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Crowding, an environmental stressor, blocks long-term memory formation in *Lymnaea*

Pascaline De Caigny and Ken Lukowiak*
Hotchkiss Brain Institute, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1
*Author for correspondence (e-mail: lukowiak@ucalgary.ca)

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SUMMARY

Crowding is an environmental stressor. We found that this stressor altered (i.e. prevented) the ability of *Lymnaea* to form long-term memory (LTM) following operant conditioning of aerial respiratory behaviour. The ability to form LTM was compared between snails that had been crowded (20 snails per 100 ml of pond water) and those maintained in uncrowded conditions (two snails per 100 ml of pond water). Crowding either immediately before or after two different operant conditioning procedures – the traditional training procedure and the memory augmentation procedure – blocked LTM formation. However, if crowding is delayed by more than 1 h following training or if crowding stops 1 h before training, LTM results. If memory is already formed, crowding does not block memory recall. Pond water from a crowded aquarium or crowding with clean shells from dead snails, or a combination of both, is insufficient to block LTM formation. Finally, crowding does not block intermediate-term memory (ITM) formation. Since ITM is dependent on new protein synthesis whereas LTM is dependent on both new protein synthesis and altered gene activity, we hypothesize that crowding alters the genomic activity in neurons necessary for LTM formation.

Key words: long-term memory, *Lymnaea*, crowding, stress, block of memory formation.

INTRODUCTION

Stress may be thought of as any significant condition (i.e. the disturbance of homeostasis) that necessitates physiological, psychological and/or behavioural readjustment or modification that is necessary for the well-being of the organism (Selye, 1973; McEwen and Sapolsky, 1996; Kim and Diamond, 2002). Stressors can take the form of either physical (e.g. heat shock) or psychological (e.g. public speaking) challenges and have the ability to alter the processes of memory formation and recall (Yerkes and Dotson, 1908; Shors, 2006; Joels et al., 2006; Martens et al., 2007a; Martens et al., 2007b). Depending on the specific stressor and when and how the stress is perceived, memory formation and/or its recall may be enhanced or impaired (e.g. de Quervain et al., 1998; Bowman et al., 2003; Cahil et al., 2003; Joels et al., 2006; Martens et al., 2007a; Orr and Lukowiak, 2008). Stress has the ability to modulate memory formation and memory recall as it is a dynamic brain process (Hebb, 1949; Lewis, 1979). There are various indeterminate factors influencing whether memory formation and/or memory recall will be augmented or impaired by stress (Bowman et al., 2003; Luine, 2002; Vanitallie, 2002; Martens et al., 2007a; Martens et al., 2007b). A long-term goal of our research is to determine how a specific environmental stressor, crowding, modifies memory formation using a model system in which it is possible to study memory formation at the single-cell level. As a first step in this process, the present study examines whether crowding alters long-term memory (LTM) formation at the behavioural level.

Crowding is a stressor that alters genomic and behavioural activity in both vertebrates and invertebrates (Boranic and Poljak-Blazi, 1983; Roman et al., 2004; Reber et al., 2006; Holt, 2006). As a step towards elucidating the causal neuronal mechanisms underlying how stress modifies memory, we devised a series of experiments utilizing the *Lymnaea* model system to determine whether crowding either affects the ability to form LTM or hinders its ability to be recalled. Previously, crowding in *Lymnaea* was shown to significantly alter genomic activity, specifically affecting growth rate, embryonic development and reproduction, and retarding growth (Colton, 1908; Crabb, 1929; Forbes and Crampton, 1942; Noland and Carriker, 1946; Voronezhskaya et al., 2004).

Lymnaea is a model system used to elucidate the neuronal mechanisms underlying memory formation (Lukowiak et al., 2003a; Lukowiak et al., 2003b; Birmingham et al., 2004; Parvez et al., 2006; Lukowiak et al., 2008). In particular, aerial respiratory behaviour has proven to be very tractable in attempts to uncover the causal mechanisms of LTM formation, as this behaviour is driven by a three-neuron central pattern generator (CPG) whose sufficiency and necessity has been shown (Syed et al., 1990; Syed et al., 1992; Lukowiak et al., 2003b). In addition, RPeD1, one of the three CPG neurons, has been shown to be a necessary site for the molecular processes required for LTM formation, reconsolidation, extinction and forgetting (Scheibenstock et al., 2002; Sangha et al., 2003c; Sangha et al., 2003d; Sangha et al., 2005; Lattal et al., 2006). Importantly, using this model system, we have recently demonstrated that stress has the ability to modify memory formation (Martens et al., 2007a; Martens et al., 2007b; Orr and Lukowiak, 2008). In the present study we report that crowding, during a critical 1 h period just prior or immediately after operant conditioning, prevents LTM but does not block intermediate-term memory (ITM) formation or recall of an already formed LTM.

