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Novel neural correlates of operant conditioning in normal and differentially reared *Lymnaea*

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SUMMARY

The aerial respiratory behaviour of the mollusc *Lymnaea stagnalis* is an important homeostatic behaviour that can be operantly conditioned. The central pattern generator underlying this behaviour, as well as motorneurons innervating the respiratory orifice, the pneumostome, have been identified and their activity can be monitored in the semi-intact preparation using electrophysiological recordings. In this study, we used both intact animals and semi-intact preparations to identify novel changes in the respiratory central pattern generator following operant conditioning. In addition, we reared animals in the absence of this respiratory behaviour throughout development, to investigate whether previous experience and activity-dependent plasticity during development are essential to allow neural plasticity in the adult. We found that animals raised normally (allowed to perform aerial respiratory behaviour) exhibited the expected reduction in aerial respiratory behaviour following operant conditioning. Then, using the semi-intact preparation, we identified novel neural changes within the network as a result of the conditioning. These included specific changes at the level of the central pattern generator interneurons, as well as the motor output. In the differentially reared intact animals, there was no behavioural reduction as a result of operant conditioning, although their baseline respiratory behaviour was already significantly reduced as a result of their differential rearing. There were, however, significant differences found in the network parameters in the semi-intact preparation, similar to those observed in normally reared animals. We thus provide evidence for neural plasticity within the network in the absence of significant behavioural changes in differentially reared animals, and show that plasticity was not dependent on previous activity of the network during development.

Key words: learning, memory, invertebrate, aerial respiration, central pattern generator, semi-intact preparation, mollusc.

INTRODUCTION

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 predicts future outcomes and modifies its behaviour accordingly. The freshwater pond snail *Lymnaea stagnalis* is a useful model for investigating the neural correlates and cellular mechanisms of operant conditioning (Lukowiak et al., 1996). Specifically, the aerial respiratory behaviour of *Lymnaea* can be operantly conditioned and resultant neural changes are manifested within the neural network, the respiratory central pattern generator (CPG) regulating this behaviour (Spencer et al., 1999; Spencer et al., 2002; McComb et al., 2005; Lowe and Spencer, 2006).

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). *Lymnaea*'s aerial respiratory behaviour can be operantly conditioned to demonstrate long-term memory (LTM) (Lukowiak et al., 1996; Lukowiak et al., 1998). This is accomplished by the application of an aversive stimulus to the pneumostome area with each attempted pneumostome opening. This results in the immediate closure of the pneumostome and cessation of aerial respiration. Thus, 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).

Like most rhythmic behaviours, this aerial respiratory behaviour is controlled by a CPG, the cellular components of which have been identified (Syed et al., 1990; Syed and Winlow, 1991). This particular respiratory CPG consists of three monosynaptically connected interneurons located within the central ganglionic ring: 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 (Syed et al., 1990; Syed et al., 1991; Syed and Winlow, 1991). RPeD1 is a large neuron and chemosensory information concerning oxygen partial pressure is thought to be relayed to RPeD1 (Bell et al., 2008; Inoue et al., 2001; Syed and Winlow, 1991; Wedemeyer and Schild, 1995). IP3 interneuron activity produces a pneumostome opening *via* monosynaptic, excitatory connections with the pneumostome opener motorneurons, the visceral I and J (VI/J) cells. VD4 activity produces pneumostome closing. The respiratory CPG cycle is initiated by hypoxia-induced patterned activity in RPeD1 (Inoue et al., 2001).

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 (Hermann and Bulloch, 1998) have previously demonstrated in this way, that aerial respiratory behaviour in *Lymnaea* develops independently of experience. However, it has not yet been determined whether the ability of the CPG to change during operant conditioning is independent of previous behaviour and experience. One main aim of this study was to investigate the plasticity of the

respiratory CPG in differentially reared animals, that is, in animals reared with no prior experience of the aerial respiratory behaviour. We sought to determine if the ability to exhibit higher-order plasticity (i.e. associative learning) is dependent on experience during development.

Previous operant conditioning studies in *Lymnaea* have identified a number of neuronal changes occurring within the respiratory CPG. In particular, RPeD1 has been identified as an important locus of learning and memory, and there is evidence that gene transcription in RPeD1 is necessary for formation of LTM (Scheibenstock et al., 2002). Lowe and Spencer (Lowe and Spencer, 2006) also demonstrated that artificially silencing RPeD1 in a semi-intact preparation reduced the number of training sessions required to produce LTM. However, it is clear from previous studies that the neural correlates underlying this behaviour are dispersed throughout the network and may include changes in other CPG interneurons such as the IP3 interneuron, as well as motorneuron connections (Spencer et al., 1999; McComb et al., 2005). The second main goal of this study was to utilize the semi-intact preparation, in which both pneumostome behaviour and CPG neural activity can be simultaneously monitored (Lowe and Spencer, 2006), to identify novel neural correlates of operant conditioning of the aerial respiratory behaviour.

MATERIALS AND METHODS

Specimens

Normally reared *Lymnaea*

Specimens of *Lymnaea stagnalis* L., 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, OH, USA). The breeding containers were open to the atmosphere and animals were freely able to perform aerial respiration at any time. The snails were kept on a light:dark cycle of 12 h:12 h and a diet consisting of Spirulina algae flake food (Nutrafin Max; Rolf C. Hagen, 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 were unable to perform aerial respiration throughout development. Clear, plastic breeding containers with fine mesh walls were submerged in well-aerated, artificial pond water aquariums. Special care was taken to ensure that no air bubbles were trapped in the enclosures. A single egg sack was hatched under each enclosure and snails were raised to adulthood (3–6 months) without ever experiencing aerial respiration. The snails were kept on the same light:dark cycle and the same diet as normally reared animals. These snails were age-matched (3–6 months) to normally reared snails but as reported previously, some 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. No differences in behaviour between the smaller and larger adults were found in this study, as also found previously by Hermann and Bulloch (Hermann and Bulloch, 1998).

Procedures

Operant conditioning

Snails were selected and randomly assigned to one of three groups: naïve, yoked or conditioned. During the training sessions and the memory test, naïve animals were allowed to freely perform aerial respiration. The operantly conditioned group received a punishing tactile stimulus that was contingent on the animal opening its pneumostome at the air–water interface to perform aerial respiration. The stimulus was of sufficient intensity to cause immediate pneumostome closure. Yoked animals also received the tactile stimuli, but these were not contingent on pneumostome opening. Instead, they were applied to the pneumostome area and timed according to the stimulus given to the conditioned animal to which it was yoked.

Snails were individually identified by a series of markings on their shells and placed in an 800 ml beaker with well-aerated pond water. The snails were given 10 min to habituate and explore the new environment. 100% nitrogen gas was bubbled into the water for 10 min prior to and for the duration of each session in order to induce the hypoxic ventilatory response. Generally, there was a 10-fold reduction in oxygen content after the 10 min period. After the acclimatization period, 300 ml of water were siphoned out of the beaker at which point the snails entered the 30 min pre-observation session, during which all animals were allowed to freely perform aerial respiration. The number of pneumostome openings and total breathing time were monitored and calculated for each animal. We did not wish the differentially reared animals to surface between training sessions as this has been shown to affect learning and memory (Sangha et al., 2003). Thus, the beaker was capped for both normally reared and differentially reared animals between training sessions, preventing them from surfacing. Thus, in this modified procedure, the discriminative stimulus was aerial respiration and not the application of the first contingent physical stimulus to signify the beginning of each training session. One hour after the pre-observation period, the animals entered the first of four 30 min training sessions. The number of attempted pneumostome openings was recorded for the conditioned animals while the number of pneumostome openings and total breathing time were determined for both naïve and yoked control groups. Each of the four training sessions was separated by 1 h to allow consolidation of memory (Lukowiak et al., 2000). Eighteen 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 and the post-training number of pneumostome openings and total breathing time were determined for all animals.

