferroni corrected one-way ANOVA.

Results were considered significantly different if a P value of less than 0.05 was achieved. All data analyses were carried out using GraphPad Prism (v3.0; GraphPad Software Inc., California, USA). In all figures, the error bars represent the standard error of the mean.

RESULTS

The overall goal of this study was to determine if neural and behavioural plasticity is dependent on previous experience. *Lymnaea stagnalis* reared under normal conditions are able to modify their aerial respiratory behaviour following associative learning and demonstrate plasticity in their CNS. It is not yet known whether differentially reared *Lymnaea*, animals prevented from performing aerial respiration during development, are capable of this higher order learning and plasticity. This was addressed by using an operant conditioning paradigm to assess learning and memory in differentially reared *Lymnaea*.

Aerial Respiration in the Intact *Lymnaea*

I first sought to determine the differences in aerial respiration of normally reared, 'respiring' *Lymnaea* and differentially reared, 'non-respiring' *Lymnaea* in my experimental setup. Aerial respiration was first quantified to establish baseline respiratory behaviour under hypoxic conditions. I then applied the operant conditioning paradigm to determine whether or not training could reduce aerial respiratory behaviour in differentially reared *Lymnaea*, as it does in normally reared *Lymnaea* (Lukowiak *et al.*, 1996).

The aerial respiratory behaviour of normally reared *Lymnaea stagnalis* can be operantly conditioned to demonstrate LTM (Lukowiak *et al.*, 1996). In the past, this was accomplished by giving the intact animals four 0.5-hour training sessions over the span of two days followed by a memory test on day three. In between training sessions, the animals were allowed to freely open their pneumostome to perform aerial respiration. However, for this study, four training sessions were given in one day with the memory test 18-hours after the final session. Also, the animals were kept submerged under water in between training sessions by means of a perforated barrier, and were thus unable to surface to perform aerial respiration. Therefore, in this paradigm, the discriminative stimulus was aerial respiration and not the application of the first contingent poke signifying the beginning of each training session. The first task was to determine whether or not the animals could be operantly conditioned with this modified training paradigm.

Within the respiring and non-respiring *Lymnaea*, three groups of animals were used in this study. Naïve animals were allowed to freely perform aerial respiration. Operantly conditioned, experimental animals were 'punished' by contingent application of a tactile stimulus to every pneumostome opening. Yoked animals also received a tactile stimulus, but this was not contingent on a pneumostome opening. Each animal received a pre- and post-

observation session in which the animals could freely perform aerial respiration. LTM was operationally defined as a significant reduction in the number of pneumostome openings and total breathing time in the post-observation session compared to the pre-observation session.

Analysis of Aerial Respiration in Naïve Lymnaea

All animals were subjected to hypoxic stress by bubbling nitrogen gas in the water. I first investigated the respiratory response of the naïve respiring and non-respiring animals to hypoxic artificial pond water. Analysis of the respiratory parameters indicated a significant difference in the response between the two groups (Fig. 6). Naïve respiring animals raised in normal, open-environment conditions performed aerial respiration more often in the pre-observation session than their naïve counterparts raised in an enclosed environment ($n = 16$, paired t-test, $P < 0.001$). Naïve respiring animals also performed aerial respiration for a longer duration in the pre-observation session compared to the naïve non-respiring animals ($n = 16$, paired t-test, $P < 0.001$). This behaviour was consistent across the four training sessions and the memory test, and did not change from the pre- to the post-observation session for either group. Taken together, non-respiring animals performed aerial respiration significantly less than respiring animals in hypoxic conditions.

Effects of Operant Conditioning on Aerial Respiration

I next aimed to determine how the ability to modify aerial respiratory behaviour was affected by rearing conditions. For these experiments, I used the yoked and experimental groups of animals. Similar to the naïve animals, respiring and non-respiring animals demonstrated significant differences in their respiratory behaviour in the pre- observation session (Fig. 7). That is, respiring animals performed aerial respiration more often (one-way ANOVA, $F(60,3) = 18.89$, $P < 0.0001$) and for a longer duration (one-way ANOVA, $F(60,3) = 31.42$, $P < 0.0001$) than their non-respiring counterparts in the pre-observation session. A two-way repeated measures ANOVA of the number of pneumostome openings [$F(150,5) = 4.93$, $P = 0.0003$] and the total breathing time [$F(150,5) = 12.38$, $P < 0.0001$] revealed a significant interaction between the different groups and the conditioning stimulus. A Bonferroni *post hoc* test indicated that only the experimental respiring animals showed a significant reduction in the number of pneumostome openings ($P < 0.01$) and total breathing time ($P < 0.001$) from the pre- to the post-observation session. Thus, there was an effect of the operant conditioning paradigm on the respiratory behaviour of experimental respiring animals only (Fig. 7). As such, these experimental respiring animals suppressed the hypoxic ventilatory response despite a hypoxia-

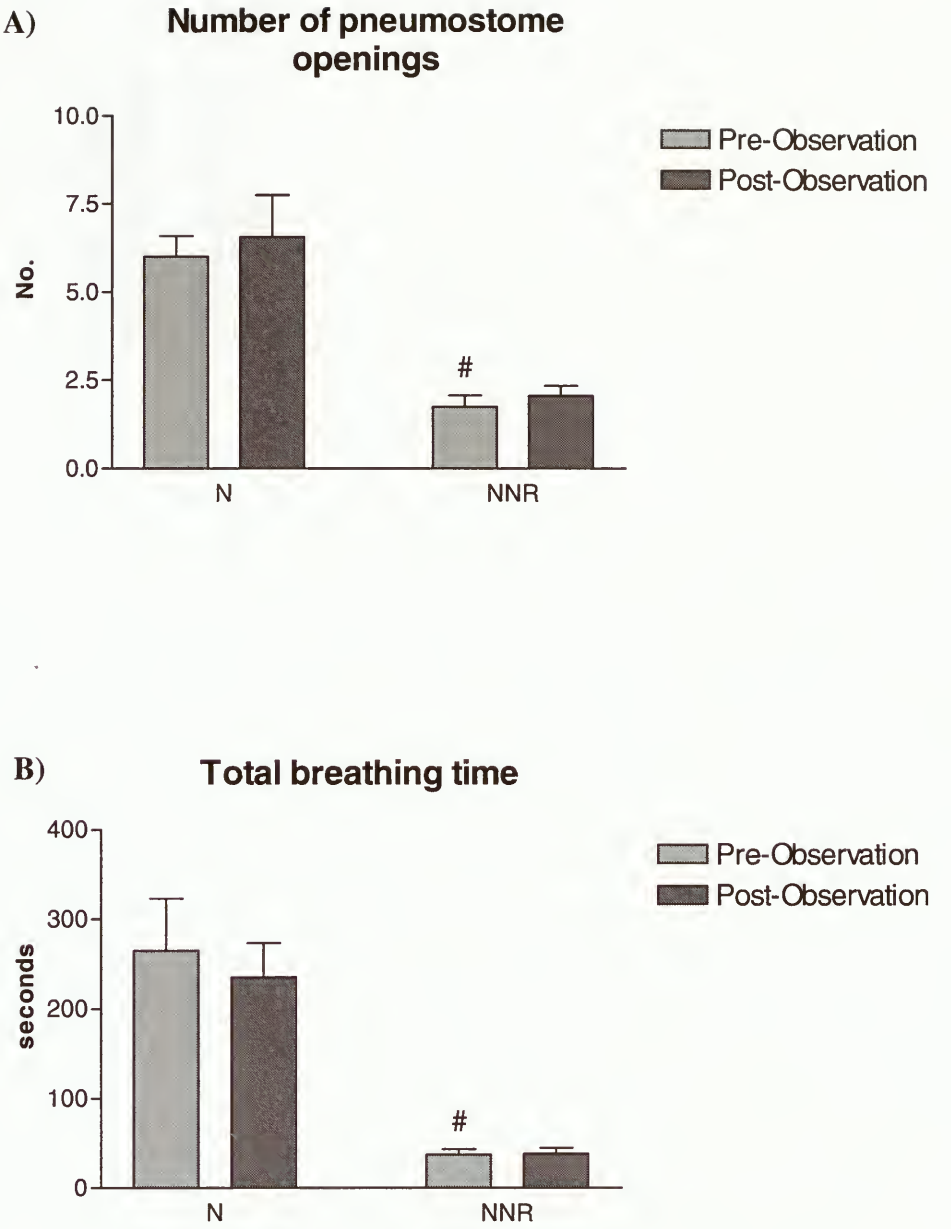


Figure 6: Aerial respiratory behaviour of the intact naïve controls in the pre- and post-observation session. **(A)** The naïve non-respiring animals performed aerial respiration significantly less often [number of pneumostome openings in the pre-observation session: naïve respiring (N), 6.0 ± 0.6, naïve non-respiring (NNR), 1.8 ± 0.3] and **(B)** for a shorter duration [total breathing time (seconds) in the pre-observation session: naïve respiring, 265 ± 58, naïve non-respiring, 37 ± 6] compared to naïve respiring controls (indicated by '#'). However, there were no differences in aerial respiration from the pre- to post-observation session for either group.
(number of animals = 16 per group) (#, $P < 0.001$)

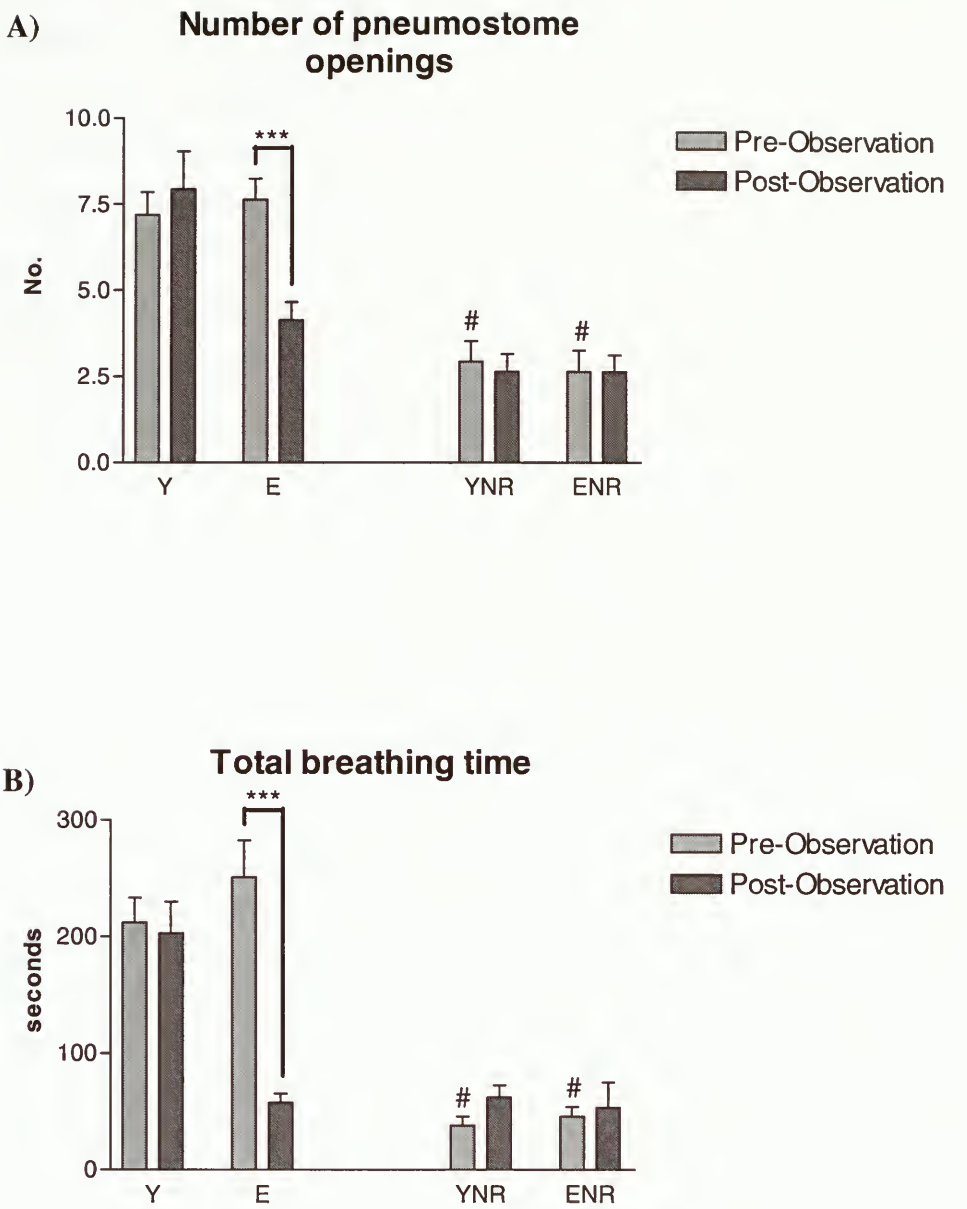


Figure 7: Aerial respiratory behaviour of the operantly conditioned intact animals and their yoked counterparts in the pre- and post-observation sessions. **(A)** In the pre-observation sessions, yoked and experimental respiring animals performed aerial respiration significantly more often than yoked and experimental non-respiring animals (indicated by '#') [number of pneumostome openings in the pre-observation session: yoked respiring (Y), 7.2 ± 0.7, experimental respiring (E), 7.6 ± 0.6, yoked non-respiring (YNR), 2.9 ± 0.6, experimental non-respiring (ENR), 2.6 ± 0.6]. **(B)** Yoked and experimental respiring animals performed aerial respiration for a longer duration compared to yoked and experimental non-respiring animals (indicated by '#') [total breathing time (seconds) in the pre-observation session: yoked respiring, 212 ± 21, experimental respiring 251 ± 31, yoked non-respiring, 37 ± 6, experimental non-respiring, 46 ± 8]. Training produced a significant reduction in both the number of pneumostome openings and the total breathing time in experimental respiring animals, but not in experimental non-respiring animals.
(number of animals = 16 per group) (*** = within group, $P < 0.001$; # = between groups, $P < 0.001$)

induced drive to perform aerial respiration. That is, since there were no differences in the yoked respiring group of animals, it is evident that the (non-contingent) stimulus itself did not cause a reduction in aerial respiratory behaviour.