MATERIALS AND METHODS

Model system

Pond snails, *Lymnaea stagnalis* (L.), obtained from a colony originally formed from snails collected from polders near Utrecht, The Netherlands and maintained at Vrije Universiteit, Amsterdam,

The Netherlands, were raised in a facility at the University of Calgary, Alberta, Canada. Adult snails with shells of length 23–26 mm were used in experiments and were fed lettuce *ad libitum*. Snails were maintained in aquaria with normal oxygen levels (6 ml $O_2\,l^{-1}$) at room temperature (~20°C).

Aerial respiratory behaviour and operant conditioning

The traditional training procedure

Snails were removed from their home aquaria and placed into a 1-litre beaker containing 500 ml of hypoxic pond water (PW; <0.1 ml $O_2\,l^{-1}$). PW was made hypoxic by bubbling N_2 gas through the water for 20 min prior to introducing the snails. The animals were given a 10-min acclimatization period prior to a 30-min training session. By subjecting the snails to a hypoxic challenge, the animals increased their rate of aerial respiration (Lukowiak et al., 1996; Lukowiak et al., 1998). The animals were operantly conditioned by applying a gentle tactile stimulus with a wooden applicator to their pneumostome as the pneumostome began to open. The stimulus was strong enough to cause the snails to close their pneumostome yet gentle enough that the snails did not perform the full-body withdrawal response. This pneumostome closer response is a graded part of the whole-snail escape response (Orr et al., 2007). Every time the snail opened its pneumostome and received the stimulus during the training period, the time was recorded for use in future yoked control experiments. All behavioural experiments were done 'blind' such that the person performing the training paradigm was unaware of the status of the cohort being tested.

In order to cause LTM, the traditional training procedure utilized in the present study consisted of two 30-min training sessions (TS1 and TS2) with a 1 h interval between the sessions (Lukowiak et al., 1998), after which time the snails were randomly selected to be returned to either their home aquaria or to crowded conditions for a specified time (see below). The snails were then tested for memory (MT; i.e. a 'savings test') using a similar test to that used during training sessions.

The memory augmentation procedure

A second, faster training procedure that results in LTM formation was also used in the present study (Martens et al., 2007a). In this procedure, snails were exposed to a noxious, aversive 25 mmol l^{-1} KCl stimulus immediately prior to a single 30 min TS as described in the traditional training procedure. Snails were placed in individual Petri dishes (37 mm) containing 4 ml of 25 mmol l^{-1} KCl for 30–35 s. This volume was sufficient to cover the foot of the snails but was not enough to submerge them. The KCl bath caused snails to withdraw into their shells. The snails were then placed in the hypoxic training beaker for acclimatization for 10 min, followed by the 30 min TS (see above). This procedure results in LTM that persists for up to 36 h (Martens et al., 2007a).

Change of context

In some experiments, a 'carrot context' was also employed. This consisted of bubbling N_2 through a 750 ml Erlenmeyer flask containing blended carrot and then through the water in the training beaker (Haney and Lukowiak, 2001). 'Change-of-context' testing was employed as an internal control to test for injury or unresponsiveness, as LTM recall is context-specific.

Crowded conditions

In crowded conditions, snails were maintained for a specified period of time (1–24 h) at a density of 20 snails per 100 ml of PW (normal density is two snails per 100 ml of PW). Snails can be maintained at these and greater densities for 2 to 3 months without an increase in mortality (Crabb, 1929; Forbes and Crampton, 1942; Noland and Carriker, 1946) although growth may be compromised. Maintaining snails at these densities for 24 h was not considered to be harmful. We added naïve (i.e. untrained) snails to the home 'crowded aquaria' to create crowding.

Crowded pond water (CPW)

In some experiments, only 'crowded pond water' (CPW) was used. In order to obtain CPW snails were crowded for 24 h at a density of 20 snails per 100 ml of PW. The water was then used during experiments. Thus, the experimental snails did not directly experience the crowded conditions.

Empty shells

Another control condition used for some experiments was generated by placing clean shells from deceased snails in the training beaker with experimental snails at the identical density to that used in the crowding experiments (20 snails per 100 ml of PW).

Crowded pond water and empty shells

As a final control procedure, we combined CPW with empty shells.

Yoked control procedure

The yoked control procedure is similar to the operant conditioning training procedure. The difference is that the yoked control snails are 'poked' in their pneumostome area, not when they attempt to open their pneumostome but at the exact time as the snail to which they are yoked opens its pneumostome and receives the tactile stimuli. Thus, these yoked control snails receive exactly the same number of tactile stimuli delivered at the same time as the operant conditioned snails during the training sessions. However, during the memory test (MT), yoked control snails receive the tactile stimulus when they attempt to open their pneumostome. Thus, when the behaviour of yoked control snails is compared with the behaviour of operantly conditioned snails, we compare the response of each respective group in MT.