Dissection of semi-intact preparations

A similar approach to that of Lowe and Spencer (Lowe and Spencer, 2006) was used to dissect the semi-intact preparations. Briefly, snails were anaesthetized in a *Lymnaea* saline solution (composition in mmol l^{-1}: 51.3 NaCl, 1.7 KCl, 4.1 $CaCl_2 2H_2O$; 1.5 $MgCl_2 6H_2O$, 5 Hepes buffer, pH to 7.9 with NaOH) containing 30% Listerine (Pfizer Canada, Toronto, Canada) for 3 min. The anaesthetic agent in Listerine is menthol and its use does not affect memory formation (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 easily view the openings. A medial incision from the base of the mantle to the head was made to expose the inner cavity. The oesophagus 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. All preparations were then given 20–30 min to recover from surgery prior to electrophysiological and behavioural analysis.

Electrophysiological recordings

Intracellular recordings were simultaneously obtained from the RPeD1 and the VI cell in semi-intact preparations using standard

electrophysiological techniques (Spencer et al., 1999; Spencer et al., 2002). Located on the dorsal surface, one VI cell is morphologically distinct and is easily identified as the largest of the pneumostome opener motorneurons. Cell penetration was aided by proteolytic enzymatic treatment (Protease, Type IX; Sigma-Aldrich, MO, USA) over the surface of the right pedal and the visceral ganglia. Glass microelectrodes with a resistance of 20–60 MΩ were pulled on a Kopf electrode puller (Model 730; David Kopf Instruments, CA, USA) and back filled with saturated K_2SO_4. Signals were obtained using a Neuro Data IR283A amplifier (Cygnus Technology, PA, USA) connected to a PowerLab/4SP digital acquisition system (AD Instruments, CO, USA) and Chart recording software (v4.2; AD Instruments).

Data and statistical analysis

In the intact animal, the respiratory parameters were quantified across all sessions. For intact animal data, statistical analysis incorporated a repeated measures design. A two-way repeated measures analysis of variance (two-way RM-ANOVA) was used to test for a possible interaction effect between two independent variables: the treatment group and the training sessions. All *post-hoc* analyses were carried out using a Tukey–Kramer test.

In the semi-intact preparation, all parameters were quantified over a 5–10 min recording session and significance was established using a two-way ANOVA design with rearing conditions as one factor and training as the second factor. Tukey–Kramer *post-hoc* tests were conducted to determine significance and results were considered significantly different if a P value of less than 0.05 was achieved. All data analyses were carried out using GB-Stat (Dynamic Microsystems, MD, USA). In all figures, the error bars represent the standard error of the mean.

RESULTS

The two main goals of this study were to define novel changes in the network properties after operant conditioning of the respiratory behaviour of *Lymnaea*, and also to determine whether animals reared in the absence of this behaviour can demonstrate behavioural or neural changes associated with the training procedure. This allowed us to determine whether neural and behavioural plasticity are dependent on previous experience.

For intact animal experiments, each animal received a pre- and post-observation session before and after training respectively, during which the animals could freely perform aerial respiration. Long-term memory (LTM) was operationally defined as a significant reduction in the number of pneumostome openings and total breathing time in the post-observation session compared with the pre-observation session. Operantly conditioned animals were 'punished' by contingent application of a tactile stimulus to every pneumostome opening, whereas yoked animals received a tactile stimulus that was not contingent on a pneumostome opening. Naïve animals received no stimulation and were allowed to breathe freely in each training session.

A two-way RM-ANOVA of all intact animal groups (naïve, yoked and conditioned) revealed a significant interaction effect between the two independent variables (treatment group and session) for both the number of openings ($F_{(5,90)}$=2.96; P=0.01) and total breathing time ($F_{(5,90)}$=7.43; P<0.0001) for the pre-test session and post-test session. Further *post-hoc* analysis indicated significant differences in various groups as discussed below.

Analysis of the aerial respiratory behaviour in normal and differentially reared naïve animals

Hermann and Bulloch (Hermann and Bulloch, 1998) have previously shown that differentially reared *Lymnaea* are capable of performing aerial respiration when permitted to do so. We first sought to determine what differences existed in the aerial respiration of normally and differentially reared *L. stagnalis* using our experimental set-up. Aerial respiration was quantified in naïve animals to determine baseline respiratory behaviour under hypoxic conditions.

Post-hoc analysis of the respiratory parameters in naïve animals indicated a significant difference in the response between the two groups. Normally reared naïve animals performed aerial respiration more often than their differentially reared naïve counterparts in the pre-observation session (P<0.01; Fig. 1A). Normal naïve animals also performed aerial respiration for a longer duration compared with the naïve differentially reared animals in the pre-observation session (P<0.01; Fig. 1B). 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

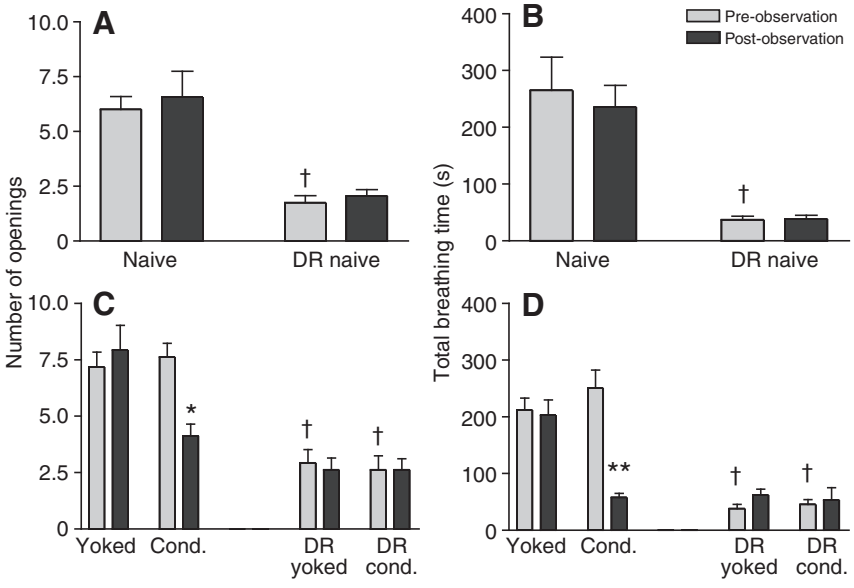

Fig. 1. Respiratory behaviour of intact animals before and after the operant conditioning. (A) Differentially reared naïve animals showed significantly fewer pneumostome openings (DR naïve: 1.8±0.3; †P<0.01) in the pre-observation period than normally reared naïve animals (6.0±0.6). (B) Differentially reared animals also showed a reduced total breathing time (37±7 s; †P<0.01) compared with normally reared naïve animals (265±58 s). (C) In the pre-observation period, the differentially reared yoked and conditioned animals showed significantly fewer pneumostome openings (DR yoked: 2.9±0.6, DR cond.: 2.6±0.6; †P<0.01) than the normally reared yoked and conditioned (Cond.) animals (Yoked: 7.2±0.7, Cond.: 7.6±0.6). Only the normally reared conditioned animals showed a significant reduction in the number of pneumostome openings from the pre- to the post-observation session, following the operant conditioning (*P<0.05). (D) Only normally reared conditioned animals showed a significant reduction in total breathing time from the pre- to the post-observation period, following the training (**P<0.01). N=16 for each group.

(P>0.05). However, there were no differences in the qualitative nature of the pneumostome openings between normally and differentially reared animals [as also previously shown by Hermann and Bulloch (Hermann and Bulloch, 1998)]. Taken together, in hypoxic conditions, differentially reared animals performed aerial respiration significantly less often than normally reared animals.

Effects of operant conditioning on aerial respiration of intact animals

We next aimed to determine how the ability to modify aerial respiratory behaviour was affected by rearing conditions. *Post-hoc* analysis indicated that prior to training, again, normally reared animals exhibited more respiratory behaviour than the differentially reared animals (pre-observation session: P<0.01; Fig. 1C). The same was true when total breathing time was considered (P<0.01; Fig. 1D). Importantly, only the normally reared conditioned animals showed a significant reduction in the number of pneumostome openings (P<0.05) and total breathing time (P<0.01) from the pre- to the post-observation session. The differentially reared conditioned animals did not show a similar reduction in aerial respiratory behaviour as a result of the operant conditioning. There were no significant changes in the number of pneumostome openings (Fig. 1C) or total breathing time (Fig. 1D) in differentially reared animals from the pre- to the post-observation session. There were no significant differences in the yoked control groups, demonstrating that the non-contingent stimulus did not cause any reduction in aerial respiratory behaviour.