This associative learning was further monitored by the construction of a learning curve (Fig. 8). Learning has been operationally defined in this model as the significant reduction in number of attempted pneumostome openings from training session 1 (TS1) to training session 4 (TS4) (Spencer *et al.*, 1999, 2002). If learning occurred, then LTM was defined as the significant reduction in attempted pneumostome openings from TS1 to the memory test (MT). The experimental respiring animals demonstrated both learning and LTM with the modified training paradigm (one-way ANOVA, $F(15,2) = 117.9$, $P < 0.0001$).

The non-respiring experimental animals, however, did not show a similar reduction in aerial respiratory behaviour as a result of operant conditioning. There were no changes in the number of pneumostome openings or total breathing time from the pre- to the post-observation session (Fig. 7). Furthermore, these non-respiring animals did not show a significant reduction in the number of attempts to open their pneumostome from TS1 to TS4 or to the MT (Fig. 8). Therefore, by definition, experimental non-respiring animals were unable to learn and form LTM using the same paradigm as the experimental respiring animals. Interestingly, the experimental respiring animals reduced their aerial respiratory activity to the initial level observed in the non-respiring animals. In other words, there were no significant differences in the respiratory parameters between the experimental respiring post-observation sessions and the non-respiring pre-observation sessions.

Summary of Intact Data

In intact *Lymnaea*, the results showed that only the respiring animals displayed plasticity in their aerial respiratory behaviour, whereas the non-respiring animals did not. That is, operant conditioning produced a reduction in aerial respiratory behaviour in the conditioned respiring animals only, in response to the aversive stimulus.

Behavioural and Neural Correlates of Learning and Memory in the Semi-Intact Preparation

Next, I wanted to investigate the neural correlates of operant conditioning using a semi-intact preparation. I also wanted to conclusively determine whether or not non-respiring animals were capable of learning and memory. According to the parameters defined, there was no evidence to support LTM formation in the intact non-respiring experimental animals. However,

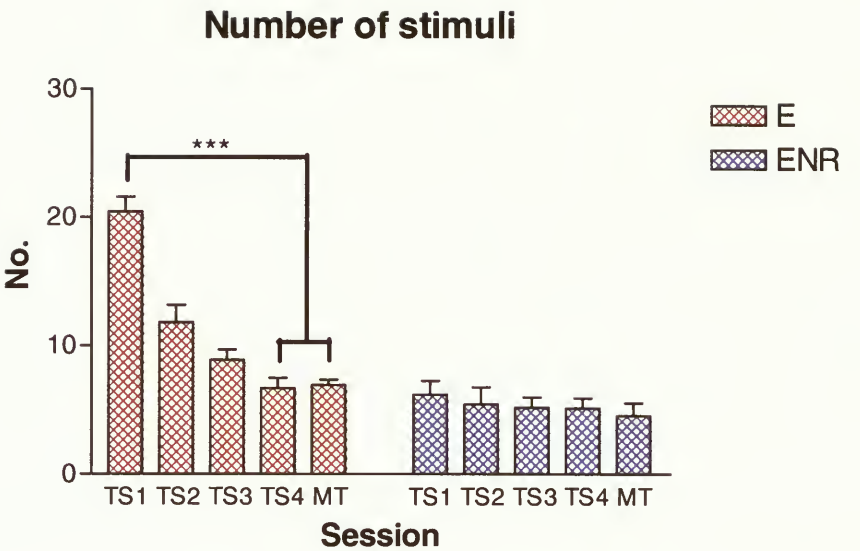


Figure 8: Number of attempted pneumostome openings across the four training sessions and the memory test. While experimental respiring (E) animals demonstrated both learning and memory (number of pokes: TS1, 20.4 ± 0.6, TS4, 6.7 ± 0.8, MT, 6.9 ± 0.6), experimental non-respiring (ENR) animals did not show a reduction in aerial respiratory behaviour as a result of operant conditioning.
(number of animals = 16 per group) (***, $P < 0.001$)

it was possible that conditioning produced partial effects, behavioural and/or neural, that would be evident in the semi-intact preparation.

Following the post-observation session, the snails were immediately dissected and CPG and pneumostome activity were simultaneously monitored. In this experiment, RPeD1 and VI cell activity were recorded by means of dual-channel intracellular electrophysiology. I monitored RPeD1 because it is the cell that receives excitatory input from the pneumostome/osphradial area and initiates the CPG respiratory rhythm. Since IP3 is located on the ventral surface of the CNS and RPeD1 is located on the dorsal surface, its activity was indirectly assessed as characteristic bursting patterns in the VI cell (Syed *et al.*, 1990, 1991). Located on the dorsal surface, VI is morphologically distinct and is easily identified as the largest of the pneumostome opener motor neurons.

The experimental protocol in the semi-intact preparation was designed to both observe the neural and behavioural correlates of the respiratory behaviour and to monitor the effects of the reinforcing stimulus used previously to condition the intact animals. The paradigm is presented as a schematic in Figure 9. Briefly, CPG and pneumostome activity were simultaneously monitored for a five-minute pre-test period, followed by a contingent application of the stimulus to the open pneumostome, and a subsequent five-minute post-test period. Data are reported as the pre-test values of any given parameter and the change in parameter value elicited by the reinforcing stimulus. Also, as in the whole animal, data were separately analyzed for the naïve animals and the yoked and experimental animals. However, in addition to the naïve respiring and naïve non-respiring *Lymnaea* that underwent the intermittent hypoxic sessions, a separate group was also used straight from the differential rearing containers. This was done to observe the first 'bouts' of aerial respiration in the animals' existence. These animals were denoted 'straight-from-tank non-respiring'.

Analysis of Pneumostome Openings & IP3 Bursting Activity in Naïve Semi-Intact Preparations

The first task was to determine the baseline differences in behavioural and neural activity between respiring and non-respiring naïve preparations. I investigated pneumostome opening and corresponding IP3 activity in the VI cell in the naïve semi-intact preparations in order to determine whether the behaviour correlated with cellular activity. Figures 10a and 10b illustrate the number of pneumostome openings and IP3 bursts in the naïve animals during the five-minute pre-test session. As expected, the naïve non-respiring groups displayed significantly fewer pneumostome openings than the naïve respiring preparations (one-way ANOVA, $F(45,2) = 33.64$, $P < 0.0001$). Accordingly, the number of IP3 bursts recorded from the VI cell were also

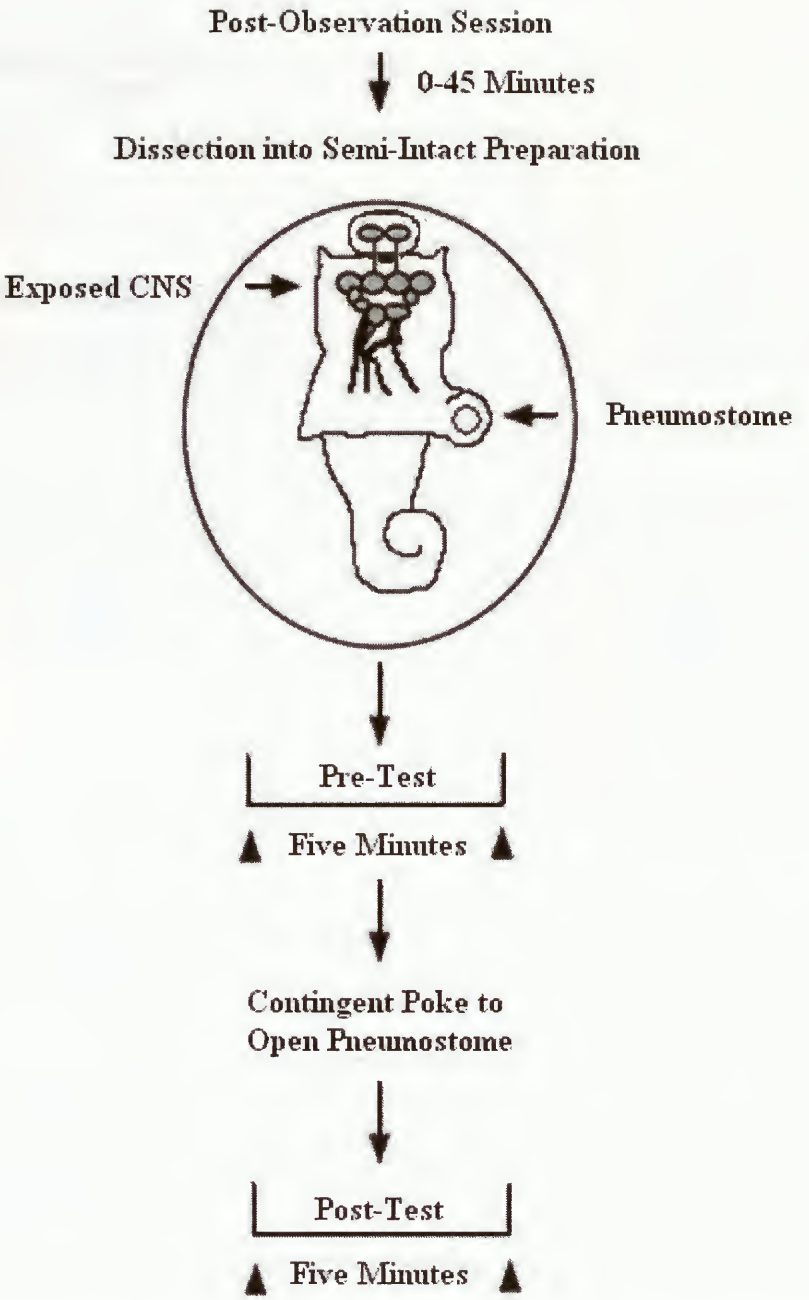


Figure 9: Diagrammatic representation of the experimental protocol used for the semi-intact preparation. Following the intact animal post-observation session, the animals were dissected into the semi-intact preparation. CPG activity was monitored via intracellular recordings. (Modified from Spencer *et al.*, 2002)

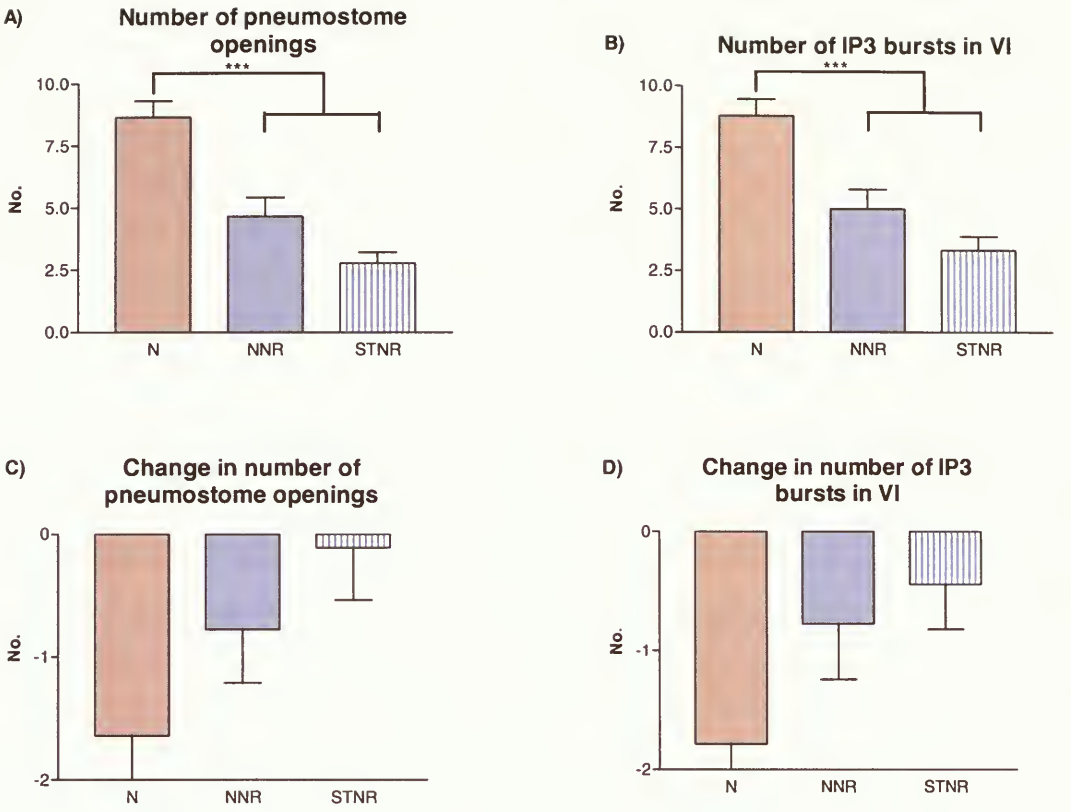


Figure 10: Pneumostome behaviour and IP3 parameters in the naïve semi-intact preparations. I analyzed both the number of pneumostome openings and the number of IP3 bursts recorded from the VI cell. **(A)** Naïve respiring animals performed aerial respiration more often than naïve non-respiring and straight-from-tank non-respiring animals [number of pneumostome openings in the pre-test session: naïve (N), 8.6 ± 0.7, naïve non-respiring, (NNR) 4.7 ± 0.7, straight-from-tank non-respiring (STNR), 2.8 ± 0.4]. **(B)** The number of IP3 bursts was significantly higher in the naïve respiring animals compared to non-respiring animals [number of IP3 bursts in the pre-test session: naïve, 8.8 ± 0.7, naïve non-respiring, 5.0 ± 0.8, straight-from-tank non-respiring, 3.3 ± 0.6]. **(C,D)** Graphs depicting the change in behaviour and cellular activity following the application of the reinforcing stimulus. All preparations showed a reduction in the number of pneumostome openings and IP3 bursts, although the changes were insignificant.
(number of preparations: N = 14, NNR = 9, STNR = 9) (*, $P < 0.05$; ***, $P < 0.001$)

significantly reduced in the naïve non-respiring preparations compared to naïve respiring preparations (one-way ANOVA, $F(45,2) = 25.00$, $P < 0.0001$). In response to the reinforcing stimulus at the end of the pre-test session, all of the naïve animals showed a further reduction in the number of pneumostome openings and the number of IP3 bursts in the five-minute post-test session. As these preparations were naïve and not previously exposed to the stimulus, the change was statistically insignificant. With regards to the straight-from-tank non-respiring *Lymnaea*, these preparations opened their pneumostome significantly less often than naïve respiring preparations (one-way ANOVA, $F(29,2) = 22.58$, $P < 0.0001$) and demonstrated significantly fewer IP3 bursts in the VI cell (one-way ANOVA, $F(29,2) = 17.18$, $P < 0.001$). However, there were no significant differences between the naïve and straight-from-tank non-respiring preparations. Thus, straight-from-tank non-respiring animals were immediately capable of performing aerial respiration although they were prevented from doing so during development.