Breathing observation procedure

The total breathing time (TBT) of snails was also observed before and after crowding to ascertain whether the combination of stresses (crowding and the hypoxic challenge) results in abnormal aerial respiratory behaviour. The breathing observations consisted of placing snails in hypoxic water for a 10-min acclimatization period. The snails were then gently pushed below the surface of the water. We monitored each snail and recorded the time each snail kept its pneumostome open during the 30-min observation session. These snails were then either crowded or placed in their eumoxic home aquaria for 24 h, at which point another observation session was performed. TBT was then compared between the two sessions. Tactile stimuli were not delivered to the pneumostome during these observation sessions, thus allowing snails to perform aerial respiration as often as necessary. In both operant conditioning procedures used here (described above) snails received the tactile stimulus to their pneumostome as soon as they attempted to open the pneumostome. We are thus not able to compare the TBT with the number of attempted openings.

Operational definition of memory

In order for memory to be present, the number of attempted pneumostome openings (i.e. the number of tactile stimuli

delivered) during the MT must be significantly less (P<0.05) than the number of attempted pneumostome openings administered during the TS. ITM (persisting up to 3 h) is dependent on *de novo* protein synthesis, whereas LTM (lasting at least 24 h) depends on both *de novo* protein synthesis and gene transcription (Sangha et al., 2003b; Sangha et al., 2003c; Parvez et al., 2006; Martens et al., 2007a).

Statistical methods

Parametric data were analyzed using a repeated-measures analysis of variance (ANOVA) followed by a Tukey–Kramer *post-hoc* test. For groups that did not pass the normality test, a Friedman's test was conducted, followed by a Dunn's Multiple Comparison *post-hoc* test. When groups were not matched, a Kruskal–Wallis test was performed, followed by a Dunn's Multiple Comparison *post-hoc* test. Other data were analyzed using a one-way ANOVA test and a Tukey–Kramer *post-hoc* test. *Post-hoc* tests were conducted to determine which measurement(s) differed. When comparing TBT, a paired *t*-test was used. In all cases, significance was considered to be at least P<0.05.

RESULTS

Total breathing time before and after crowding

We first determined whether crowding altered TBT (Fig. 1). A 30 min hypoxic pre-observation (Pre-obs) session was performed, during which TBT was calculated. Following the Pre-obs session, snails were placed into the crowded condition (20 snails per 100 ml of PW) for 24 h before being challenged to a second hypoxic session (Post-obs 1). There was a significant decrease in TBT in this session (Fig. 1). We then determined whether this decrease in TBT was permanent; immediately following the Post-obs 1 session, snails were placed into an uncrowded home aquarium (two snails per 100 ml of PW) for 24 h prior to a third hypoxic challenge session (Post-obs 2). In Post-obs 2, the TBT of the snails did not differ from the TBT observed in the Pre-obs session but was significantly greater than the TBT during the Post-obs 1 session (Fig 1).

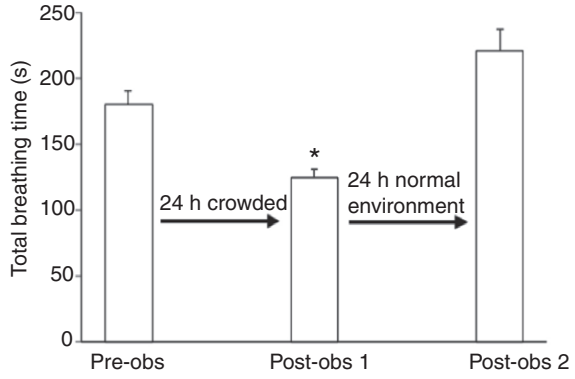

Fig. 1. Crowding reversibly alters total breathing time (TBT). A naïve cohort of snails (N=23) received a 30-min hypoxic challenge (10–12 snails/500 ml; Pre-obs). The mean TBT (± s.e.m.) is plotted on the *y*-axis. These snails were then placed into crowded conditions (20 snails/100 ml) for 24 h and were then given a second hypoxic challenge (Post-obs 1). Snails were then placed into an uncrowded aquarium (two snails/100 ml) for 24 h. A Kruskal–Wallis (non-parametric ANOVA) was performed on these data (KW=19.326; P<0.01) followed by a Dunn's Multiple Comparison test. TBT was significantly less in Post-obs 1 compared with Pre-obs (*P<0.01). TBT in Post-obs 2 was significantly greater than in Post-obs 1 (P<0.01). There was no statistical difference between Pre-obs and Post-obs 2 (P>0.05).