The associative learning demonstrated by the normally reared conditioned group was further analyzed by the construction of a learning curve (Fig. 2). Learning has been operationally defined in this model as the significant reduction in number of attempted pneumostome openings from training session 1 (TS1) to TS4 (Lukowiak et al., 1996; Spencer et al., 1999; Spencer et al., 2002; Lowe and Spencer, 2006). If learning occurred, then LTM is defined as the significant reduction in attempted pneumostome openings from TS1 to the memory test (MT) (Lukowiak et al., 1996; Lowe and Spencer, 2006). The two-way RM-ANOVA showed a significant interaction effect ($F_{(20,360)}$=11.69; P<0.0001). *Post-hoc* analysis showed that the normally reared conditioned animals demonstrated both learning and LTM with our training procedure (P<0.01). However, the differentially reared 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. 2). Therefore, by our definition, these animals were unable to learn and form LTM using the *same training* as used for the normally reared conditioned animals. Interestingly, however, the normally reared conditioned animals reduced their aerial respiratory activity following training to the initial pre-training level observed in the differentially reared animals. In other words, there were no significant differences in the level of respiration between the normally reared animals in the *post*-observation sessions and the differentially reared animals in the *pre*-observation sessions. These data suggest that the level of respiration observed following training in the normally reared animals but *prior* to training in the differentially reared animals may be a minimum level of respiration required in this hypoxic environment.

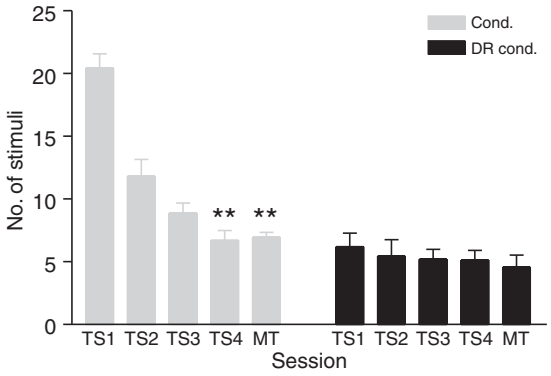

Fig. 2. Only the normally reared conditioned animals demonstrated a reduction in behaviour across the training sessions and memory test. Whereas normally reared conditioned animals (Cond.) showed a significant reduction (**P<0.01) in the number of attempted openings from session 1 (TS1: 20.4±0.6) to session 4 (TS4: 6.7±0.8) and the memory test (MT: 6.9±0.6), differentially reared conditioned (DR cond.) animals did not show a reduction in aerial respiratory behaviour as a result of the operant conditioning (TS1: 6.2±1.1, TS4: 5.1±0.8, MT: 4.6±1.0).

Behavioural and neural correlates of learning and memory in the semi-intact preparation

Despite the demonstration above that differentially reared animals were unable to show a reduction in their respiratory behaviour following the operant conditioning, it is possible that their initial level of respiratory activity was too low to be further reduced by the training procedure. Therefore, when analyzing the neural correlates of the normally reared animals in a semi-intact preparation, we chose also to analyze the differentially reared animals in order to determine, despite the lack of behavioural change, whether they demonstrated any evidence of neural changes associated with the training. According to the parameters defined above, there was no evidence to support LTM formation in the intact differentially reared conditioned animals. However, it was possible that conditioning produced partial effects, behavioural and/or neural, that would become evident when examined in the semi-intact preparation.

Following the post-observation session, all animals were immediately dissected into a semi-intact preparation and the CPG, motorneuron and pneumostome activity was simultaneously monitored. In this experiment, VI motorneuron and RPeD1 cell activity were monitored. RPeD1 is the cell that receives excitatory input from the peripheral chemoreceptor cells in the pneumostome/osphradial area and initiates the CPG respiratory rhythm (Inoue et al., 2001; Bell et al., 2007; Bell et al., 2008). Since the pneumostome opener cell, IP3 interneuron, is located on the ventral surface of the CNS and RPeD1 is located on the dorsal surface, IP3 interneuron activity was indirectly assessed as characteristic bursting patterns in the VI motorneuron. Input 3 was first described by Benjamin and Winlow (Benjamin and Winlow, 1981) as a characteristic synaptic input onto various cells, despite the unknown identity of the source of the input. Syed et al. (Syed et al., 1990) later identified the IP3 interneuron and demonstrated that activity recorded in this cell produced the characteristic activity previously described as input 3 onto the identified follower cells. Since then, the VI cell has been widely used to indirectly monitor the activity of the IP3 interneuron (Syed et al., 1990; Syed et al., 1991; Spencer et al., 2002; McComb et al., 2005), as simultaneous recordings from RPeD1 and IP3 interneuron are not possible because of their locations on different surfaces of the CNS. The characteristic bursting activity monitored in the VI cell is a result of synaptic input from the IP3 interneuron (Syed et al., 1990) and here will be defined as ‘input 3 events’.

The experimental protocol in the semi-intact preparation was designed to observe both the neural and behavioural correlates of the respiratory behaviour and to also monitor changes following presentation of a contingent, punishing stimulus to an open pneumostome (Spencer et al., 2002) in all groups.

Analysis of pneumostome openings and IP3 events in naïve semi-intact preparations

The first aim was to determine the baseline differences in behavioural and neural activity between normally and differentially reared naïve preparations over an initial 5 min period of recordings. We investigated pneumostome openings and corresponding IP3 events in the VI cell in the naïve semi-intact preparations in order to determine whether the behaviour correlated with cellular activity. Fig. 3A,B illustrates the number of pneumostome openings and IP3 events in the naïve animals during the 5 min session. As expected, the differentially reared naïve preparations displayed significantly fewer pneumostome openings than the normally reared naïve preparations ($P<0.05$; Fig. 3A). Accordingly, the number of IP3 events recorded from the VI cell was also significantly reduced in the differentially reared naïve preparations compared with normally reared preparations ($P<0.01$; Fig. 3B).

From these data, we observed 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, differentially reared naïve preparations performed aerial respiration less often than normally reared preparations.