From these data, I conclude that the semi-intact preparations behaved in a manner similar to the intact animals from which they were derived. That is, as in the intact animal, non-respiring naïve preparations performed aerial respiration less often than respiring preparations. The differences in aerial respiratory behaviour between the naïve groups were very closely associated with differences in IP3 activity; there were no significant differences in the number of pneumostome openings and IP3 bursts within the groups. Thus, I am confident that the IP3 activity was appropriately monitored via the VI cell.

The total extent of respiratory activity in the semi-intact preparations before and after the stimulus was also calculated. Previously, I saw the naïve respiring preparations opened their pneumostome more often than naïve non-respiring preparations. I now demonstrate that the naïve respiring preparations performed aerial respiration for a significantly greater duration than the straight-from-tank preparations, but not significantly more than the naïve non-respiring preparations (one-way ANOVA, $F(45,2) = 25.00$, $P = 0.0198$) (Fig. 11). Recall that as intact animals, naïve respiring animals performed aerial respiration for a greater duration than naïve non-respiring animals. This difference in respiration is addressed in the discussion section.

Analysis of Pneumostome Openings & IP3 Bursting Activity in Trained Semi-Intact Preparations

Having analyzed respiratory and IP3 activity in naïve semi-intact preparations, conditioned animals were dissected to determine the effects of operant conditioning on these parameters. Representative pre-test recordings of yoked respiring and experimental respiring animals are shown in Figure 12. I found that in the semi-intact preparations, respiratory behaviour and IP3 activity was reflective of operant conditioning in the intact animal (Fig. 13).

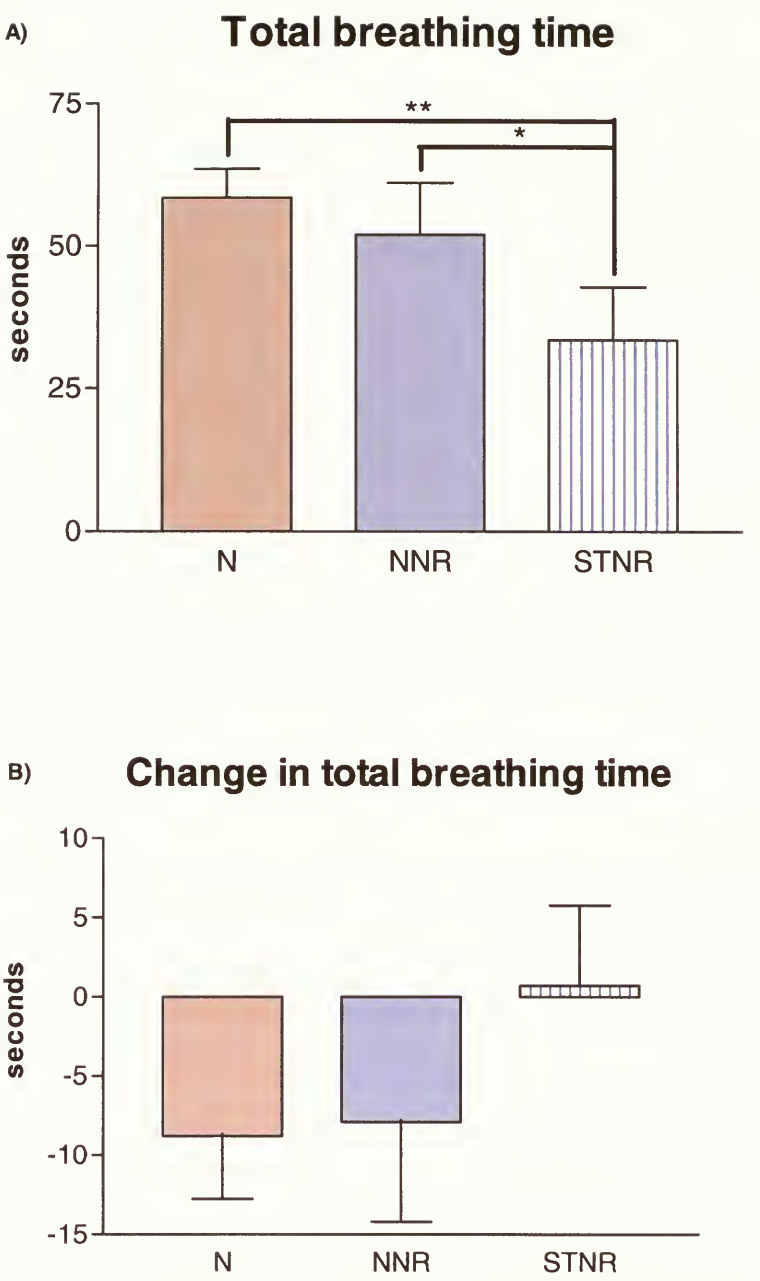


Figure 11: Pre-test total breathing time and change in total breathing time in the naïve semi-intact *Lymnaea*. Naïve respiring and naïve non-respiring animals performed aerial respiration for a significantly greater duration than straight-from-tank non-respiring animals [total breathing time (seconds) in the pre-test session: naïve (N), 58 ± 5, naïve non-respiring (NNR), 52 ± 9, straight-from-tank non-respiring (STNR), 34 ± 9]. The reinforcing stimulus produced an overall reduction in total breathing time in the naïve respiring and non-respiring animals while the straight-from-tank animals showed no response.
(number of preparations: N = 14, NNR = 9, STNR = 9) (*, $P < 0.05$; **, $P < 0.01$)

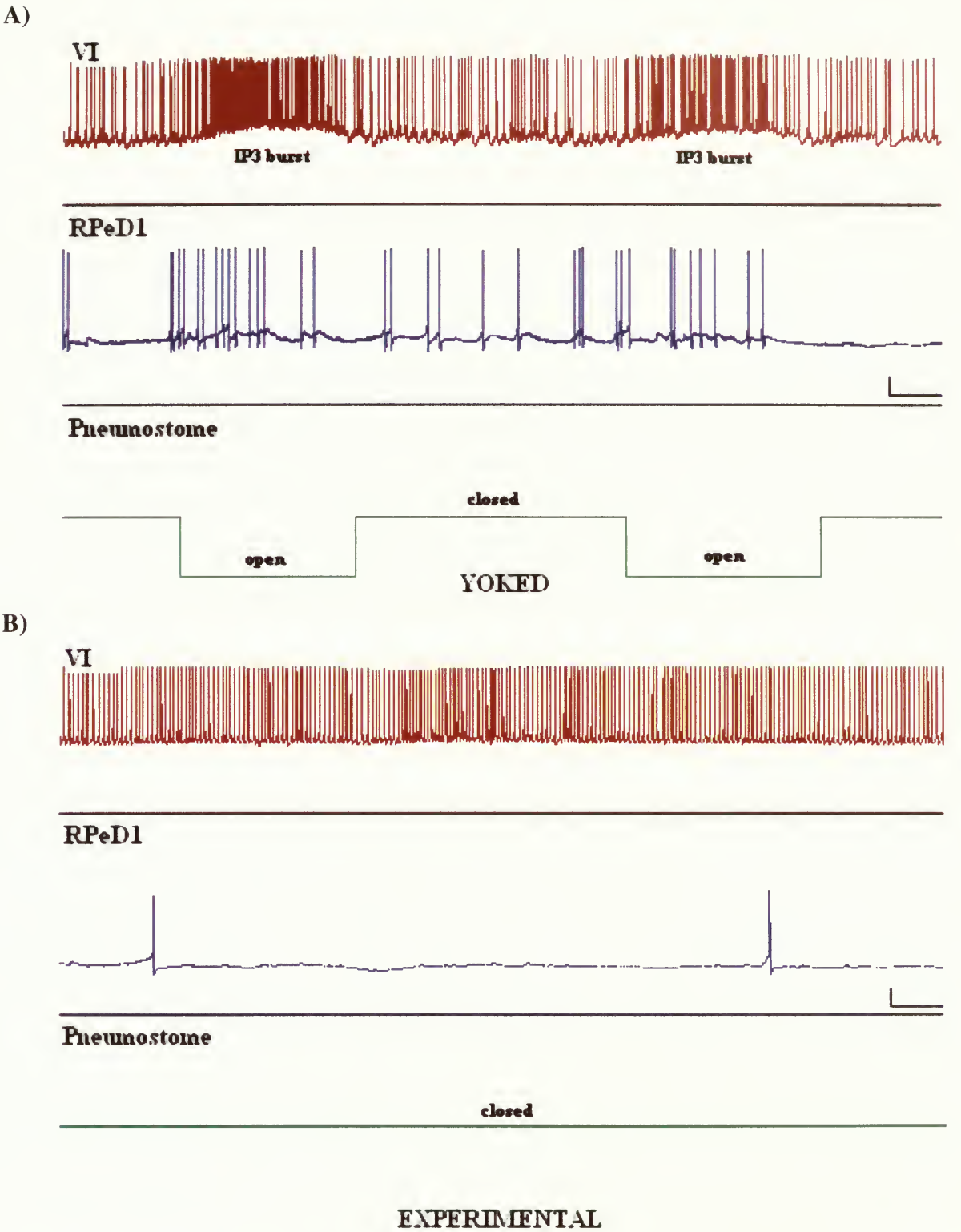


Figure 12: Representative pre-test electrophysiology recordings from (A) yoked respiring and (B) experimental respiring animals.
(vertical bar = 15mV, horizontal bar = 5sec)

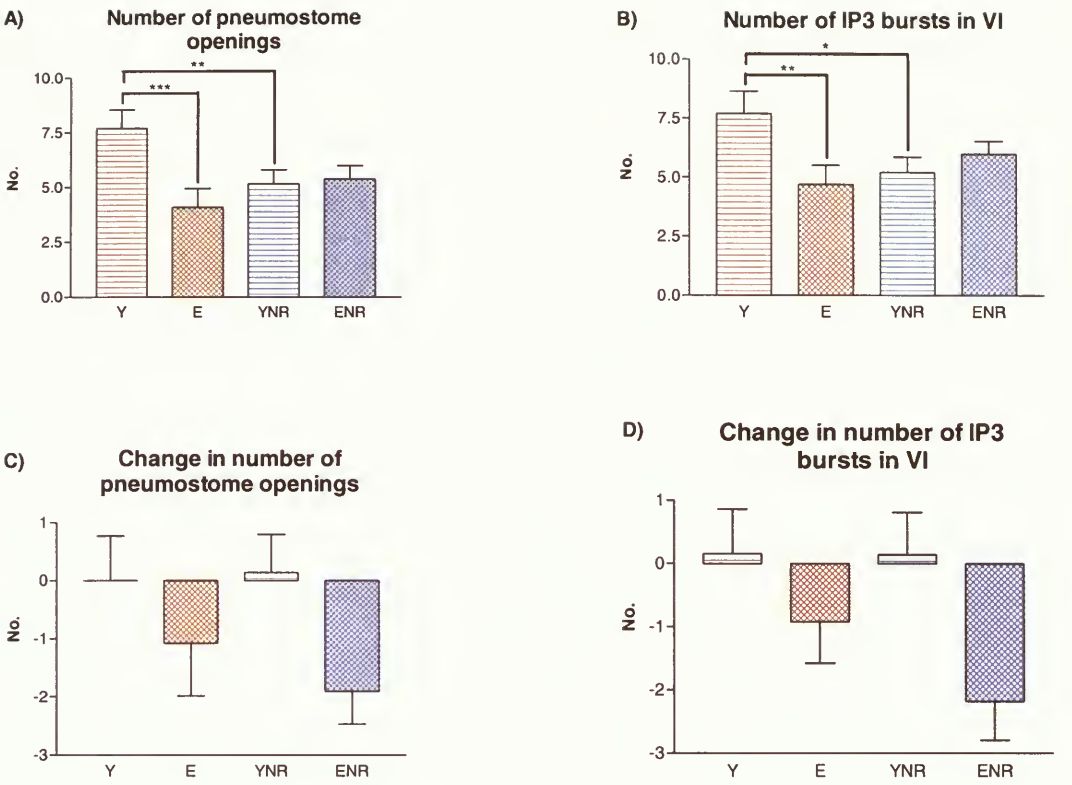


Figure 13: Pneumostome and IP3 parameters in the yoked and experimental semi-intact preparations. **(A)** Experimental respiring preparations performed aerial respiration significantly less often than yoked respiring controls in the pre-test session. The experimental non-respiring preparations, however, did not demonstrate a reduction in aerial respiration compared to corresponding yoked controls [number of pneumostome openings in the pre-test session: yoked respiring (Y), 7.7 ± 0.8, experimental respiring (E), 4.1 ± 0.9, yoked non-respiring (YNR), 5.1 ± 0.6, experimental non-respiring (ENR), 4.6 ± 0.6]. **(B)** IP3 activity was also significantly reduced in the experimental respiring animals compared to yoked respiring preparations, but not the non-respiring yoked and experimental preparations [number of IP3 bursts in the pre-test session: yoked respiring, 7.7 ± 1.0, experimental respiring, 4.7 ± 0.8, yoked non-respiring, 5.2 ± 0.6, experimental non-respiring, 6.0 ± 0.5]. **(C,D)** The contingent poke to the pneumostome produced a further reduction in aerial respiration and IP3 activity in the experimental preparations compared to yoked controls.
(number of preparations: Y = 13, E = 13, YNR = 14, ENR = 11) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

That is, the experimental respiring animals opened their pneumostome significantly less than yoked respiring controls, but not significantly different from the non-respiring yoked and experimental animals (one-way ANOVA, $F(60,3) = 5.083$, $P = 0.0033$). The number of IP3 bursts, which result in pneumostome openings, was also significantly reduced in the experimental respiring semi-intact preparations (one-way ANOVA, $F(60,3) = 3.719$, $P = 0.0161$). And although statistically insignificant, the reinforcing stimulus caused a further reduction in both the number of IP3 bursts and number of pneumostome openings in the experimental respiring. The yoked respiring snails, on the other hand, did not respond positively or negatively to the contingent poke. Because these animals received many (non-contingent) pokes during the intact animal training, the yoked animals may have habituated to the stimulus. Comparing the non-respiring yoked and non-respiring experimental snails in the pre-test session, the results suggested the operant conditioning paradigm was ineffective; there was no significant difference in the number of pneumostome openings or IP3 bursts in the pre-observation session. But, as was observed in the experimental respiring animals, the experimental non-respiring animals displayed a reduction in the number of pneumostome openings following the reinforcing stimulus whereas the yoked non-respiring animals did not. In this manner, the experimental non-respiring behaved in similar to experimental respiring preparations in response to the stimulus. Thus, experimental non-respiring animals demonstrated some evidence of behavioural and neural plasticity.