TBT is significantly less in eumoxia than it is in hypoxia (Lukowiak et al., 1996). Therefore, in the present study, we determined whether crowding caused TBT to be suppressed in eumoxia. Experiments were repeated as above except that the TBT of the snails was observed in eumoxia (N=20). The TBT in eumoxic conditions 24 h before crowding was 74.8±8.2 s. Following 24 h of crowding, TBT was significantly decreased to 51.3±3.1 s (P<0.01). TBT following 24 h in the uncrowded condition was 77.6±8.5 s, which was not significantly different to that of the Pre-obs session (P>0.05). Thus, crowding causes significant decreases in TBT in both hypoxia and eumoxia, but this change is not permanent. Together these data demonstrate that crowding is a stressful stimulus, as the normal homeostatic response to hypoxia is altered.

Crowding and the traditional training procedure

A cohort of 30 naïve snails was randomly distributed into two groups: (1) an operant conditioning group and (2) a yoked control group. The operant conditioning group was subjected to the traditional training procedure (TS1 and TS2) and was tested (MT) for LTM 24 h later. The yoked control group received identical training except that, during each TS, they received the non-contingent tactile stimulus. Following the traditional training procedure, the operantly conditioned group demonstrated LTM while the yoked control snails did not (Fig. 2A). Thus, as previously reported, this training procedure results in associative learning and LTM.

We next tested whether crowding of snails for 24 h before the traditional training procedure (24 h OC) would alter the ability of snails to form LTM. Futhermore, following 24 h of crowding and receiving the traditional training procedure, results reveal that LTM was not present 24 h later. MT was significantly greater than TS2 but not significantly different from TS1. However, TS2 was significantly less than TS1, indicating that the crowding did not alter the ability of snails to learn and remember for at least 1 h (Fig. 2B).

Having shown that, following the traditional training procedure, crowding prior to training blocked LTM formation, we next tested whether crowding after the traditional training procedure would also block LTM formation. Thus, another cohort of naïve snails (N=22) was given the traditional training procedure and placed into crowded conditions for 24 h (24 h OC) immediately after TS. When MT was tested, LTM was not present. MT was significantly greater than TS2 but not significantly different from TS1. However, TS2 was again significantly less than TS1 (Fig. 2C). Therefore, these data indicate that crowding before or after the traditional operant conditioning training procedure blocks LTM formation in *L. stagnalis*. Moreover, crowding before training does not appear to interfere with the ability to learn or to remember for 1 h, as TS2 was significantly different from TS1.

Crowding and the memory augmentation procedure

Martens et al. demonstrated that snails subjected to the KCl bath prior to a single 30 min TS, which normally only results in a memory persisting for 2–3 h (i.e. ITM), exhibited memory 24 h later (i.e. LTM) (Martens et al., 2007a). We replicated those results (Fig. 3A). The snails demonstrated LTM, since the number of attempted pneumostome openings in MT was significantly less than the number of attempted openings in TS1. We then challenged this cohort of snails with a change-of-context test (CT). The snails did not demonstrate memory in the new context, as the number of attempted pneumostome openings in CT was significantly greater than MT but was not different from TS1. These data reveal that the decreased

number of attempted pneumostome openings in TS was not the result of sickness or other side effects caused by the KCl bath. Another cohort of snails was exposed to the memory augmentation procedure to determine whether memory is observed 2 h after TS1. As expected, MT was significantly less than TS1, suggesting that memory is detected 2 h after training. This 2 h memory was also context specific, as changing the context (CT) resulted in the snails behaving as though they did not possess memory, i.e. CT was significantly greater than MT but not significantly different from TS1 (Fig. 3B).