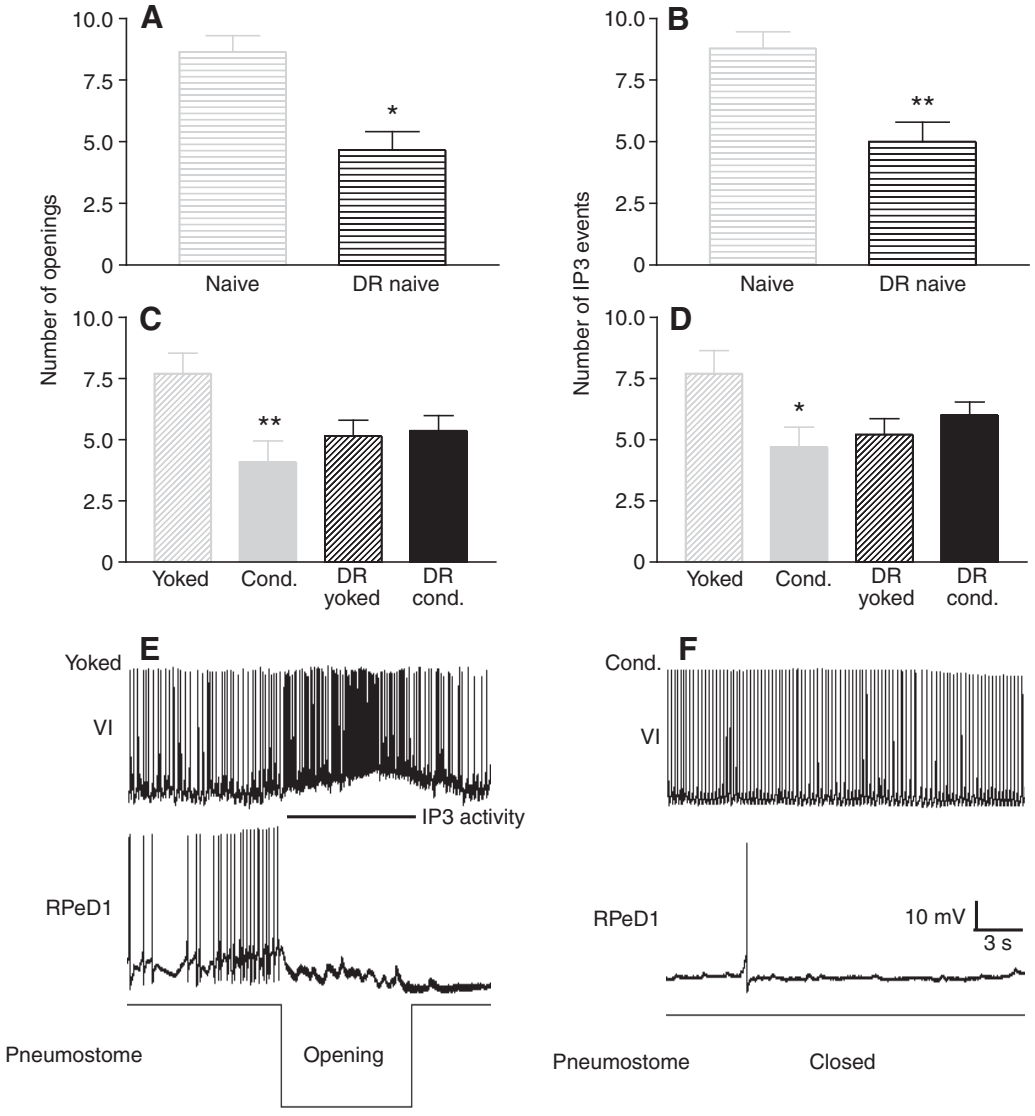

Fig. 3. Pneumostome opening behaviour coincides closely with the number of IP3 events recorded in the VI cell in semi-intact preparations and reflects the behaviour of the intact animal. Both the number of pneumostome openings (A,C) and the number of IP3 events in the VI cell (B,D) were recorded. (A) The differentially reared naïve preparations showed significantly fewer pneumostome openings (DR naïve: 4.7±0.7; *$P<0.05$) than the normally reared naïve preparations (8.6±0.7). (B) Accordingly, the number of IP3 events was significantly lower (**$P<0.01$) in the differentially reared naïve preparations (5.0±0.8) compared with the normally reared naïve preparations (8.8±0.7). (C) Normally reared conditioned (Cond.) preparations performed aerial respiration significantly less often (4.1±0.9) than normally reared yoked controls (7.7±0.8; **$P<0.01$). The differentially reared conditioned (DR cond.) preparations, however, did not demonstrate a significant reduction in aerial respiration compared with their corresponding yoked controls (DR yoked: 5.1±0.7, DR cond.: 5.4±0.6; $P>0.05$). (D) The number of IP3 events was also significantly reduced in the normally reared conditioned animals (4.7±0.8) compared with normally reared yoked preparations (7.7±1.0; *$P<0.05$), but not in the differentially reared conditioned preparations compared with their yoked controls (DR yoked: 5.2±0.6, DR cond.: 6.0±0.5). (E) Representative electrophysiological recordings in a normally reared yoked control preparation showing an IP3 event in the VI and RPeD1 cells and corresponding pneumostome opening. (F) Representative electrophysiological recordings in a normally reared conditioned preparation showing the absence of an IP3 event or pneumostome opening.

Analysis of pneumostome openings and IP3 events in conditioned semi-intact preparations

Having analyzed respiratory activity and associated IP3 events in naïve semi-intact preparations, and having determined that this semi-intact preparation was an appropriate representation of the behaviour of the intact animal, conditioned and yoked animals were next dissected to determine the effects of operant conditioning on the pneumostome opening parameters and cellular activity.

We found that in the semi-intact preparations, pneumostome opening behaviour and IP3 events reflected operant conditioning in the intact animal (Fig. 3C,D). A two-way ANOVA revealed a significant interaction effect between rearing condition and training, for both the number of openings ($F_{(1,47)}$=6.29; P<0.05) and the number of IP3 events ($F_{(1,47)}$=6.02; P<0.05). The normally reared conditioned preparations opened their pneumostome significantly less often than their yoked controls (P<0.01), but not significantly less than the differentially reared yoked and conditioned animals (Fig. 3C). The number of IP3 events (recorded in the VI cell) that produce pneumostome openings was also significantly reduced in the normally reared conditioned preparations compared with their yoked controls (P<0.05). As found in the intact animals, there were no significant differences between differentially reared yoked and conditioned preparations in either the number of pneumostome openings (Fig. 3C) or IP3 events (Fig. 3D). Overall, these results suggested that the semi-intact preparation is a good model for studying the behavioural and neural correlates of learning and memory. As in the whole animal, normally reared conditioned preparations performed aerial respiration less than their yoked controls. Concurrently, IP3 events were also reduced in these preparations.

Novel neural correlates of learning and memory in the semi-intact preparation

A number of studies have suggested a multi-loci model of learning and memory (e.g. Benjamin et al., 2000) in which modifications induced by learning are dispersed throughout the neural circuitry mediating the behaviour. Therefore, a number of novel cellular parameters were analyzed to determine where neuronal changes that underlie behavioural changes may occur in this system, and to compare such changes between normally reared and differentially reared preparations.

IP3 events recorded from the VI cell

IP3 activity (and IP3 events recorded in the VI cell), result in pneumostome opening (Syed et al., 1991; Syed and Winlow, 1991). We have already shown that the number of IP3 events recorded in the VI cell is reduced in conditioned preparations. We next analyzed three other IP3 event parameters and found them to be altered in normally reared conditioned preparations, compared with yoked controls. These parameters included the intensity of the IP3 events