LTM was also evident in the experimental respiring *Lymnaea* when the total breathing time was considered (Fig. 14). Experimental respiring preparations performed aerial respiration significantly less than the yoked respiring preparations (one-way ANOVA, $F(60,3) = 9.747$, $P < 0.0001$). However, like the naïve preparations, the total breathing time was comparable between the respiring yoked and non-respiring yoked (and experimental) preparations. There was also no significant difference between the total breathing time of yoked non-respiring and experimental non-respiring preparations, but the latter demonstrated a greater reduction in the five-minute post-test. This was further evidence to suggest behavioural plasticity in the differentially reared *Lymnaea*.

Overall, the results suggested that the semi-intact preparation was a good model for studying the behavioural and neural correlates of learning and memory. As in the whole animal, experimental respiring preparations performed aerial respiration less than the yoked respiring controls. Concurrently, IP3 activity was also reduced in these preparations. However, unlike the whole animal, the experimental non-respiring preparations did demonstrate some evidence of LTM following the application of the stimulus compared to yoked non-respiring controls.

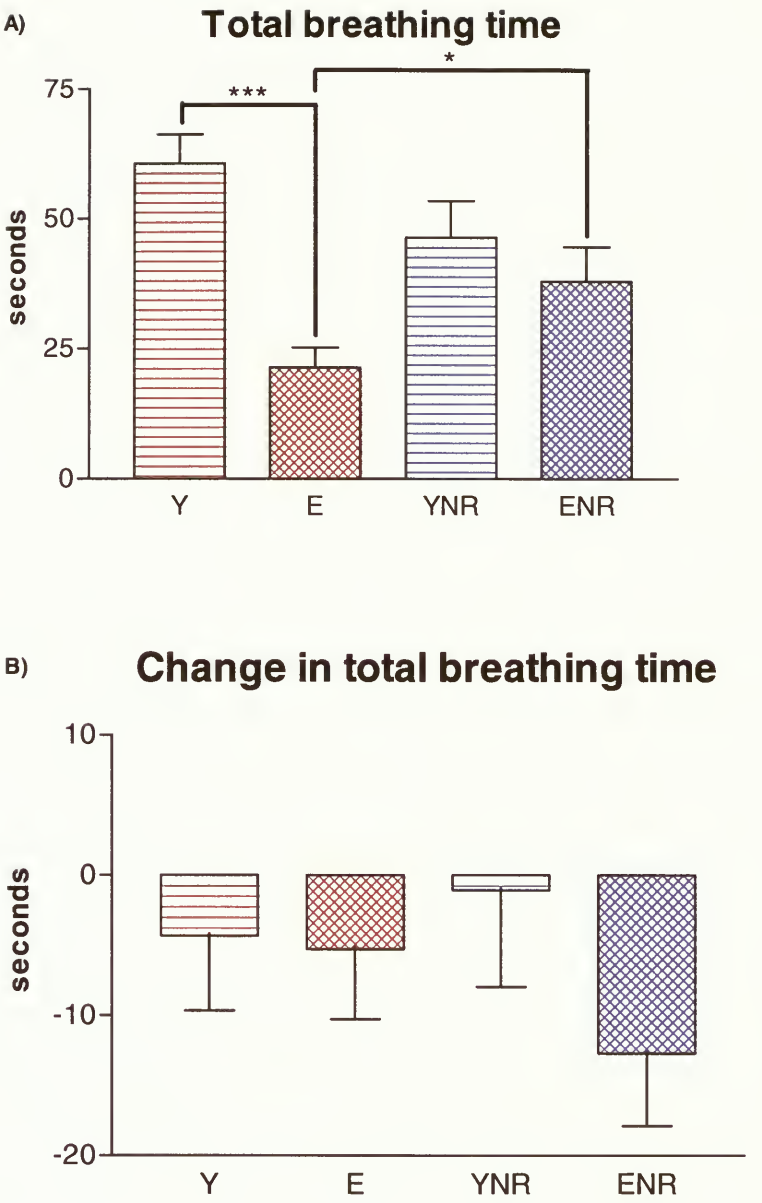


Figure 14: Total breathing time and change in total breathing time in the yoked and experimental semi-intact *Lymnaea*. **(A)** Experimental respiring preparations performed aerial respiration less than yoked respiring controls. The experimental non-respiring preparations, however, did not demonstrate a reduction in aerial respiration compared to yoked non-respiring preparations and performed respiration for a significantly greater duration compared to experimental respiring preparations [total breathing time (seconds) in the pre-test session: yoked respiring (Y), 60 ± 6, experimental respiring (E), 21 ± 4, yoked non-respiring (YNR), 46 ± 7, experimental non-respiring (ENR), 38 ± 6]. **(B)** There was no significant change in total breathing time in response to the conditioning stimulus.
(number of preparations: Y = 13, E = 13, YNR = 14, ENR = 11) (*, $P < 0.05$; ***, $P < 0.001$)

A number of studies have suggested a multi-loci model of learning and memory (e.g. Benjamin *et al.*, 2000). That is, modifications induced by learning are dispersed throughout the neural circuitry mediating the behaviour. Therefore, a number of other behavioural and cellular parameters were analyzed to determine where neuronal changes that underlie behavioural changes may occur in this system, and to compare these changes from respiring to non-respiring preparations. These included a number of IP3 parameters and RPeD1, motor neuron, and pneumostome activity.

Latency to Pneumostome Opening Following the Reinforcing Stimulus

It has been shown in *Lymnaea* that a reinforcing stimulus can induce behavioural and neural changes associated with LTM (Spencer *et al.*, 2002). The latency to the 'next' pneumostome opening in the post-test following the application of the contingent stimulus was determined for the trained and untrained preparations (Fig. 15). This parameter was used as an indication of the behavioural response to the stimulus. There were no significant differences in the response to the reinforcing stimulus amongst the naïve preparations. However, statistical analysis indicated that the experimental respiring preparations displayed a greater latency to the next pneumostome opening compared to the yoked respiring preparations (one-way ANOVA, $F(47,3) = 4.487$, $P = 0.0075$). The experimental non-respiring preparations also demonstrated this increased latency compared to the yoked non-respiring preparations, although the difference was not significant. These results were again indicative, though not conclusive, of behavioural plasticity in the differentially reared *Lymnaea*. Interestingly, the naïve preparations took longer to attempt aerial respiration than the yoked preparations, again suggesting the yoked preparations 'ignored' the stimulus. Taken together, experimental preparations suppressed the hypoxic ventilatory drive for a longer duration compared to controls following the application of the contingent poke.

IP3 Activity as Recorded from the VI Cell

IP3 activity results in pneumostome opening (Syed *et al.* 1991; Syed and Winlow, 1991). I have already shown that the number of IP3 burst is reduced in experimental preparations. I analyzed three other IP3 parameters and found them to be altered in experimental respiring preparations compared to control preparations. These parameters included the intensity of the

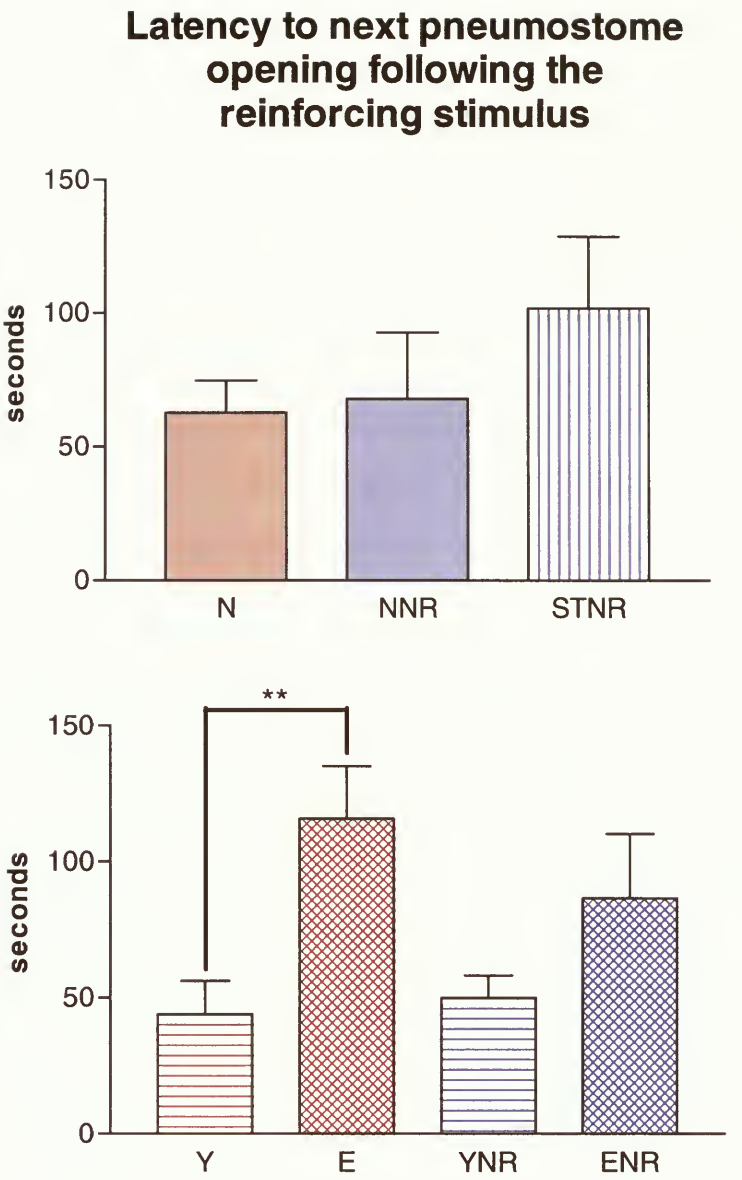


Figure 15: Latency to next pneumostome opening following contingent application of the stimulus. There is no significant difference in the response to the stimulus among the naïve respiring and non-respiring preparations [latency (seconds) to next pneumostome opening: naïve respiring (N), 63 ± 12, naïve non-respiring (NNR), 68 ± 25, straight-from-tank non-respiring (STNR), 102 ± 27]. There was, however, an increase in latency in experimental preparations compared to yoked preparations [latency (seconds) to next pneumostome opening: yoked respiring (Y), 44 ± 12, experimental respiring (E), 116 ± 19, yoked non-respiring (YNR), 50 ± 8, experimental non-respiring (ENR), 87 ± 24].
(number of preparations: N = 14, NNR = 9, STNR = 9, Y = 13, E = 13, YNR = 14, ENR = 11)
(**, $P < 0.01$)

IP3 bursts, the latency in IP3 burst-to-pneumostome opening, and the correlated IP3 and pneumostome activity.

While I did not use a tension transducer to measure the force of each pneumostome opening, IP3 burst frequency was used as an indirect measure of the intensity of the pneumostome opening (Fig. 16). A high burst frequency qualitatively resulted in a large, 'willing' pneumostome opening while a low burst frequency produced a smaller pneumostome opening. There was no significant difference in IP3 burst frequency between the naïve respiring and non-respiring preparations (one-way ANOVA, $F(45,2) = 1.177$, $P > 0.05$). There was, however, a significant difference in the yoked and experimental respiring preparations (one-way ANOVA, $F(60,3) = .3070$, $P < 0.0001$). The operant conditioning affected the intensity of the IP3 activity such that burst frequency, as recorded from the VI cell, was significantly reduced in the experimental respiring preparations compared to the yoked respiring preparations. The experimental non-respiring preparations did not behave like the experimental respiring preparations in the pre-test session but demonstrated a reduction in IP3 burst frequency following the stimulus. Thus, the reinforcing stimulus induced changes in IP3 activity in the experimental non-respiring preparations such that resultant pneumostome openings were smaller in the post-test session.