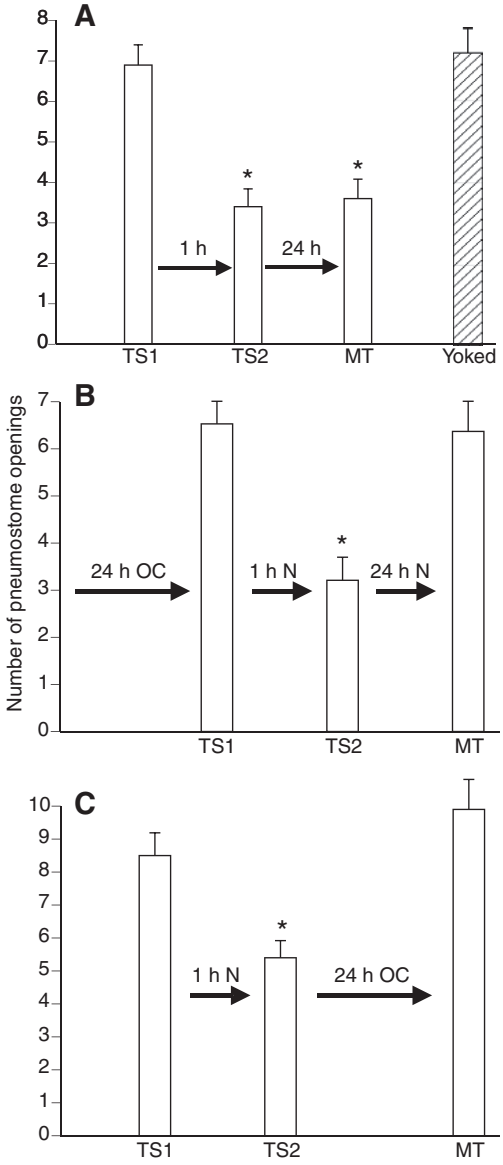

Fig. 2. The traditional training procedure and crowding. (A) The traditional training procedure results in LTM, i.e. MT is significantly less than TS1 (*P<0.01) but not significantly greater than TS2 (P>0.05). Yoked control snails also do not demonstrate LTM as Yoked is not significantly different from TS1 (P>0.05) but is significantly greater than TS2 (P<0.01). (B) A cohort of naïve snails (N=19) were placed in crowded conditions for 24 h prior to TS1. In TS2 (separated by an hour, in which the snails were placed in their normal aquarium; N), the snails received a tactile stimulation each time they began to open their pneumostome. Twenty-four hours after TS2, the memory test (MT) was performed. As can be seen, LTM is not formed; that is, when the data were analyzed (one-way ANOVA) we found that while TS2 is significantly smaller from TS1 (*P<0.01; demonstrating learning and a 1 h memory), MT is significantly greater than TS2 (P<0.01) but not significantly different from TS1 (P>0.05). (C) A cohort of naïve snails was placed in crowded conditions for 24 h immediately after TS2. A similar analysis as in A was done on these snails (N=22). TS2 is significantly less than TS1 (*P<0.01) and also the MT (P<0.01). Values are means ± s.e.m.

Crowding immediately after the training procedure prevents the formation of LTM

To determine whether crowding also blocks LTM formation using the memory augmentation procedure, as it did during the traditional training procedure, the effect of crowding immediately after the memory augmentation procedure was also examined (Fig. 4).

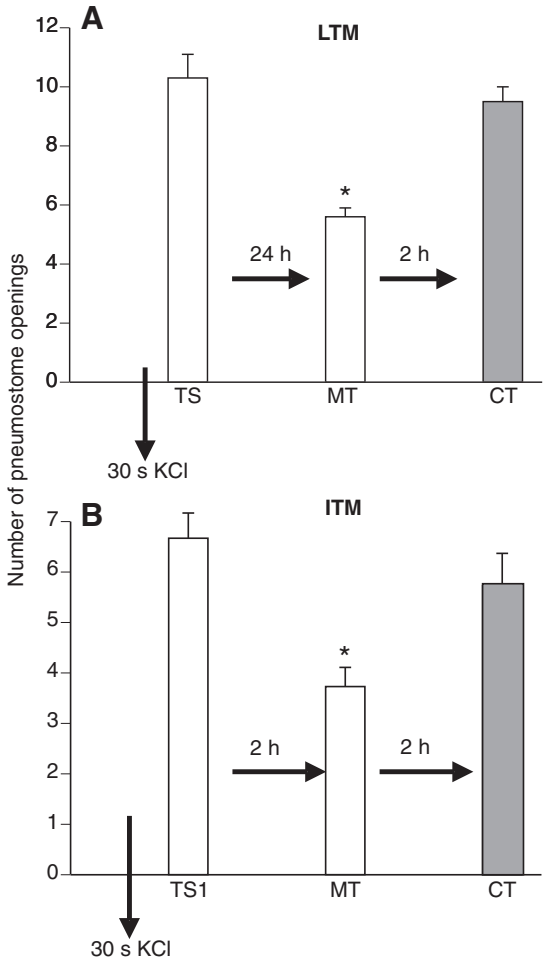

Fig. 3. Long-term (LTM) and intermediate-term (ITM) memory formation following the memory augmentation procedure. (A) A group of naïve snails (N=20) received the KCl bath immediately before a 30-min TS in hypoxic pond water. We tested for memory 24 h later (MT). Each time the snail attempted to open its pneumostome it received a tactile stimulus to the pneumostome. These snails were then challenged (2 h later) to a change-of-context (carrot context) session (CT). The data were subjected to a repeated-measures ANOVA ($F_{19,2}$=22.183; P<0.01) followed by a Tukey–Kramer comparison test. The number of attempted openings in MT is significantly less than in TS (*P<0.01). The number of attempted openings in CT is not significantly different from in TS (P>0.05) but is significantly greater than MT (P<0.01). (B) As in A, except memory was tested 2 h after TS. MT is significantly less than TS, showing that ITM had formed (*P<0.05). Furthermore, there was no significant difference between the response when the context was changed (CT) and TS (P>0.05). The analysis in B was obtained using a Friedman's Test followed by a Dunn's Multiple Comparison test. Values are means ± s.e.m.