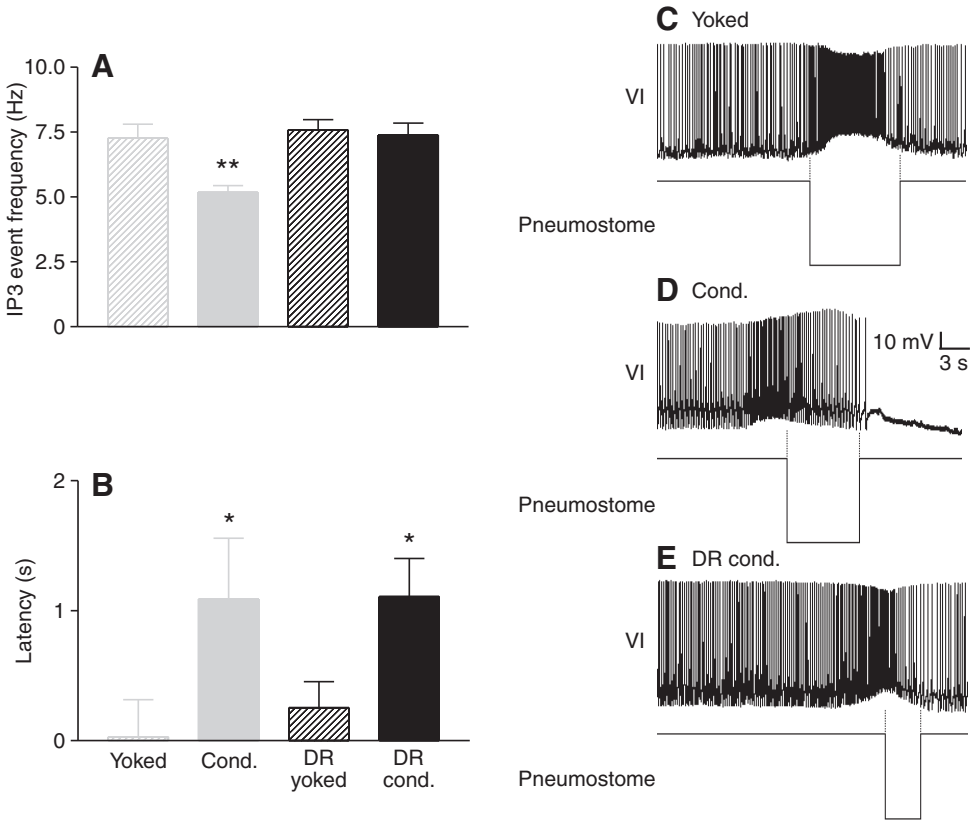

Fig. 4. Conditioning induces novel changes in the latency from IP3 events to pneumostome opening in normal and differentially reared preparations. (A) Intra-burst spike frequency of IP3 events was significantly reduced (**P<0.01) in normally reared conditioned (Cond.) preparations (5.2±0.3 Hz) compared with the normally reared yoked controls (7.3±0.5 Hz), but was not altered in the differentially reared preparations (DR yoked: 7.6±0.4 Hz, DR cond.: 7.4±0.5). (B) The latency from IP3 events in the VI motorneuron to the pneumostome opening was determined for all preparations. A significant increase in the latency was observed in both normally reared conditioned preparations (Cond.: 1.1±0.5 s) and differentially reared conditioned preparations (DR cond.: 1.1±0.3 s) compared with their corresponding yoked controls (Yoked: 0.0±0.3 s, DR yoked: 0.3±0.2 s; *P<0.05). (C–E) Representative recordings showing IP3 events in the VI cell and pneumostome openings in (C) normally reared yoked control, (D) normally reared conditioned, and (E) differentially reared conditioned preparations, demonstrate the reduced IP3 event frequency and increased latency to openings in conditioned preparations.

(intra-burst spike frequency), the latency from the IP3 event to pneumostome opening, and the coincident IP3 event and pneumostome activity.

Although we did not use a tension transducer to measure the force of each pneumostome opening, the intra-burst frequency of the IP3 event was used as an indirect measure of the intensity of the pneumostome opening. Generally, a high intra-burst frequency results in a qualitatively large pneumostome opening, whereas a low intra-burst spike frequency produces a smaller pneumostome opening. Owing to the aversive nature of the stimuli, we hypothesized that conditioned preparations would demonstrate a reduction in the intra-burst spike frequency of the IP3 event. Firstly, there was no significant difference in intra-burst spike frequency of the IP3 event between the normally reared (6.2±0.4 Hz) and differentially reared (6.6±0.4 Hz) naïve preparations ($P>0.05$), as well as no qualitative difference in the pneumostome opening. However, a two-way ANOVA revealed a significant interaction between rearing condition and training ($F_{(1,47)}=4.90$; $P<0.05$). There was a significant difference between the conditioned and yoked normally reared preparations ($P<0.01$; Fig. 4A). The training affected the intensity of the IP3 event such that intra-burst spike frequency was significantly reduced in the normally reared conditioned preparations compared with the normally reared yoked preparations. This difference was not evident in the preparations of the differentially reared animals ($P>0.05$).

Latency from the IP3 event in the VI cell, to pneumostome opening

There is a direct monosynaptic connection between the VI motorneurons and the pneumostome opener muscles (Syed et al., 1991). We next wanted to measure the latency from the IP3 event in the VI cell to the resulting pneumostome opening, to see if it was affected by conditioning. Wolpaw (Wolpaw, 1997) has previously shown a reduction in motorneuron conduction velocity following operant conditioning of the H-reflex. We, therefore, hypothesized an increase in the latency in conditioned preparations. In the naïve groups, there was no difference in this latency between the normally reared preparations (0.8±0.2 s) and the differentially reared preparations (0.4±0.5 s). However, on analysis of the conditioned and yoked control groups, a two-way ANOVA revealed a significant effect of training only on the latency ($F_{(1,46)}=8.40$; $P<0.01$), but no significant interaction between training and rearing ($P>0.05$). In the normally reared conditioned animals, there was a significant difference in the pneumostome response (Fig. 4B). In the yoked controls, a pneumostome opening was recorded as soon as the IP3 event was observed, while in the conditioned groups, there was a significant lag time in the pneumostome opening. The differentially reared conditioned preparations also demonstrated a similar increase in this latency compared with their controls. This was the first significant result that indicated that the differentially reared animals showed neural changes in response to the conditioning.

Coincident IP3 events and pneumostome activity

The respiratory motor program dictates that IP3 events produce a pneumostome opening (Syed and Winlow, 1991). Thus, IP3 events were also scored for coincident pneumostome activity in the semi-intact preparation. In other words, we asked the question: if IP3 events were observed in the VI cell and/or RPeD1, was there a corresponding pneumostome opening? Although all normally and differentially reared naïve and yoked preparations demonstrated >90% coincident activity, the number of IP3 events that did not result in pneumostome openings was significantly increased in conditioned preparations. A two-way ANOVA revealed a significant effect of training ($F_{(1,117)}=29.91$; $P<0.0001$), although the interaction between training and rearing did not reach significance ($P=0.06$). Interestingly, *post-hoc* analysis revealed a significant reduction in coincident activity in conditioned preparations from both normally and differentially reared animals, again showing that differentially reared animals exhibited neural changes associated with the operant conditioning (Fig. 5).

The motorneuron firing frequency (inclusive of IP3 events) was next determined for the 5 min recording session. Statistical analysis indicated no differences in VI frequency between the normally and differentially reared naïve preparations and no difference in activity between yoked and conditioned preparations (normally reared naïve: 3.0±0.2 Hz, normally reared yoked: 3.2±0.3 Hz, normally reared conditioned: 2.8±0.2 Hz, differentially reared naïve:

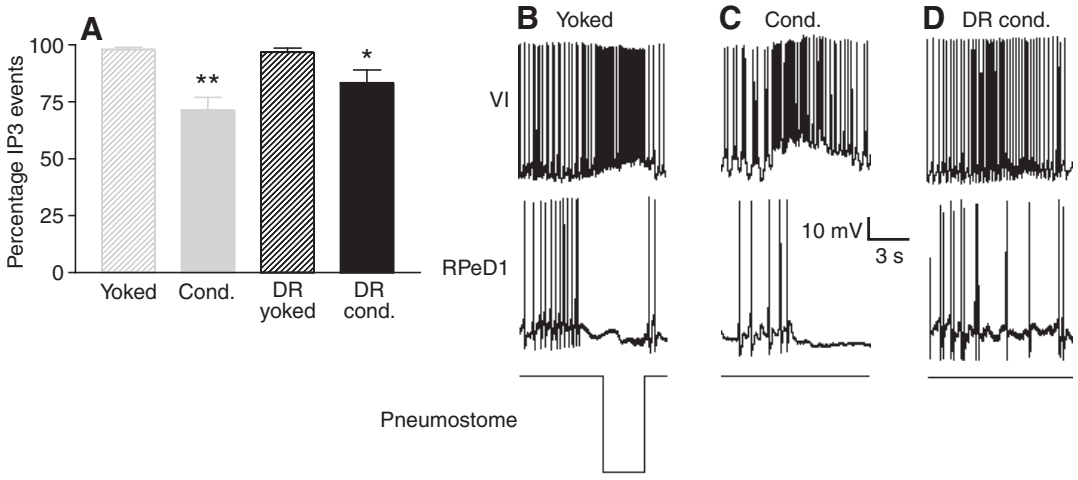

Fig. 5. Coincident IP3 events and pneumostome activity were significantly reduced in both normal and differentially reared conditioned preparations. Coincident activity was monitored by determining the percentage of IP3 events that produced a pneumostome opening. (A) In both normally reared and differentially reared conditioned (Cond.) preparations, significantly fewer IP3 events resulted in pneumostome openings compared with the yoked controls (Yoked: 98±1%, Cond.: 71±6%, DR yoked: 97±2%, DR cond.: 84±6%; $^{**}P<0.01$; $^{*}P<0.05$). (B) Representative electrophysiological recordings in a normally reared yoked control preparation showing a coincident IP3 event with pneumostome opening. (C,D) Representative recordings from a normally reared (C) and differentially reared (D) conditioned preparation illustrate IP3 events that did not result in pneumostome openings.

3.6±0.2 Hz, differentially reared yoked: 3.7±0.3 Hz, differentially reared conditioned: 3.6±0.3 Hz; $P>0.05$). Thus, the overall motorneuron activity was not affected either by operant conditioning or differential rearing.