There is a direct monosynaptic connection between the VI motor neurons and the pneumostome opener muscles (Syed *et al.*, 1991). I wanted to determine the latency in IP3 activity (in the VI cell) and the resultant pneumostome opening and see if it was affected by conditioning (Fig. 17a). There was no difference in this latency between the naïve groups (one-way ANOVA, $F(45,2) = .4718$, $P > 0.05$). In both the respiring and non-respiring conditioned animals though, there was a significant difference in the pneumostome response (one-way ANOVA, $F(60,3) = 3.783$, $P = 0.0149$). In the yoked groups, a pneumostome opening was recorded as soon as IP3 activity was observed while in the experimental groups, there was a significant lag time in the pneumostome opening. Also, the experimental respiring preparations were the only group to show a further increase in the latency following the reinforcing stimulus (Fig. 17b). Thus, operant conditioning affected the latency in the pneumostome opening response following IP3 activity. The respiratory motor program dictates that IP3 bursting activity produces a pneumostome opening (Syed and Winlow, 1991). Thus, IP3 activity was also scored for correlated pneumostome activity in the pre-test session (Fig. 18). In other words, if IP3 activity was observed in the VI cell and/or RPeD1, was there a corresponding pneumostome opening? While all naïve and yoked respiring and non-respiring preparations demonstrated > 90% coincident activity, the number of IP3 bursts that did not result in pneumostome openings in

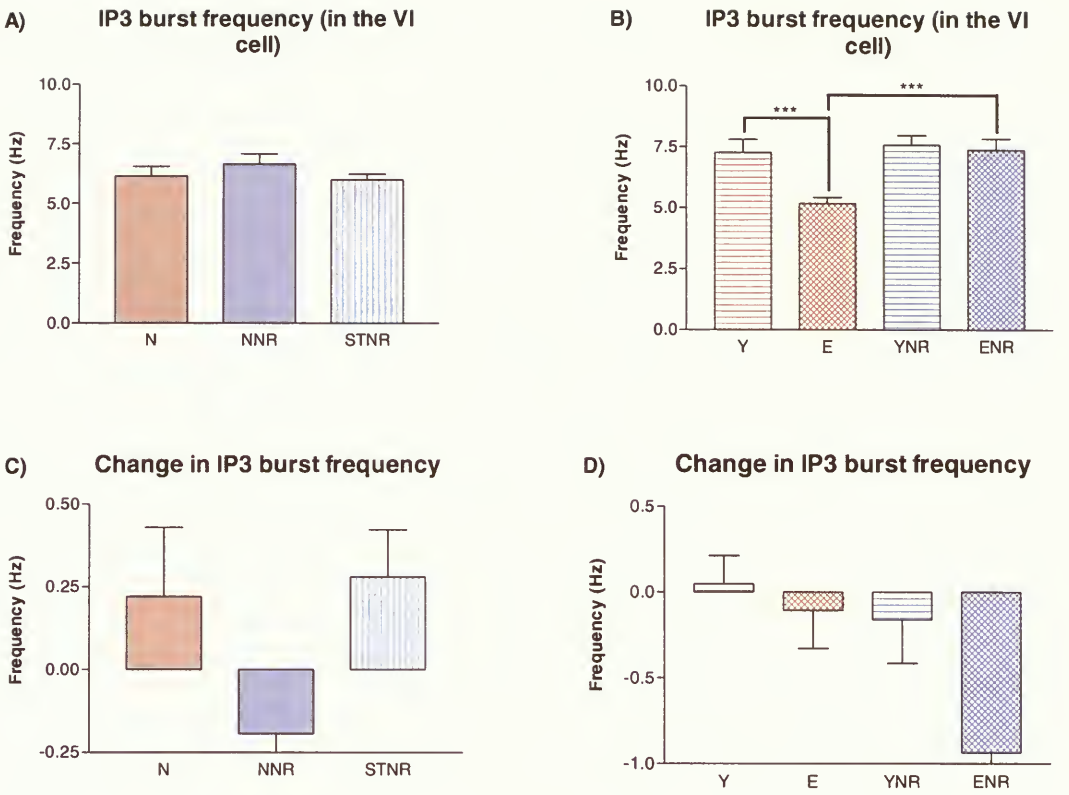


Figure 16: Pre-test IP3 burst frequency and change in IP3 burst frequency, as recorded from the VI cell. **(A)** There were no differences in IP3 burst frequency amongst the naïve preparations [IP3 Hz: naïve respiring (N), 6.2 ± 0.4, naïve non-respiring (NNR), 6.6 ± 0.4, straight-from-tank non-respiring (STNR), 6.0 ± 0.2]. **(B)** The intensity of IP3 activity was reduced in experimental respiring preparations compared to yoked respiring preparations [IP3 Hz: yoked respiring (Y), 7.3 ± 0.5, experimental respiring (E), 5.2 ± 0.2, yoked non-respiring (YNR), 7.6 ± 0.4, experimental non-respiring (ENR), 7.4 ± 0.4]. The same was not true of non-respiring preparations. **(C,D)** The experimental non-respiring preparations demonstrated the greatest reduction in IP3 burst frequency following the reinforcing stimulus.
(number of preparations: N = 14, NNR = 9, STNR = 9, Y = 13, E = 13, YNR = 14, ENR = 11)
(***, $P < 0.001$)

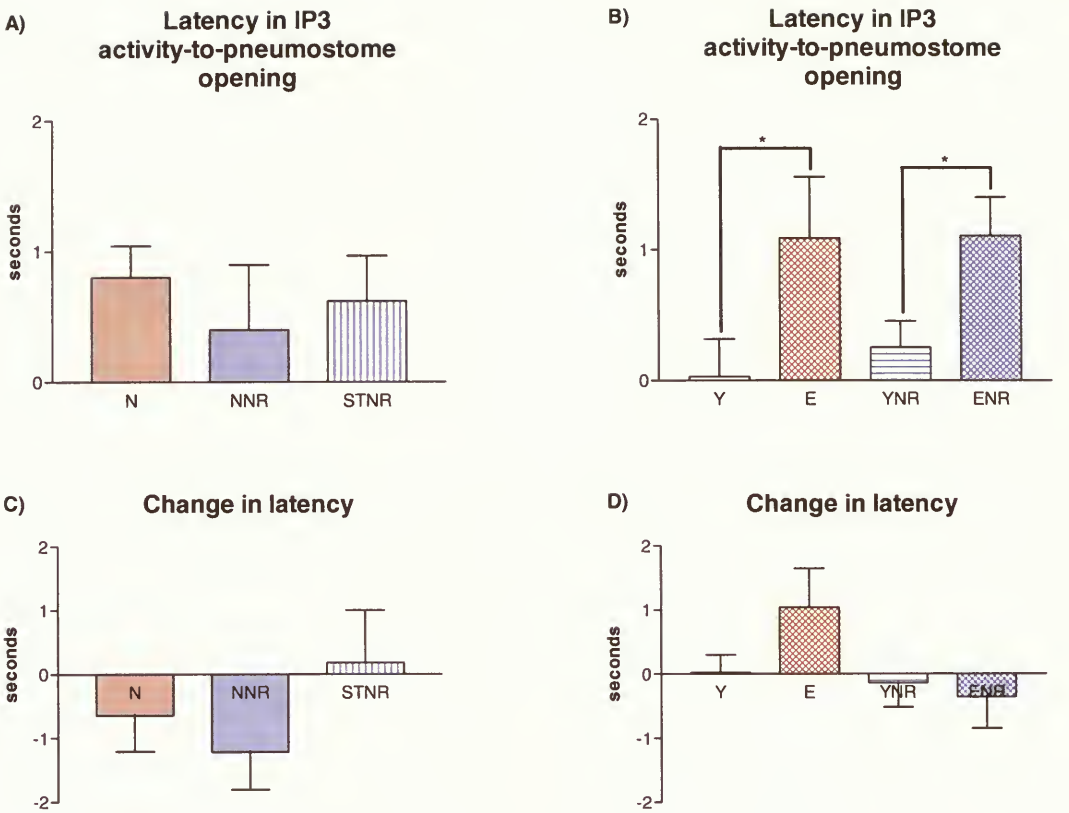


Figure 17: Latency in IP3 bursting activity (in the VI cell) to pneumostome opening and change in parameter following reinforcing stimulus. **(A)** There were no differences in latency amongst the naïve respiring and non-respiring *Lymnaea* [latency in IP3 burst-to-pneumostome opening (seconds): naïve respiring (N), 0.8 ± 0.2, naïve non-respiring (NNR), 0.4 ± 0.5, straight-from-tank non-respiring (STNR), 0.6 ± 0.3]. **(B)** A pneumostome opening was recorded at the same time as the IP3 burst in yoked animals, while there was a lag in the response in experimental animals [latency in IP3 burst-to-pneumostome opening (seconds): yoked respiring (Y), 0.0 ± 0.3, experimental respiring (E), 1.1 ± 0.5, yoked non-respiring (YNR), 0.3 ± 0.2, experimental non-respiring (ENR), 1.1 ± 0.3]. **(C,D)** There were no significant changes in the latency in response to the reinforcing stimulus, though the experimental respiring animals were the only group to show an increase.
(number of preparations: N = 14, NNR = 9, STNR = 9, Y = 13, E = 13, YNR = 14, ENR = 11)
(*, $P < 0.05$)

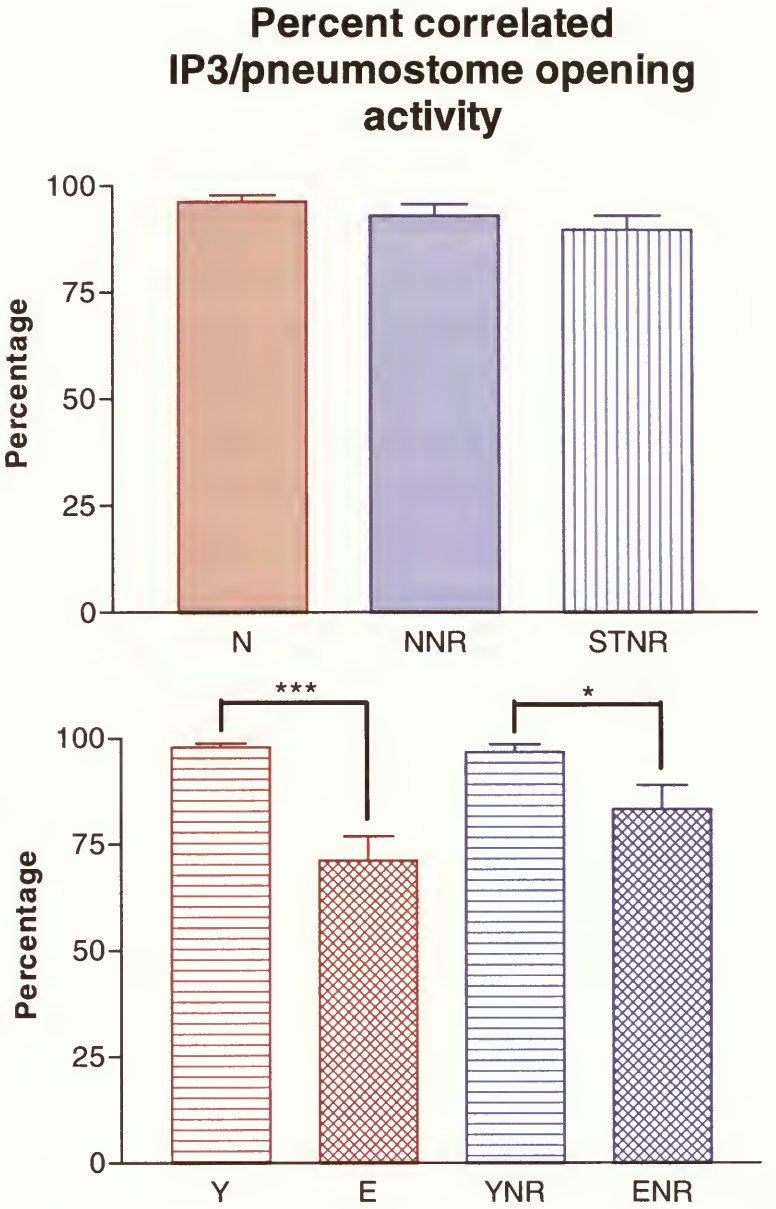


Figure 18: Percentage of IP3 bursts monitored in either the VI cell or RPeD1 that produced a pneumostome opening. Over 90% of IP3 activity produced pneumostome openings in naïve and yoked preparations. But in experimental preparations, a greater number of IP3 bursts did not result in pneumostome openings [percent correlation of IP3 and pneumostome activity: naïve respiring (N), 96 ± 8, naïve non-respiring (NNR), 93 ± 12, straight-from-tank non-respiring (STNR), 90 ± 14, yoked respiring (Y), 98 ± 6, experimental respiring (E), 71 ± 29, yoked non-respiring (YNR), 97 ± 9, experimental non-respiring (ENR), 84 ± 28].
(number of preparations: N = 14, NNR = 9, STNR = 9, Y = 13, E = 13, YNR = 14, ENR = 11)
(*, $P < 0.05$; ***, $P < 0.001$)

the experimental preparations was increased in conditioned preparations (one-way ANOVA, $F(181,6) = 9.164$, $P < 0.0001$). These results suggested a change in the respiratory motor program in the experimental respiring and non-respiring animals such that IP3 activity did not always result in a pneumostome opening.

The motor neuron firing frequency, inclusive of IP3-induced bursting activity, was also determined for the five-minute pre-test session. Statistical analysis indicated no difference in VI frequency between the naïve respiring and naïve non-respiring preparations and no difference in activity between yoked and experimental preparations ($P > 0.05$). Furthermore, there was no significant effect of the reinforcing stimulus on VI frequency ($P > 0.05$). Thus, motor neuron activity was not affected by operant conditioning.

Taken together, IP3 activity was influenced by both rearing conditions and operant conditioning. With respect to the rearing conditions, the only difference observed was that non-respiring preparations demonstrated fewer IP3 bursts in the VI cell compared to respiring preparations. This was reflective of the fewer number of pneumostome openings in both the intact animal and the semi-intact preparation. Operant conditioning affected the intensity of the IP3 burst, the latency in response, and correlated IP3 and pneumostome activity. Also, while the experimental non-respiring preparations did not demonstrate any behavioral evidence of learning and memory, the cellular parameters suggested some aspects of plasticity. Particularly, the latency and correlation parameters were significantly affected by operant conditioning in the experimental non-respiring preparations.