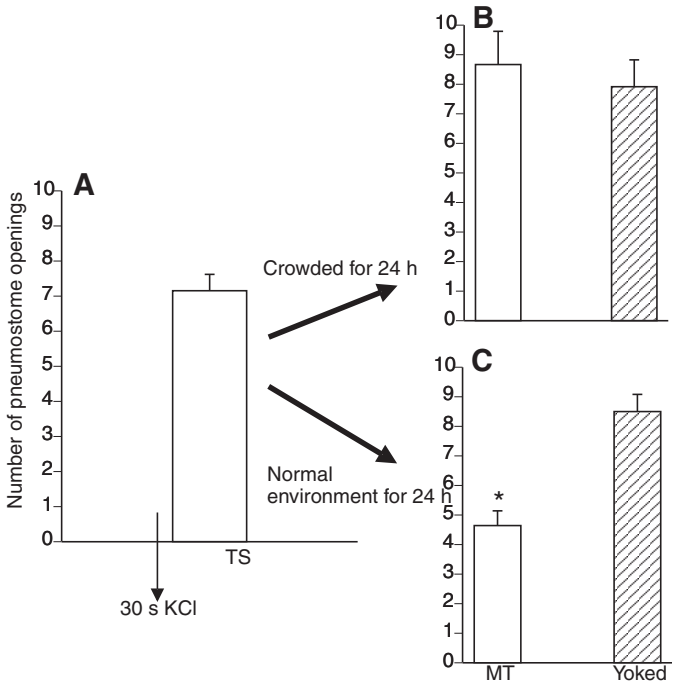

Fig. 4. Crowding, the memory augmentation procedure and LTM formation. Snails (N=52; 26 operantly conditioned, 26 yoked controls) were described in Fig. 3A. (A) Snails that received the operant conditioning procedure were randomly divided into two groups immediately following TS. One group (N=12) was subjected to crowded conditions for 24 h, while the other group (N=14) was maintained for 24 h in the control, uncrowded conditions. All snails were then tested for memory (MT). The data were subjected to a one-way ANOVA ($F_{51,4}$=5.107 P<0.01) followed by a Tukey–Kramer comparison test. As can be seen (B) LTM was not present. That is, the number of attempted openings in MT was not statistically different from TS (P>0.05). (C) By contrast, snails not subjected to crowding exhibited LTM. The number of attempted openings in MT of this group was statistically different from TS (*P<0.01). Yoked control snails subjected to either crowding or uncrowded conditions showed no statistical difference from TS (P>0.05 for each comparison) nor were they statistically different from each other (P>0.05). Values are means ± s.e.m.

Following TS1, the operantly conditioned snails (N=26) were randomly divided into two groups: 12 snails were placed into a crowded aquarium for 24 h, while the remaining 14 were placed into an uncrowded aquarium for 24 h. Snails subjected to crowding (Fig 4B) did not exhibit LTM, as the number of attempted pneumostome openings in MT was not significantly different from the number of attempted openings in TS. However, LTM was present in those snails that were housed in an uncrowded aquarium for 24 h (Fig 4C). Moreover, the number of attempted openings in MT of the snails subjected to crowded conditions was significantly greater than the number of attempted openings in MT of the snails in uncrowded conditions. To further demonstrate that crowding blocked LTM formation, yoked control snails were subjected to similar crowded and uncrowded conditions following TS (Fig 4). The response in MT of the yoked control snails was statistically similar between the two groups, revealing that crowding had no effect on how yoked control snails respond in MT compared with yoked control snails maintained in uncrowded conditions. Thus, we conclude that the immediate crowding of snails for 24 h following training prevents LTM formation.

We next tested whether delaying crowding for 1 h following TS also blocked LTM formation using the memory augmentation procedure. Following 1 h in uncrowded conditions, the trained snails (N=23) were subjected to crowded conditions for 23 h before testing MT for LTM. Results reveal that LTM was present (Fig. 5). To control for possible crowding side effects, the snails were subjected to the carrot context (CT) 2 h later. In CT, the snails behaved as naïve snails. Finally, we also subjected another cohort (N=23) of snails to the yoked control procedure and waited 1 h before subjecting these snails to crowded conditions. Results reveal that the number of attempted openings in yoked control snails was statistically the same as in TS but was significantly different from the number of attempted openings in MT.

Crowding does not block recall

Fig. 5A reveals that delaying crowding by 1 h was sufficient to allow LTM to form. These data also show that, once LTM has formed, crowding for 23 h before MT did not alter the ability of snails to access that memory.