Taken together, IP3 events were influenced by both rearing conditions and operant conditioning. With respect to the rearing conditions, the only difference observed was that differentially reared naïve preparations demonstrated fewer IP3 events in the VI cell compared with normally reared naïve preparations. This was reflective of the fewer number of pneumostome openings in both the intact animal and the semi-intact preparation of differentially reared animals. Operant conditioning affected the intensity (intra-burst frequency) of the IP3 events, the latency from IP3 events to pneumostome response, and coincident IP3 events and pneumostome activity. Furthermore, whereas the differentially reared conditioned preparations did not demonstrate any behavioural evidence of learning and memory, the cellular parameters strongly suggested aspects of neural plasticity associated with operant conditioning.

Neural and behavioural changes following application of the punishing stimulus

Following the 5 min recording session, a punishing stimulus was applied to all preparations (naïve, yoked and conditioned) on the next pneumostome opening. The recordings were then continued for a further 5 min, and pneumostome behaviour and neural activity were analyzed in the post-stimulus session and compared with the pre-stimulus session. This analysis determined whether certain behavioural or neural parameters were affected by presentation of the punishing contingent stimulus.

Only conditioned preparations demonstrated reduced pneumostome openings and reduced IP3 events following the punishing stimulus

A two-way ANOVA revealed a significant effect of training on the number of openings ($F_{(1,47)}=4.36$; $P<0.05$), as well as the number of IP3 events ($F_{(1,47)}=6.42$; $P<0.05$) after presentation of the punishing stimulus, although there was no significant interaction effect between rearing condition and training ($P>0.05$). Following the application of the punishing stimulus, it was shown that conditioned preparations from both normally and differentially reared animals showed an overall reduction in the number of pneumostome openings as well as a corresponding overall reduction in the number of IP3 events (Fig. 6). Yoked control groups did not show this reduction in activity. These data indicated that despite the lower incidence of overall respiratory activity in the differentially reared animals, they behaved in a similar manner to the normally reared animals in response to the punishing stimulus. That is, despite no overall change in behaviour as a result of the conditioning in the intact animals, they responded to the punishing stimulus in a similar manner to the normally reared conditioned group in the semi-intact preparation.

Latency to pneumostome opening following the punishing stimulus

It has been shown in *Lymnaea* that a punishing stimulus can induce behavioural and neural changes associated with LTM (Spencer et al., 2002). The latency to the 'next' pneumostome opening following the application of the punishing stimulus was then determined for the conditioned and yoked preparations. This parameter was used as an indication of the behavioural response to the stimulus. Owing to the aversive nature of the stimulus, we hypothesized that the conditioned groups would demonstrate a greater latency than their controls.

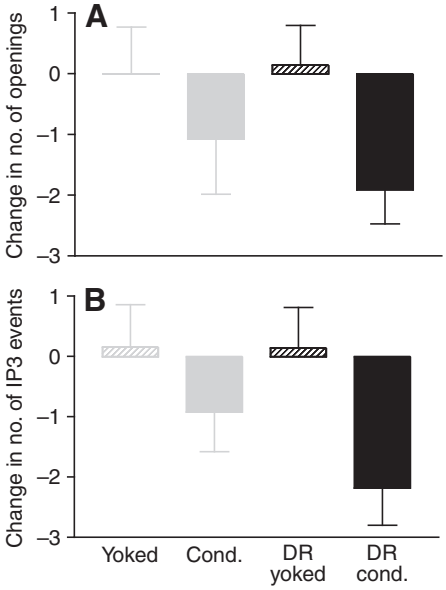

Fig. 6. Following the contingent application of the punishing stimulus to the semi-intact preparations, only conditioned preparations showed a reduction in behaviour and neural activity. (A) A two-way ANOVA revealed a significant effect of training on the change in pneumostome openings after presentation of the punishing stimulus ($F_{(1,47)}=4.36$; $P<0.05$). In both the normally reared and differentially reared conditioned preparations, there was a reduction in the number of pneumostome openings after application of a punishing stimulus to an open pneumostome in the semi-intact preparation. Yoked preparations did not demonstrate a reduction in respiratory activity (Yoked: 0.00±0.77, Cond.: –1.01±0.91, DR yoked: 0.14±0.65, DR cond.: –1.91±0.56). (B) A two-way ANOVA revealed a significant effect of training on the change in number of IP3 events after the punishing stimulus ($F_{(1,47)}=6.42$; $P<0.05$). There was a corresponding reduction in the number of IP3 events in the normally reared and differentially reared conditioned groups compared with their yoked controls after the punishing stimulus (Yoked: 0.15±0.71, Cond.: –0.92±0.66, DR yoked: 0.14±0.67, DR cond.: –2.18±0.62).

There were no significant differences in the response to the punishing stimulus amongst the naïve preparations (normally reared naïve: 63±12 s, differentially reared naïve: 68±25 s; $P>0.05$). However, analysis of the conditioned and yoked groups using a two-way ANOVA revealed a significant effect of training on the latency from the stimulus to the next opening ($F_{(1,47)}=11.34$; $P<0.005$), though there was no significant interaction effect between rearing conditions and training ($P>0.05$). *Post-hoc* analysis indicated that the normally reared conditioned preparations displayed a significantly greater latency to the next pneumostome opening than their yoked preparations ($P<0.05$; Fig. 7A). The differentially reared conditioned preparations also demonstrated a trend towards an increased latency compared with their yoked preparations, although the difference did not reach significance. These results were again indicative, though not conclusive, of behavioural plasticity in the differentially reared *Lymnaea*. Taken together, conditioned preparations suppressed the hypoxic ventilatory drive longer than controls following the application of the contingent punishing stimulus.

RPeD1 impulse activity

There were also significant short-term changes in RPeD1 activity. Following application of the contingent punishing stimulus, RPeD1