RPeD1 Impulse Activity

Previously, RPeD1 activity was found to be reduced in operantly conditioned semi-intact preparations of *Lymnaea* compared to controls (Spencer *et al.*, 1999, 2002). The data presented here somewhat support the previous findings (Fig. 19b). RPeD1 firing frequency was lower in experimental preparations compared to their yoked controls, albeit not significantly (one-way ANOVA, $F(52,3) = 1.611$, $P = 0.1979$). Furthermore, there was a negative change in RPeD1 firing frequency in the experimental respiring preparations following the reinforcing stimulus but there was no significant difference in the overall firing frequency in the post-test compared to the pre-test (Fig. 19). Significance was likely not established due to a smaller sample size than used previously and the use of a different training paradigm. There were, however, significant short-term changes in the RPeD1 activity (Fig. 20). Following application of the contingent stimulus, RPeD1 ceased firing until sufficient excitatory input re-initiated rhythmogenesis and/or it recovered from inhibition. Experimental respiring preparations demonstrated a significantly

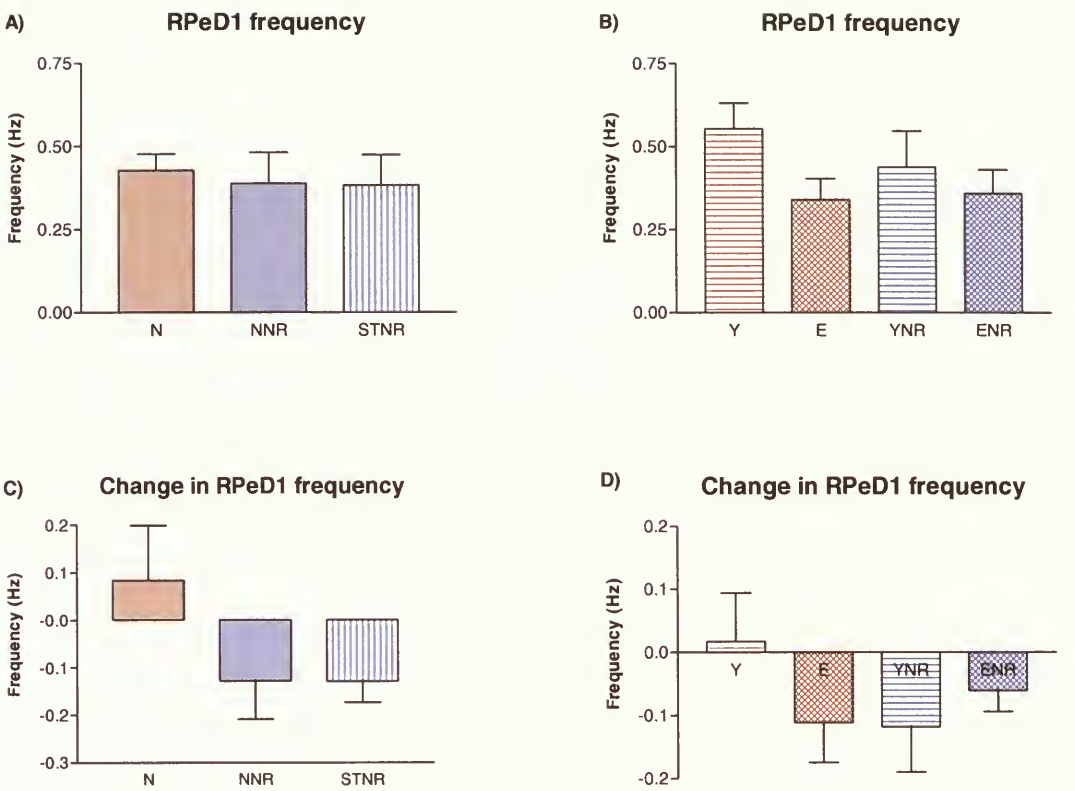


Figure 19: RPeD1 frequency in the pre-test session and the change in frequency following the reinforcing stimulus. **(A)** There were no differences in RPeD1 firing frequencies among the naïve preparations. **(B)** Experimental preparations demonstrated a slower RPeD1 frequency compared to yoked, although the results were not significant [RPeD1 frequency (Hz): naïve respiring (N), 0.43 ± 0.05, naïve non-respiring (NNR), 0.39 ± 0.09, straight-from-tank non-respiring (STNR), 0.38 ± 0.09, yoked respiring (Y), 0.55 ± 0.08, experimental respiring (E), 0.34 ± 0.06, yoked non-respiring (YNR), 0.44 ± 0.11, experimental non-respiring (ENR), 0.36 ± 0.07]. **(C,D)** There were no significant effects of the stimulus on the change in RPeD1 firing frequency.
(number of preparations: N = 14, NNR = 9, STNR = 9, Y = 13, E = 13, YNR = 14, ENR = 11)

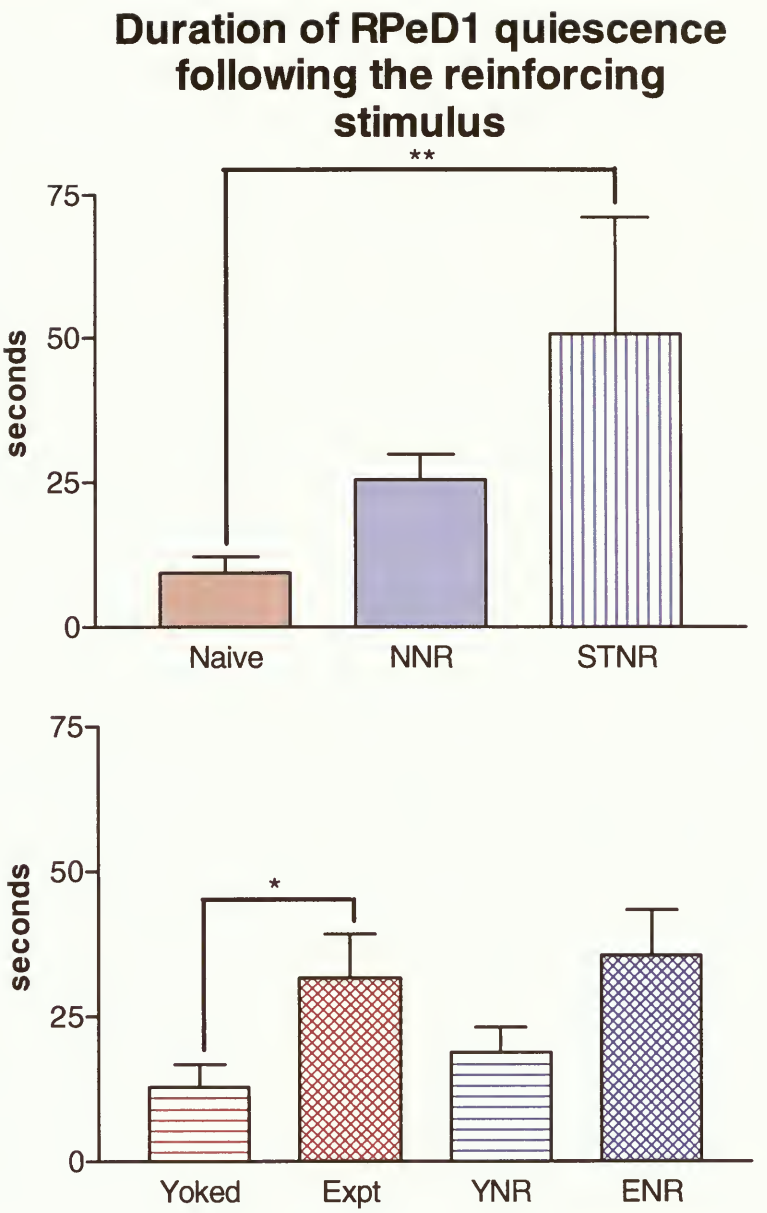


Figure 20: Latency to the 'next' action potential in RPeD1 following the application of the contingent poke. RPeD1 remained quiescent for a significantly greater duration in the straight-from-tank non-respiring naïves compared to respiring naives. Operant conditioning also resulted in an increased latency to the next action potential, although the results were only significant when comparing yoked and experimental respiring preparations [latency to next action potential in RPeD1 (seconds): naïve respiring (N), 9 ± 2, naïve non-respiring (NNR), 25 ± 4, straight-from-tank non-respiring (STNR), 51 ± 20, yoked respiring (Y), 13 ± 4, experimental respiring (E), 32 ± 7, yoked non-respiring (YNR), 19 ± 4, experimental non-respiring (ENR), 36 ± 8]. (number of preparations: N = 14, NNR = 9, STNR = 9, Y = 13, E = 13, YNR = 14, ENR = 11) (*, $P < 0.05$; **, $P < 0.01$)

increased recovery time in RPeD1 impulse activity compared to their respective yoked controls (one-way ANOVA, $F(47,3) = 4.487$, $P = 0.0075$). With respect to non-respiring preparations, a similar trend was observed in experimental non-respiring preparations but significance was not established. Interestingly, RPeD1 remained quiescent for a significantly longer duration in straight-from-tank non-respiring preparations compared to naïve respiring preparations.

Summary of Semi-Intact Data

Overall, further analysis of the neural network properties in the semi-intact preparation revealed changes associated with conditioning dispersed throughout the CPG controlling aerial respiration. Operant conditioning produced a reduction in both the number and intensity of IP3 bursts, and a change in the motor program controlling pneumostome opening, the efficacy of IP3 activity in producing a pneumostome opening, and RPeD1 impulse activity. With respect to non-respiring preparations, the strongest evidence for plasticity was demonstrated by the significant differences in latency of pneumostome opening response and correlated pneumostome/IP3 activity between yoked and experimental non-respiring preparations. The only significant effects of differential rearing as observed in naïve semi-intact preparations were the number of pneumostome openings and corresponding IP3 bursts observed in the pre-test session. Other network parameters in naïve non-respiring preparations were essentially identical to naïve respiring preparations, indicating a conserved ontogenetic mechanism regulating development and function of the respiratory CPG.

Analysis of CPG Network Properties in the Isolated CNS

The periphery is known to provide chemosensory input from the pneumostome and osphradial area and, thereby, modulate neural output (Inoue *et al.*, 2001). The aim of this section was to ascertain the effects of peripheral input on CPG activity by comparing data obtained from the isolated CNS to data obtained from the semi-intact preparation. Furthermore, by making the appropriate comparisons, I was able to determine the effects of operant conditioning on CPG output as well as the effects of differential rearing on network activity in the isolated CNS.

The CNS were removed from the semi-intact preparation and as in the semi-intact preparation, two sets of comparisons were conducted. Respiring naïve ganglia were compared to non-respiring naïve and straight-from-tank ganglia to determine the intrinsic cellular differences between the two developmentally dissimilar groups. Also, yoked ganglia were compared to

experimental ganglia to determine the effects of operant conditioning on cellular activity. The results are tabulated and presented in Table 1 (Appendix I).

IP3 Activity as Recorded from the VI Cell

As in the semi-intact preparations, the Bonferroni's corrected one-way ANOVA indicated there were differences in the number of IP3 bursts between the groups in the isolated ganglia (one-way ANOVA, $F(92,5) = 4.728$, $P = 0.0007$). There were significantly fewer IP3 bursts in naïve non-respiring and straight-from-tank non-respiring ganglia compared to naïve respiring ganglia ($P < 0.05$). There were also significantly fewer IP3 bursts in experimental respiring ganglia compared to yoked, results consistent with Spencer *et al.* (1999) ($P < 0.01$). Similarly, there were fewer IP3 bursts in experimental non-respiring ganglia compared to yoked non-respiring ganglia ($P < 0.05$). Taken together, the probability of intrinsic IP3 bursting activity was altered by both differential rearing and operant conditioning.

Interestingly, I now show differences in IP3 burst frequency among the groups in the absence of peripheral input (one-way ANOVA, $F(93,5) = 11.10$, $P < 0.0001$). That is, IP3 intensity was higher in the naïve non-respiring ganglia compared to naïve respiring ganglia ($P < 0.001$). This difference was not present in the semi-intact preparation (Fig. 16). Also unlike the semi-intact preparation, in the absence of peripheral input there were no differences in IP3 burst frequency in experimental ganglia compared to yoked ganglia and experimental non-respiring ganglia compared to yoked ganglia. These results strongly suggested a role of the periphery in modulating CPG output in both differential rearing and operant conditioning.

VI motor neuron activity was also examined in the isolated ganglia and the results were consistent with semi-intact activity (one-way ANOVA, $F(91,5) = 1.417$, $P = 0.2256$). There were no differences in VI firing frequency between the naïve respiring and naïve non-respiring ganglia. Furthermore, operant conditioning did not produce any changes in the VI frequency. I also determined that VI frequency was higher in the isolated ganglia compared to the semi-intact preparation, suggesting a peripheral inhibition of activity (VI frequency (Hz): naïve respiring and non-respiring semi-intact before poke, 3.2 ± 0.2, naïve respiring and non-respiring isolated ganglia, 3.8 ± 0.1; t-test, $P = 0.0096$, $df = 53$). Taken together, differential rearing or operant conditioning did not affect VI firing frequency, and the overall firing frequency is regulated by peripheral input.

RPeD1 Impulse Activity

RPeD1 parameters were hypothesized to be different between the non-respiring and respiring ganglia since excitatory peripheral input from the pneumostome/oshpradial ganglia would not be present in the non-respiring animals during development. Such differences were not observed in the semi-intact preparations but were observed in the isolated ganglia. The resting membrane potential was lower in respiring ganglia compared to non-respiring ganglia (resting membrane potential (mV): naïve, yoked, and experimental respiring, -57.5 ± 0.6, naïve, straight-from-tank, yoked, and experimental non-respiring, -55.4 ± 0.7; t-test, P = 0.0066, df = 61). All ganglia were included for this comparison since previously it was shown that operant conditioning does not affect this particular intrinsic membrane property (Spencer *et al.*, 1999). RPeD1 frequency was also analyzed and determined to be different between the various groups (one-way ANOVA, F(91,5) = 1.417, P = 0.2256). The firing frequency was higher in the naïve respiring compared to naïve non-respiring and straight-from-tank non-respiring ganglia (P < 0.0001). However, RPeD1 frequency in the isolated ganglia was significantly lower in operantly conditioned respiring ganglia compared to yoked respiring controls (P < 0.01). There was no significant difference between experimental non-respiring and yoked non-respiring ganglia using a Bonferroni's corrected one-way ANOVA, but a P value of 0.07 using a t-test indicated a trend for experimental non-respiring to demonstrate a reduced RPeD1 frequency compared to yoked non-respiring ganglia. I also found that RPeD1 activity was higher in the isolated CNS compared to the semi-intact CNS (RPeD1 frequency (Hz): naïve respiring and non-respiring semi-intact before poke, 0.41 ± 0.05, naïve respiring and non-respiring isolated ganglia, 0.71 ± 0.06; t-test, P = 0.0007, df = 51). This result suggested an inhibitory role of the periphery in regulating RPeD1 activity and has been reported previously by McComb *et al.* (2003).