Immediate crowding for 1 h is sufficient to block LTM formation

The consolidation process necessary for LTM following the memory augmentation procedure occurs within 1 h following TS (Martens et al., 2007a). This led us to ask if crowding snails for only 1 h immediately after training was sufficient to impair LTM formation. Immediately after training, snails were subjected to crowded conditions for 1 h, after which they were subjected to uncrowded conditions for 23 h. Results reveal that LTM was not present (Fig. 5B). Yoked control snails subjected to the same crowding challenge gave similar results.

Crowding before training can also block LTM formation

A naïve cohort of 56 snails was subjected to crowded conditions for 24 h prior to TS1 to determine whether crowding before operant training blocked LTM. The snails were trained using the memory augmentation procedure. Results reveal that LTM was not present 24 h later, as the number of attempted openings in MT was not significantly different from TS1 (Fig. 6A). Yoked control snails showed a similar response. Thus, crowding for 24 h prior to training altered the snails' ability to form LTM.

As 1 h of crowding immediately after training was sufficient to block LTM formation, we wished to determine whether crowding for 1 h immediately prior to training was sufficient to block LTM. A naïve cohort of snails was subjected to crowded conditions for 1 h just prior to training using the memory augmentation procedure. LTM was not present in snails that had been exposed to crowded conditions 1 h immediately prior to training, as the number of attempted openings in MT was not significantly different from TS1 (Fig. 6B). The response of the yoked control snails was also shown to be the same. Thus, crowding for only 1 h before TS is sufficient to block LTM formation.

Crowding and ITM formation

LTM formation in *Lymnaea*, requires both altered gene activity and new protein synthesis, while ITM, which persists for 2–3 h in *Lymnaea*, is dependent only on new protein synthesis (Sangha et al., 2003a; Sangha et al., 2003b; Lattal et al., 2006). We therefore asked if crowding also compromises ITM formation following the memory augmentation procedure. Immediately following training, the snails were placed in crowded conditions for 1 h, after which they were returned to normal conditions for a further 1 h before testing for ITM. Results revealed that ITM was present. To control

for the possibility that the decreased number of attempted openings in MT was not a reflection of memory but rather a side effect of crowding, the context of the memory test was changed. In this test, snails behaved as though they were naïve. Finally, the yoked control snails, which were subjected to the same crowding challenge and tested at the same time as the operantly conditioned snails, also showed no evidence of memory.

We then determined whether crowding prior to training had a deleterious effect on ITM formation following the memory augmentation procedure (Fig. 7B). Snails were kept in crowded conditions for 24 h before TS. Following TS they were maintained in uncrowded conditions for 2 h before MT. We found that ITM