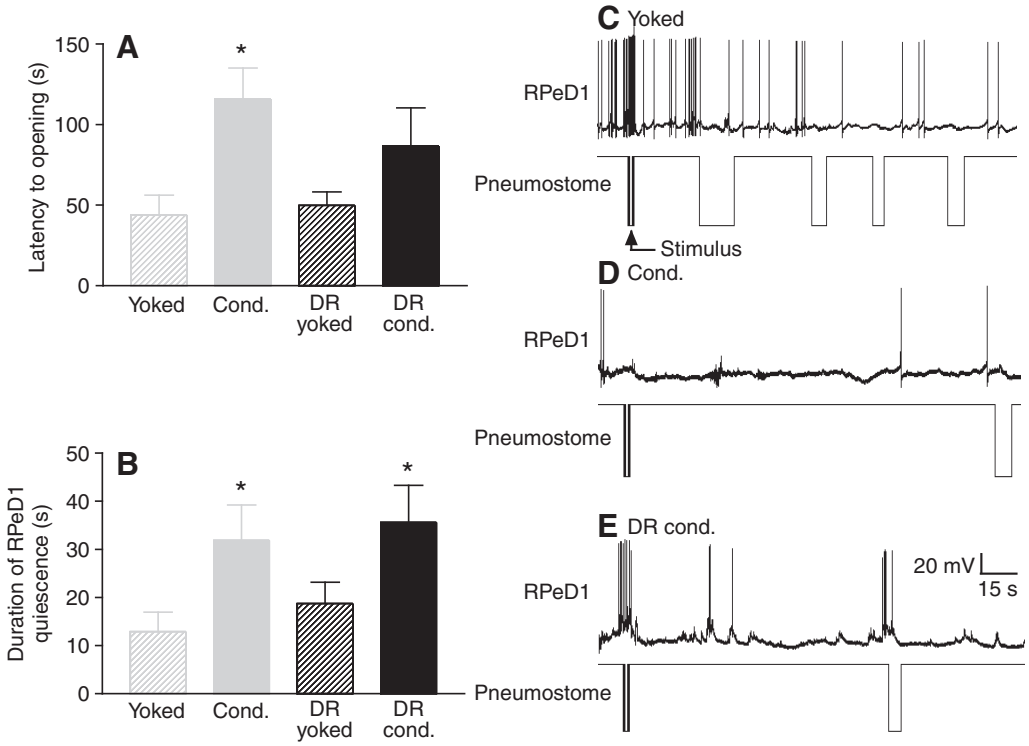

Fig. 7. Immediate behavioural and neural responses occur following the punishing stimulus in the semi-intact preparation. (A) The latency from the stimulus to the next pneumostome opening was used as an indication of the preparation's behavioural response to the aversive stimulus. A two-way ANOVA revealed a significant effect of training on the latency following the punishing stimulus (P<0.005). There was a significant increase in this latency in normally reared conditioned preparations (Cond.: 116±19 s) compared with yoked preparations (Yoked: 44±12 s; *P<0.05). Although differentially reared preparations also showed the same trend, this difference was not significant (DR yoked: 50±8 s, DR cond.: 87±24 s). (B) RPeD1 responds to a mechanical stimulus to the pneumostome with an inhibition of firing. The latency from the stimulus to the next action potential in RPeD1 was thus used as an indication of the neural response to the aversive stimulus. A two-way ANOVA revealed a significant effect of training on the latency following the punishing stimulus (P<0.005). Operant conditioning resulted in a significantly increased latency to the next action potential in both normally reared conditioned (32±7 s) and differentially reared conditioned preparations (36±8 s) compared with their corresponding yoked controls (Yoked: 13±4 s, DR yoked: 19±4 s; *P<0.05).
(C–E) Representative traces showing RPeD1 activity and pneumostome openings from (C) a normally reared yoked control, (D) a normally reared conditioned, and (E) a differentially reared conditioned preparation, illustrating increased latencies in RPeD1 firing and pneumostome openings in the conditioned preparations following the punishing stimulus.

ceased firing until sufficient excitatory input re-initiated rhythmogenesis and/or it recovered from inhibition. It has previously been shown that RPeD1 is more quiescent in isolated ganglia dissected from conditioned animals (Spencer et al., 1999), so we hypothesized an increase in latency to resumed RPeD1 activity in conditioned preparations. Indeed, a two-way ANOVA again revealed a significant effect of training on the latency to resumed RPeD1 activity ($F_{(1,47)}$=9.01; P<0.005), though there was no significant interaction effect between rearing conditions and training (P>0.05). Normally reared conditioned preparations demonstrated a significantly increased recovery time in RPeD1 impulse activity compared with their respective yoked controls (P<0.05; Fig. 7B). With respect to differentially reared conditioned preparations, they too demonstrated an increased RPeD1 quiescence compared with the differentially reared yoked preparations (P<0.05). Thus, differentially reared conditioned preparations demonstrated similar neural responses to the punishing stimulus as the normal 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 events in the VI motorneuron, a change in the motor program controlling pneumostome opening as well as RPeD1 impulse activity. With respect to differentially reared preparations, the strongest evidence for plasticity was demonstrated by the significant difference between differentially reared yoked and conditioned preparations in latency to pneumostome opening following an IP3 event, correlated pneumostome activity and IP3 events, and the duration of RPeD1 quiescence following the punishing stimulus.

DISCUSSION

In this study, the freshwater mollusc *Lymnaea stagnalis* was used to further our understanding of how network properties change as a result of associative learning, as well as to investigate whether or not this plasticity is dependent on previous experience during development. Among the many benefits of using *Lymnaea* is the ability to perform experiments on both the intact animal and the semi-intact preparation; 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, identified neurons directly involved in the behaviour. Using this approach, we showed that animals prevented from performing aerial respiration during

development did not demonstrate any behavioural reduction when tested as intact animals, though they did demonstrate many aspects of neuronal network plasticity in the semi-intact preparation as a result of the conditioning procedure. We also further identified novel changes in both CPG and motorneuron properties following the operant conditioning.

Hermann and Bulloch (Hermann and Bulloch, 1998) have previously shown that *Lymnaea* could be raised from eggs to adulthood without ever experiencing aerial respiration. *Lymnaea* is a pulmonate mollusc (Harris, 2003), in which respiration occurs partially across the somatic epidermis (cutaneous respiration) but also *via* a primitive lung (aerial respiration) (Syed et al., 1991). Thus, during differential rearing, cutaneous respiration appeared sufficient to meet the metabolic demands of these animals and maintain homeostasis in normoxic (aerated) pond water. When allowed to do so, differentially reared *Lymnaea* surfaced and performed aerial respiration, showing that the behaviour is genetically programmed and is activity and experience independent (Hermann and Bulloch, 1998). They did, however, show qualitative and quantitative differences in aerial respiration; the most relevant to this study being that in hypoxic conditions, the normally reared animals demonstrated the hypoxic ventilatory response (increased aerial respiratory behaviour) whereas the differentially reared animals did not. Likewise, in our study, we found that the differentially reared snails exhibited a significant reduction in both number of pneumostome openings and total breathing time in hypoxic conditions, compared with normally reared animals. Hermann and Bulloch (Hermann and Bulloch, 1998) reasoned that differentially reared animals did not experience hypoxic conditions during development as their rearing tanks were aerated and normoxic, and the oxygen saturation of haemocyanin, the haemolymph oxygen carrier in *Lymnaea* (Dawson and Wood, 1982; Dawson and Wood, 1983), was estimated to be 100%. However, it is known that developmental hypoxia in vertebrates reduces the adult hypoxic ventilatory response, so we cannot rule out that developmental hypoxia led to the reduced hypoxic ventilatory response of the differentially reared animals shown here. It has previously been suggested that developmental hypoxia may lead to aberrant function or number of chemoafferant neurons (Erickson et al., 1998; Joseph et al., 2000), so it is also possible that in the differentially reared animals, developmental hypoxia may have led to reduced peripheral sensory input to RPeD1.

The reasons and underlying mechanisms as to why the differentially reared animals exhibited a reduced hypoxic ventilatory response are currently unknown. One reason may be a reduced metabolic requirement for oxygen, possibly due to increased anaerobic metabolism in the differentially reared animals. Another may be a change in synaptic functioning or network properties of the hypoxic-sensing neurons as a result of the differential rearing and/or presumed CPG network inactivity. Bell et al. (Bell et al., 2007) recently showed that peripheral chemoreceptor cells in the osphradium of *Lymnaea* are oxygen-sensing neurons with monosynaptic connections with RPeD1. Furthermore, this synapse exhibits hypoxia-induced short-term facilitation, at least *in vitro*. It has been shown that both lesioning of the osphradial nerve (Bell et al., 2007) as well as RPeD1 nerve crush (Haque et al., 2006) reduces aerial respiratory drive as well as reduces movements to the water surface. It is thus plausible that disruptions in the synaptic connections of these cells during differential rearing might underlie the reduced hypoxic response. Another explanation may involve nitric oxide (NO) production, as NOS inhibitors have also been shown to prevent or reduce the hypoxic ventilatory response in *Lymnaea* (Taylor et al., 2003). Whether the reduced hypoxic response in differentially reared *Lymnaea* results from a reduced requirement for oxygen, or changes to the oxygen-sensing pathway [as previously proposed by Hermann and Bulloch (Hermann and Bulloch, 1998)], remain to be determined.

The CPG aerial respiratory rhythm is also driven by mechano-sensory excitatory inputs to RPeD1 as a result of the pneumostome breaking the surface of the water (Haque et al., 2006). We hypothesize that during differential rearing of *Lymnaea*, these excitatory inputs to RPeD1 were silent and the respiratory CPG was inactive. Presumably, the CPG connections were not subjected to any activity-dependent modulation during development. In this study, we 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 the aerial respiratory behaviour in differentially reared animals and both behavioural and neural monitoring in the semi-intact preparation.

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 criteria we had previously established, differentially reared *Lymnaea* did not demonstrate learning or LTM when conditioned with the *same* procedure as normally reared *Lymnaea* (Lukowiak et al., 1996; Spencer et al., 2002). We cannot rule out that this may have resulted from muscle weakness in the pneumostome as a result of differential rearing, but we consider this unlikely, as there were no qualitative differences in the pneumostome openings of this group compared with normally reared animals [as also previously shown by Hermann and Bulloch (Hermann and Bulloch, 1998)]. It can, however, be argued that aerial respiration could not be further reduced in the differentially reared animals following conditioning since they started with such a low level of behavioural expression. This notion is further strengthened by the fact that the baseline level of respiration in the differentially reared animals *prior* to conditioning is the same level of respiration that normally reared animals maintain *following* conditioning. It is also possible (though less likely), that because they did not attempt aerial respiration as often as respiring animals, the differentially reared animals may not have received sufficient punishing stimuli to produce the operant response (Papini and Bitterman, 1990; Terry, 2003). Although Martens et al. (Martens et al., 2007) have recently demonstrated long-term memory lasting at least 24 h following a single trial of conditioning of aerial respiratory behaviour, their protocol was designed to evoke the whole-body withdrawal response, an event considered more significant than the tactile stimulation of the open pneumostome used here.