Summary of Isolated CNS Data

Intracellular electrophysiological recordings from the isolated ganglia were used to re-examine the effects of operant conditioning and development on network properties, and the influence of peripheral input on these properties. Operant conditioning resulted in a reduction in the number of IP3 bursts in experimental ganglia and a reduction in RPeD1 firing frequency compared to yoked controls. Differential rearing affected the number of IP3 bursts, IP3 burst frequency, RPeD1 firing frequency, and also RPeD1 resting membrane potential. Finally, peripheral input was shown to modulate IP3, VI, and RPeD1 firing frequency and there is evidence to suggest an important role of this input in regulating behavioural and neural output.

Data from the semi-intact preparation provided both behavioural and neural indications of learning and memory in the experimental non-respiring *Lymnaea*. However, it remained to demonstrate a reduction in aerial respiratory behaviour in the intact animals. In an attempt to increase the number of pneumostome openings during the observation sessions and to increase the number of attempted pneumostome openings during the training sessions, the duration of each session was doubled to one hour.

Effects of Increased-Duration Training Sessions on Differentially Reared Lymnaea

Operant conditioning of the experimental non-respiring *Lymnaea* using the 'double-duration' paradigm resulted in a significant reduction in aerial respiratory behaviour (Fig. 21). A one-way ANOVA revealed a significant reduction in both the number of pneumostome openings (one-way ANOVA, $F(75,5) = 12.33$, $P < 0.0001$) and the total breathing time (one-way ANOVA, $F(75,5) = 23.27$, $P < 0.0001$) in the experimental non-respiring *Lymnaea* trained with the double duration paradigm. As such, these animals suppressed their hypoxic ventilatory response in a manner similar to experimental respiring *Lymnaea*.

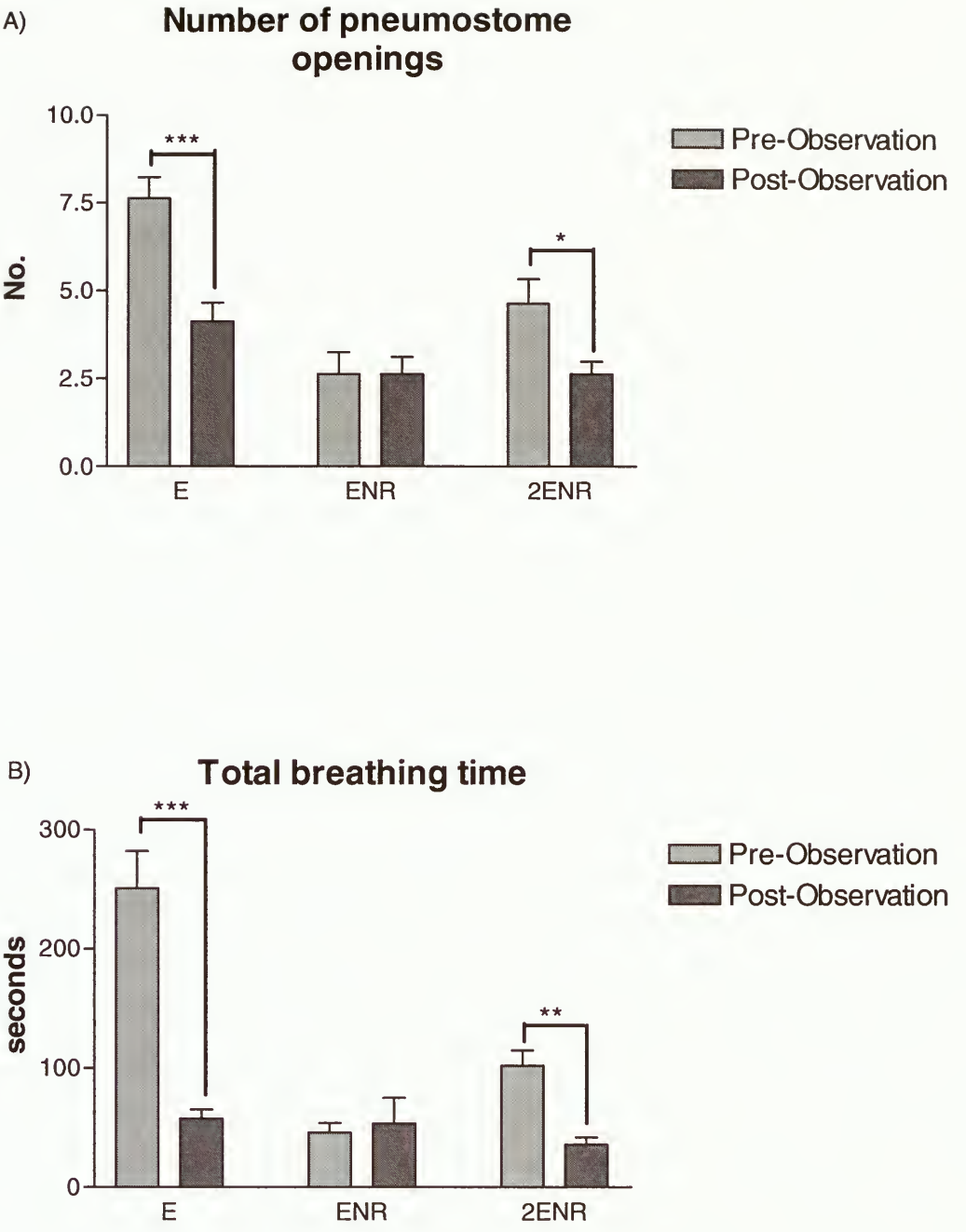


Figure 21: Aerial respiratory behaviour of the operantly conditioned intact *Lymnaea* in the pre- and post-observation sessions. Experimental respiring and experimental non-respiring data was previously presented in Figure 7. 2ENR represents experimental non-respiring animals trained with the 'double-duration' operant conditioning paradigm. There was a significant reduction in both (**A**) the number of pneumostome openings [number of pneumostome openings in the pre-observation session: experimental non-respiring (2ENR), 4.6 ± 0.7] and (**B**) the total breathing time [total breathing time (seconds) in the pre-observation session: experimental non-respiring (2ENR), 102 ± 13] following operant conditioning when the training time was doubled. (number of animals = 16 per group) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

Discussion

The freshwater mollusc *Lymnaea stagnalis* was used to further the understanding of how network properties change as a result of associative learning, and to determine whether or not this plasticity is dependent on previous experience during development. The respiratory and neural correlates of operant conditioning were first determined in normally reared *Lymnaea*. The same procedure was then applied to differentially reared *Lymnaea* to determine if these animals would demonstrate the same responses to the training paradigm. In this study, I show that animals prevented from performing aerial respiration during development can be operantly conditioned, and as such demonstrate behavioural and neural plasticity.

Respiration and the Respiratory CPG of Normally and Differentially Reared *Lymnaea*

Response to Hypoxia of Naïve Animals

Respiration is a fundamental behaviour that is under constant neural control. As an example, plasticity in the neural networks controlling respiration is necessary to adapt to both short-term and long-term hypoxic stresses. *Lymnaea stagnalis* can accomplish this by modulating its aerial respiratory behaviour (Jones, 1961); there is increased aerial respiration under hypoxic conditions and decreased aerial respiration under normoxic conditions. In these experiments, nitrogen bubbling was used to reduce the oxygen content of the artificial pond water and thereby increase the aerial respiratory drive of *Lymnaea stagnalis*. There were both qualitative and quantitative differences in the aerial respiratory behaviour of differentially reared animals in response to hypoxia. Normally reared naïve animals frequently and regularly demonstrated negative geotaxis, the directed movement toward the air-water interface (Hermann and Bulloch, 1998), for breathing purposes. The differentially reared animals did not exhibit these same behavioural responses; they did not perform aerial respiration as often as normally reared animals and generally did not resurface following the initial bouts of respiration. Interestingly, RPeD1 firing frequency was significantly lower in the isolated CNS of naïve non-respiring animals compared to naïve respiring animals. Since RPeD1 activity initiates the respiratory cycle, this reduced RPeD1 frequency likely resulted in the reduced aerial respiratory behaviour in the intact animal.

Differences in RPeD1 activity may have been due to reduced peripheral sensitivity to dissolved oxygen in differentially reared animals compared to normally reared animals. In other words, one effect of differential rearing may be atypical function of chemosensory receptors that

indicate environmental hypoxia. RPeD1 activity is regulated by peripheral input and in non-respiring animals, presumably there was reduced excitatory input to RPeD1 during development. Since non-respiring *Lymnaea* did not surface during development and experience an aerial environment, activity-dependent synaptic plasticity within the network may have been affected (Mitchell and Johnson, 2003). Furthermore, both hypoxia and hyperoxia during development impairs adult ventilatory control, partly due to aberrant function or number of chemoafferant neurons (Erickson *et al.*, 1998; Joseph, *et al.*, 2000). Hence, even in hypoxic conditions, there might be reduced sensory input to RPeD1. This may have led to reduced RPeD1 impulse activity and reduced aerial respiratory behaviour in differentially reared animals.

There may be other physiological changes during development which may have led to differentially reared animals being better adapted to hypoxic conditions. For example, a high haemocyanin content might have reduced the requirement for aerial respiration of the differentially reared animals. Hermann and Bulloch (1998) have previously argued that it is unlikely these differentially reared animals possess increased haemocyanin levels and/or enhanced oxygen use since they consider it unlikely the animals experienced hypoxia during development. The rationale was that in the normoxic rearing tanks, the oxygen saturation of haemocyanin, the haemolymph oxygen carrier in *Lymnaea*, is 100% (Dawson and Wood, 1982, 1983). However, this assumption may be incorrect and acclimatization to an environment that does not permit aerial respiration may include such a physiological adaptation. Haemolymph pO_2 of a differentially reared animal is likely lower than that of a normally reared animal. A Western blot would determine exactly the haemocyanin content in the haemolymph, but was not performed in this study.

Analysis of Respiration and CPG Activity in Naïve Semi-Intact Preparations

Naïve respiring and non-respiring animals were dissected into semi-intact preparations to determine the effects of differential rearing on respiration and CPG activity. Straight-from-tank non-respiring animals were also incorporated in this study to analyze the network parameters during the preparations first ever bouts of aerial respiration to determine if they were 'normal'. As the results suggest, the straight-from-tank preparations spontaneously performed aerial respiration when the saline was lowered to the level of the pneumostome. Furthermore, there were no qualitative differences in the respiratory behaviour or the motor program dictating pneumostome openings in these preparations compared to naïve respiring preparations. In other words, RPeD1 activity resulted in IP3 bursts that produced pneumostome openings. Thus, aerial

respiration is ontogenetically programmed and the underlying neural network is maintained, regardless of previous experience during development (Hermann and Bulloch, 1998).

Similar to the intact animals from which they were dissected, naïve non-respiring preparations performed aerial respiration less often than naïve respiring preparations. However, unlike the intact animals, there was no significant difference in total breathing time between the two groups. It is likely that this difference in respiratory activity may be reflective of the nature of the dissection and the experiment. In my semi-intact preparation, the pneumostome is propped up, raised higher than the body to prevent the body from drying during the recording session. In doing so, the pneumostome is held at the air-water interface. It has been shown that sensory input from the pneumostome and osphradial ganglion provides input to RPeD1 to initiate CPG activity (Inoue *et al.*, 2001; Wedemeyer and Schild, 1995). Thus, 'holding' the pneumostome at the air-water interface may be providing the sensory input required to induce similar levels of aerial respiration in both respiring and non-respiring naïve preparations. It is also possible that if a longer recording period had been used, the significance would be re-established, since intact respiring animals perform aerial respiration regularly during observation sessions while non-respiring animals surface less often following the initial bouts of respiration. The straight-from-tank non-respiring preparations, on the other hand, did not demonstrate a similarly high total breathing time despite continuous exposure to the air-water interface. Inexperience likely contributed to this effect as the naïve respiring and non-respiring animals had performed aerial respiration as intact animals, whereas the straight-from-tank had not. Thus, straight-from-tank may be 'learning' to perform aerial respiration and as such do not perform aerial respiration to the same extent as respiring and non-respiring naïves.

Operant Conditioning of Normally Reared *Lymnaea*

The study of learning and memory can be approached behaviourally, which emphasizes the association between the behaviour, the stimuli, and the consequence, or neurologically, which addresses the underlying neural mechanisms. A number of associative conditioning paradigms exist for *Lymnaea stagnalis*, including appetitive and aversive classical conditioning of feeding behaviour (Benjamin *et al.*, 2000) and aversive operant conditioning of aerial respiratory behaviour (Lukowiak *et al.*, 1996). Here, I evaluated the behavioural and neural correlates of operant conditioning of aerial respiration in the intact animal, the semi-intact preparation, and the isolated ganglia.

Behavioural Analysis of Operant Conditioning in Normally Reared Lymnaea

Compared to the pre-observation session, the conditioned respiring preparations demonstrated a significant reduction in both the number of pneumostome openings and the total breathing time in the post-observation session. Since these behavioural changes were not observed in the naïve and yoked controls, only the contingent application of the reinforcing stimulus to the open pneumostome acted to produce a reduction in aerial respiratory behaviour in the experimental *Lymnaea*. Consequent to the punishment, these animals attempted aerial respiration fewer times as training progressed.

In future experiments, it may not be necessary to perform the pre- and post-observation sessions to assess learning and memory. The results indicated that experimental animals that demonstrated fewer attempted pneumostome openings in training session 4 (TS4) and the memory test (MT) compared to training session 1 (TS1) also performed aerial respiration fewer times and for a shorter duration in the post-observation session compared to the pre-observation session. Thus, a significant reduction in the number of attempted pneumostome openings during the sessions is a sufficient marker of learning and memory. In this study, all experimental animals were 'good learners'. That is, there was > 50% reduction in the number of attempted pneumostome openings in TS4 and the MT compared to TS1 (Spencer *et al.*, 1999, 2002). This modified training protocol produced more 'good learners' than the original paradigm established by Lukowiak *et al.* (1996), possibly because their protocol permitted aerial respiration in between training sessions whereas mines did not. The unpunished aerial respiration in their study may have acted as interfering events in the formation of LTM. In support of this, Sangha *et al.* (2003a) have previously shown that LTM was prolonged if *Lymnaea* were prevented from freely performing aerial respiration following the final training session.

When dissected into the semi-intact preparations, the conditioned animals showed significantly fewer openings in the pre-test compared to yoked controls. These data further support the evidence that semi-intact preparations behave similarly to the intact animals from which they were dissected (Lowe, 2004). The semi-intact preparations then received a contingent poke to the open pneumostome and it was observed that the conditioned preparations did not attempt a pneumostome opening for a significantly longer duration compared to yoked controls. However, there was no further significant reduction in aerial respiratory behaviour of conditioned preparations in the post-test compared to the pre-test. Spencer *et al.* (2002) previously demonstrated a significant reduction in aerial respiratory behaviour only following the application of the reinforcing stimulus. Though seemingly contradictory, I must note the differences in training protocols. Lukowiak *et al.* (1996) permitted breathing between training

sessions and the signal (i.e. discriminative stimulus) for the animals that training had started was the first tactile stimulus. Thus, the tactile stimulus produced a subsequent reduction in aerial respiration. In the current study, respiration was not permitted between training sessions and the signal for training was the removal of the barrier and the ability to re-perform respiration. Thus, the semi-intact preparations likely expressed memory of the training as soon as the saline was lowered to the level of the pneumostome and the preparation was physically able to perform aerial respiration again.

Analysis of RPeD1 following Operant Conditioning in Normally Reared Lymnaea

The changes underlying learning and memory are generally not confined to a single locus, but rather occur at multiple sites within the neural network (Benjamin, *et al.*, 2000; Brembs, 2003; Spencer *et al.*, 1999, 2002). Thus, dual-cell electrophysiology was utilized to directly assay the electrical activity of both RPeD1 and the VI motor neuron, and IP3 activity was indirectly monitored as characteristic bursting activity within the VI cell. Operant conditioning was hypothesized to alter the synaptic inputs and/or the intrinsic membrane properties of the cells comprising the respiratory CPG.

No changes were observed in the RMP of RPeD1 between yoked and experimental preparations, which confirms previous reports by Spencer *et al.* (1999). Previously, RPeD1 frequency has been shown to decrease following the tactile stimulus in the semi-intact preparation but in this study, no changes in RPeD1 frequency were apparent as a result of the application of the stimulus (Spencer *et al.*, 2002). Again, these results may be reflective of the differences in the training paradigms employed. As explained above for the number of pneumostome openings, it is likely that in my preparations the expression of LTM was not dependent on the tactile stimulus but the ability to perform aerial respiration. In support of this, I observed a low RPeD1 firing frequency in the pre-test and did not see a significant reduction in the post-test. Nevertheless, there was a significant effect of operant conditioning on RPeD1 activity in the semi-intact preparation; RPeD1 was quiescent for a significantly longer duration in experimental preparations compared to yoked preparations, following the reinforcing stimulus. Given the recent findings by Lowe (2004) that experimental hyperpolarization of RPeD1 augmented LTM formation, I speculate that this quiescence may represent a neural encoding of LTM. However, it does not explain why the same effect was observed in the straight-from-tank non-respiring preparations, and to an extent in naïve non-respiring preparations. In this case, the quiescence may represent suppressed activity as a result of rearing conditions and response to hypoxia. I also demonstrated a significant reduction in the RPeD1 firing frequency in the isolated

CNS derived from experimental animals compared to yoked controls. Thus, RPeD1 remains an important locus in the expression of operant conditioning.

Interestingly, both operant conditioning and differential rearing resulted in a reduced aerial respiratory behaviour and reduced RPeD1 impulse activity in the isolated CNS. This is a clear indication that a decrease in RPeD1 activity leads to a decrease in behaviour. However, since differentially reared animals appear to be more tolerant of hypoxia, it is possible operant conditioning also leads to an increase in tolerance to hypoxia. Thus, a conserved neural mechanism may be responsible for producing the same behavioural output.

Analysis of IP3/VI following Operant Conditioning in Normally Reared Lymnaea

In addition to RPeD1, I provide evidence to suggest that IP3 is an important locus in the formation or expression of LTM. I show in the semi-intact preparation that the intensity and number of spontaneous IP3 bursts recorded from the VI pneumostome opener motor neuron was reduced in experimental animals compared to yoked controls. These differences corresponded well with the behaviour of the intact animal and the semi-intact preparations. Experimental animals demonstrated an 'unwillingness' to open the pneumostome despite hypoxic conditions, which was reflected in the reduced IP3 burst intensity that produced smaller and weaker pneumostome openings in the semi-intact preparations compared to controls. Experimental animals also attempted aerial respiration fewer times than controls, which was reflective of the reduced number of IP3 bursts recorded from the visceral I cell.

I also show that even in the event of an IP3 burst, a pneumostome opening was not necessarily observed in experimental preparations. In other words, the correlated bursting/pneumostome opening activity was significantly reduced following operant conditioning. Since IP3 activity was observed in the VI cell, there are likely changes in synaptic connections between IP3 and VI or VI and the pneumostome opener muscles. Previously, it has been shown that there was a loss of correlation of RPeD1 and IP3 activity following operant conditioning (McComb *et al.*, 2005a; Spencer *et al.*, 1999). Together, these results demonstrate synaptic remodeling throughout the CPG as a result of conditioning and that changes in IP3 activity may also manifest independently of changes in RPeD1 activity. In future experiments, direct recordings and manipulation of IP3 activity will demonstrate conclusively the role of the cell in producing the behavioural adaptations to operant conditioning.

There were no differences in the VI motor neuron firing frequency between the experimental and yoked preparations. However, there was a lag in pneumostome opening response following IP3 activity in the VI cell of experimental preparations, which may indicate a

change in neuromuscular transmission. This may include decreased transmitter release from the presynaptic VI cell or decreased excitability of the postsynaptic pneumostome opener muscles (Kandel, 2001). Thus, there is an additional loss of correlated activity between the VI cell and the pneumostome.

Taken together, I have identified additional correlates of operant conditioning in *Lymnaea stagnalis*. I provide evidence that there is encoding of LTM not only within the respiratory CPG but also likely within the motor neuron and its connections with the pneumostome opener muscles. These finding are consistent with recent literature; modulation has been documented to occur at all levels of the nervous system, including the CPG, the sensory, motor, and facilitating neurons, the sensory organs, and the musculature (Harris-Warrick and Marder, 1991).

Operant Conditioning of Differentially Reared *Lymnaea*

In the differentially reared *Lymnaea*, I presumed the respiratory CPG was inactive during development. Inactivity of the CPG may have led to different synaptic connectivity patterns or synaptic strengths compared to normally reared animals, as presumably these connections would not have been subjected to activity-dependent modulation during development. I thus sought to determine if previous experience and CPG activity during development was necessary for plasticity of the network in the adult. This was assessed by operant conditioning of its aerial respiratory behaviour. Having identified the correlates of operant conditioning in normally reared animals, the training paradigm was applied to the differentially reared, non-respiring *Lymnaea* and evidence of plasticity was ascertained from the ability of the differentially reared animals to modulate respiratory and neural activity.

Behavioural Analysis of Operant Conditioning in Differentially Reared Lymnaea

The first important observation was that operant conditioning of differentially reared, intact animals did not produce a significant reduction in the aerial respiratory behaviour. That is, there were no significant changes in the number of pneumostome openings or the total breathing time from the pre- to the post-observation session, or the number of attempted pneumostome openings from TS1 to TS4 and the MT. Thus, using the definition previously established, differentially reared *Lymnaea* did not demonstrate learning or LTM when conditioned with the same paradigm as normally reared *Lymnaea* (Lukowiak *et al.*, 1996; Spencer *et al.*, 2002). Accordingly, semi-intact preparations derived from experimental non-respiring animals did not

demonstrate any significant differences in number of pneumostome openings and total breathing time compared to their yoked controls. Thus, an important question that needed to be addressed was, "are differentially reared *Lymnaea* capable of associative learning?" There were some behavioural indications of learning and memory in the semi-intact preparation. For example, the experimental non-respiring preparations demonstrated the greatest reduction in aerial respiratory behaviour in the post-test session. Furthermore, these preparations showed the same increase in lag time between the application of the reinforcing stimulus and the next pneumostome opening, as did the experimental respiring group. These modifications, although statistically insignificant, were the first indications of behavioural plasticity in non-respiring animals.

It can be argued that aerial respiration could not be further reduced in the non-respiring animals following conditioning since they started with such a low level of expression of the behaviour. Furthermore, since they did not attempt aerial respiration as often as respiring animals, they may not have received enough reinforcing stimuli to produce the operant response (Papini and Bitterman, 1990; Terry, 2003). I addressed this by increasing the duration of the training sessions such that the experimental non-respiring animals performed aerial respiration for a longer duration in the pre-observation session and received a similar number of stimuli as experimental respiring animals in TS1. Indeed the results showed that non-respiring *Lymnaea* significantly reduce the total breathing time and the number of attempted pneumostome openings following operant conditioning (Fig. 21). Thus, there is behavioural data to indicate that differentially reared animals do behaviourally express learning and memory and can exhibit plasticity.

Neural Correlates of Operant Conditioning in Differentially Reared Lymnaea

Though no significant behavioural differences were noted in the intact animal or the semi-intact preparation using the half-hour training sessions, the semi-intact preparations did show some behavioural indications of learning and memory. Furthermore, it has been documented that learning and memory can occur in the absence of a clear behavioural output (Terry, 2003). Thus, I looked for evidence of plasticity within the respiratory neural network. Significant differences in neural activity as a result of operant conditioning were observed in two parameters: the latency of IP3 activity-to-pneumostome opening and percent correlated IP3 activity and pneumostome opening. These results were similar to those obtained using experimental respiring preparations. Since these changes were the only results to demonstrate significance, it may indicate that the synapses between IP3 and VI and VI and the pneumostome opener muscles are the most plastic and vulnerable to neuromodulation. Indeed by altering the

correlated activity of these cells and the muscles, there would be a behavioural reduction in aerial respiration.

Other network properties were also affected by training, albeit insignificantly. For example, RPeD1 firing frequency was reduced in experimental non-respiring preparations compared to yoked controls. This was similar to the experimental respiring preparations, which also did not demonstrate significance. However, unlike experimental respiring preparations, I did not demonstrate a significantly increased duration of RPeD1 quiescence following the reinforcing stimulus in the experimental non-respiring preparations. Also, the number of IP3 bursts and the average IP3 burst frequency were not significantly different from yoked controls. Nevertheless, the differentially reared specimens manifested a number of the neural correlates of operant conditioning. This provided evidence to support an activity- and experience-independent development and plasticity of the respiratory CPG in the differentially reared *Lymnaea stagnalis*. As such, I hypothesize that animals trained using the longer training sessions will likely demonstrate the same significant changes in network properties as experimental respiring preparations. Future studies will focus on investigating the CPG activity of non-respiring animals trained for a longer duration.

Conclusion

Dudai and Carruthers (2005) eloquently define neural plasticity as the "general hardware mechanisms that enable brains to adapt to change". In this study I demonstrated, using *Lymnaea stagnalis*, that the mechanisms underlying plasticity of aerial respiratory behaviour are not compromised by differential rearing. There was little behavioural data to suggest learning and memory using the initial paradigm, but examination of respiratory behaviour and neural network parameters in the semi-intact preparations supported the fact that operantly conditioned differentially reared animals had responded to the training paradigm. Preliminary evidence with a new paradigm further support the notion that differentially reared animals can be conditioned to reduce aerial respiratory behaviour. Future experiments can utilize this new paradigm and I can compare and contrast network changes. I conclude that *Lymnaea* that had never experienced aerial respiration during development could modify the occurrence of the behaviour. Thus, I demonstrate plasticity is not dependent on previous experience during development, but is rather ontogenetically programmed within the neural network.

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Appendix I

Cell	Parameter	N	NNR +STNR	Sig.	Y	E	Sig.	YNR	ENR	Sig.
IP3	# of IP3 bursts	13 ± 1	10 ± 1	Yes	11 ± 1	8 ± 1	Yes	13 ± 1	10 ± 1	Yes
	Burst frequency	6.2 ± 0.4	7.9 ± 0.2	Yes	6.0 ± 0.3	5.8 ± 0.3	No	8.3 ± 0.5	8.0 ± 0.3	No
VI	Firing frequency	3.9 ± 0.2	4.1 ± 0.2	No	3.8 ± 0.2	3.5 ± 0.2	No	4.2 ± 0.3	4.2 ± 0.3	No
RPeD1	RMP	-57.3 ± 1.0	-55.6 ± 0.8	No	-57.8 ± 0.7	-57.5 ± 1.1	No	-55.1 ± 1.5	-54.5 ± 1.1	No
	Firing frequency	0.97 ± 0.08	0.47 ± 0.04	Yes	0.96 ± 0.09	0.62 ± 0.06	Yes	0.62 ± 0.07	0.45 ± 0.06	No

Table 2: Network properties in isolated ganglia. For these analyses, I grouped naïve non-respiring and straight-from-tank non-respiring into one category. A Bonferroni's corrected one-way ANOVA was used to determine significance between the various comparisons.