Semi-intact preparations derived from the differentially reared conditioned animals did not initially demonstrate any significant differences in number of pneumostome openings compared with their yoked controls. However, despite this, there were behavioural indications of operant conditioning in these differentially reared semi-intact preparations. For example, statistical analysis revealed a significant effect of training on the change in opening behaviour after the punishing stimulus. Both the normally reared and differentially reared conditioned preparations demonstrated a reduction in aerial respiratory behaviour after the punishing stimulus. These modifications were the first indications of behavioural plasticity in the differentially reared animals.

Significant differences in neural activity as a result of operant conditioning were observed in three parameters in the differentially

reared preparations: the latency from IP3 events (recorded in the motorneuron) to pneumostome opening, the level of coincident IP3 events and pneumostome opening, and the duration of RPeD1 quiescence following the application of the contingent stimulus. These significant changes were also found in the normally reared conditioned preparations (but not the yoked control groups) and are thus indicative of network plasticity as a result of operant conditioning. These results are significant in that they demonstrate that neural plasticity can occur in differentially reared preparations following the operant conditioning procedure. Importantly the neural plasticity occurred both in the absence of a significant change in behaviour of the intact animal, and in the presumed absence of experience-dependent activity during development. Even though neural changes were not observed in all parameters measured in the differentially reared preparations, the fact that some were identified, may suggest that the differentially reared intact animals may have formed memory traces with less punishing stimuli than the normally reared group (though it is not known what the minimum number of stimuli are required to produce the same neuronal changes in the normally reared group). The fact that the presence of neural changes in the semi-intact preparation did not appear to correlate with behavioural changes in the differentially reared intact animals is probably due to the initially low level of behavioural expression as intact animals.

The number of novel changes that were identified in the network in this study further supports the notion that changes underlying learning and memory are generally not confined to a single locus, but rather occur at multiple sites within a neural network (Benjamin et al., 2000; Brembs, 2003; Lukowiak and Colebrook, 1988; Spencer et al., 2002). RPeD1 is the CPG neuron that receives both chemosensory (Inoue et al., 2001) and mechanosensory input (Haque et al., 2006) from the periphery and which triggers the CPG rhythm (Syed et al., 1990). It has previously been defined as an important site for LTM in operant conditioning of the aerial respiratory behaviour. Specifically, expression of new genes in the soma of RPeD1 is required for LTM formation (Scheibenstock et al., 2002) and activity levels of RPeD1 in isolated CNSs taken from trained animals are significantly reduced following conditioning (Spencer et al., 1999). Here, we showed for the first time that the *immediate* response of RPeD1 to a punishing stimulus is also significantly altered; RPeD1 was quiescent for significantly longer following the punishing stimulus in conditioned preparations compared with yoked preparations, regardless of rearing conditions. It is known that a physical stimulus to an open pneumostome in a semi-intact preparation produces temporary cessation of RPeD1 activity and interestingly, Lowe and Spencer (Lowe and Spencer, 2006) recently showed that artificially silencing RPeD1 activity between training sessions augmented LTM formation, possibly as a result of altered gene expression induced by the experimental hyperpolarization. Thus, we hypothesize from our results that RPeD1 quiescence immediately following the stimulus may represent a neural encoding of LTM. Though changes in synaptic efficacy are well accepted to play a role in the cellular encoding of learning and memory, many have suggested that altered neuronal excitability and firing behaviour of neurons is also an important or essential mechanism for learning and memory (Giese et al., 2001; Daoudal and Debanne, 2003). Indeed, invertebrate models of conditioning appeared to have led the way for this hypothesis (Alkon et al., 1982; Alkon, 1984; Brembs et al., 2002; Jones et al., 2003). However, most cellular correlates of learning have previously been associated with an increase in neuronal excitability, though there are also examples of reduced excitability and firing playing a role (Burrell et al., 2001; Lowe and Spencer, 2006). Our findings here suggest that activity changes in RPeD1 occur as a direct result of the punishing stimulus, and our previous studies suggest that this reduction in firing is a long-lasting change (Spencer et al., 1999) that is directly correlated to the reduction in behaviour (Spencer et al., 2002).

Though RPeD1 plays an important role in LTM formation, other network parameters are also probably affected (Spencer et al., 1999; McComb et al., 2005) and it is unlikely that all neural changes associated with the operant conditioning of aerial respiratory behaviour in *Lymnaea* have been identified. Here we have further identified significant changes within the CPG and its motor output and now provide evidence to suggest that IP3 events, responsible for pneumostome opening, are also important in the formation or expression of LTM. Conditioned preparations demonstrated significantly reduced intra-burst spike frequency of IP3 events in the VI cell, that produced qualitatively smaller pneumostome openings in the conditioned semi-intact preparations, compared with yoked controls. These findings strongly suggest either a change in the firing frequency of the IP3 interneuron, or a change in synaptic efficacy between the IP3 cell and the VI motorneuron. McComb et al. (McComb et al., 2005) previously identified reduced duration of IP3 events in conditioned preparations, also supporting these hypotheses. Unfortunately, it is difficult to confirm either of these hypotheses as IP3 lies on the opposite surface of the CNS and cannot be directly accessed at the same time as either RPeD1 or the VI motorneurons (Syed et al., 1990).

Another important finding of this study, suggesting a change in synaptic efficacy within the network, was that coincident IP3 events and pneumostome openings were significantly reduced in the conditioned preparations. In other words, IP3 events did not necessarily lead to pneumostome openings. Since IP3 events were observed in the VI cell, which is monosynaptically connected to the pneumostome opener muscles (Bell et al., 2008), this may indicate a change in neuromuscular transmission, such as decreased transmitter release from the VI cell or decreased excitability of the postsynaptic pneumostome opener muscle cells (Kandel, 2001). Such a change in synaptic efficacy between the VI motorneuron and the pneumostome muscles is again supported by previous findings of McComb et al. (McComb et al., 2005), who showed that *artificial* depolarization of VI failed to elicit a pneumostome opening in conditioned semi-intact preparations. Lukowiak and Colebrook (Lukowiak and Colebrook, 1988) also showed that the ability of a gill motorneuron to elicit gill withdrawal in *Aplysia* was significantly altered following classical conditioning, although in this case, a potentiation (rather than a reduction) was observed. The change in synaptic efficacy between the VI motorneuron and muscle may also be the cause of the increased latency observed when the IP3 event in the VI cell *did* produce a pneumostome opening, though we cannot rule out a reduction in conduction velocity in the motorneuron as the cause of this effect, as has been previously observed in the operant conditioning of the vertebrate H-reflex (Wolpaw, 1997). Taken together, these results demonstrate synaptic remodelling throughout the CPG as a result of conditioning and that changes in IP3 events and/or neuromuscular transmission may occur concomitant with changes in RPeD1 activity.

In summary we have identified novel neural correlates of operant conditioning in *Lymnaea stagnalis* at the single cell level, to provide evidence that there is encoding of LTM not only within the respiratory CPG but also within the VI motorneurons and their connections with the pneumostome opener muscles. These finding are consistent with recent literature that documented the occurrence

of modulation at all levels of the nervous system, including the CPG, the sensory, motor, and facilitating neurons, the sensory organs and the musculature (Baxter and Byrne, 2006; Harris-Warrick and Marder, 1991). Furthermore, we have demonstrated for the first time that network changes associated with operant conditioning of the aerial respiratory behaviour can occur in differentially reared animals that did not experience aerial respiration during development. These data strongly suggest that network plasticity in the adult CNS is not dependent on experience-dependent activity of the same network during development.

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