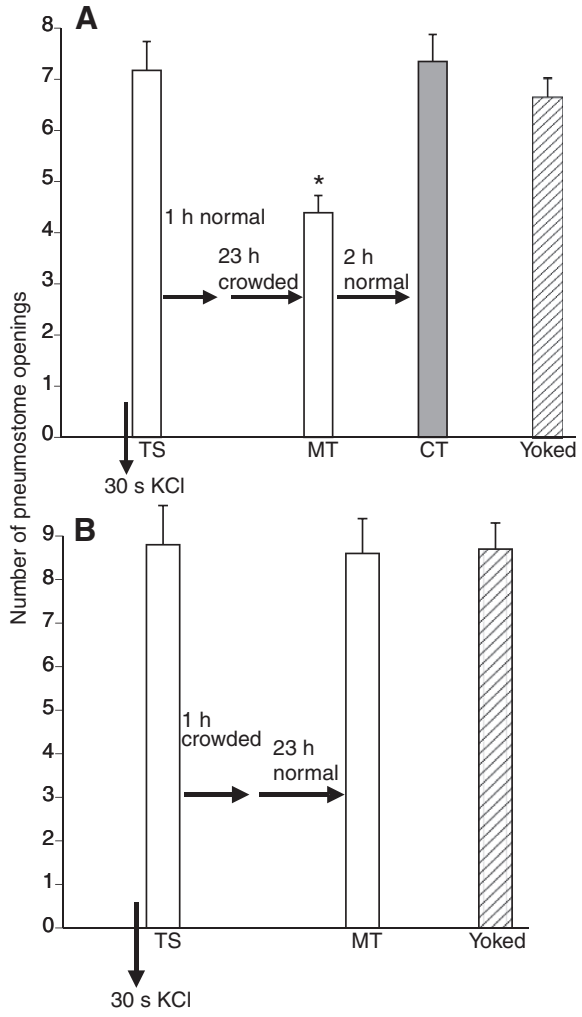

Fig. 5. Delay of crowding and LTM formation. (A) A cohort of naïve snails (N=23 operantly conditioned and N=23 yoked controls) was trained using the memory augmentation procedure and placed in an uncrowded aquarium for 1 h immediately after training. Following this period, all snails were subjected to crowded conditions for 23 h. All snails were then tested for memory (MT). Data were subjected to a repeated-measures ANOVA ($F_{45,3}$=10.982; P<0.01) followed by a Tukey–Kramer comparison test. Snails that were subjected to the operant conditioning procedure exhibited LTM (i.e. MT was significantly less than TS; *P<0.01). When these same snails were subjected to a change of context challenge (CT, carrot context) 2 h later they did not exhibit LTM. That is, CT is not significantly different from TS (P>0.05). Snails subjected to the yoked control procedure also did not exhibit LTM (i.e. yoked is not significantly different from TS, P>0.05). In addition, the response of the yoked control snails was not significantly different from the response to CT. (B) Another cohort of naïve snails (N=40; 20 operantly conditioned and 20 yoked control snails) was subjected to operant conditioning and the yoked control procedure, respectively. Immediately after their respective training procedures they were placed into a crowded aquarium for 1 h. Following this period, all snails were placed into an uncrowded aquarium for 23 h. All snails were then tested for LTM. Data were subjected to a repeated-measures ANOVA ($F_{39,2}$=0.5398; P>0.05) followed by a Tukey–Kramer comparison test. The operantly trained snails do not exhibit LTM (MT is not significantly different from TS, P>0.05). In addition, the yoked control snails subjected to the same crowded and uncrowded conditions do not exhibit LTM (Yoked is not significantly different from TS). Values are means ± s.e.m.

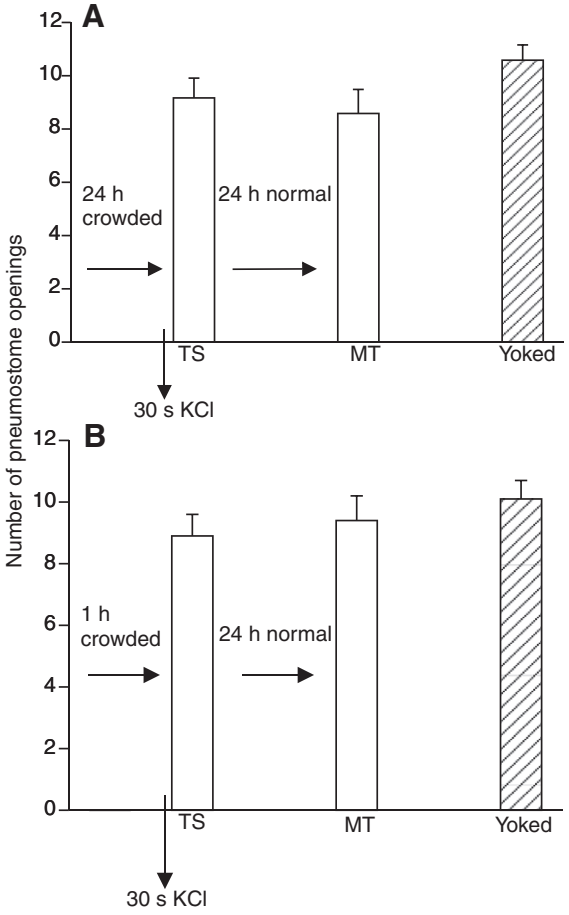

Fig. 6. Crowding before training blocks LTM formation. (A) A naïve cohort of snails (N=56; 28 operantly conditioned and 28 yoked controls) was subjected to 24 h of crowding immediately before the memory augmentation procedure. Following training, all snails were placed in an uncrowded aquarium for 24 h and then tested for LTM (MT). Data were subjected to a repeated-measures ANOVA ($F_{55,2}$=2.378; P>0.05) followed by a Tukey–Kramer comparison test. Neither the operantly conditioned snails nor the yoked control snails exhibited LTM. That is, MT is not significantly different from TS (P>0.05) nor is Yoked different from TS (P>0.05). Finally, Yoked is not significantly different from MT (P>0.05). (B) Crowding for 1 h immediately before the memory augmentation procedure is sufficient to block LTM formation. Another naïve cohort of snails (N=19; 10 operantly conditioned and nine yoked controls) was subjected to 1 h of crowding immediately before training. Following training, all snails were placed in an uncrowded aquarium for 24 h and then tested for LTM (MT). Data were subjected to a repeated-measures ANOVA followed by a Tukey–Kramer comparison test. Neither the operantly conditioned snails nor the yoked control snails exhibit LTM ($F_{18,2}$=0.8510; P=0.4364). That is, MT is not significantly different from TS (P>0.05) nor is Yoked different from TS (P>0.05) Finally, Yoked is not significantly different from MT (P>0.05). Values are means ± s.e.m.

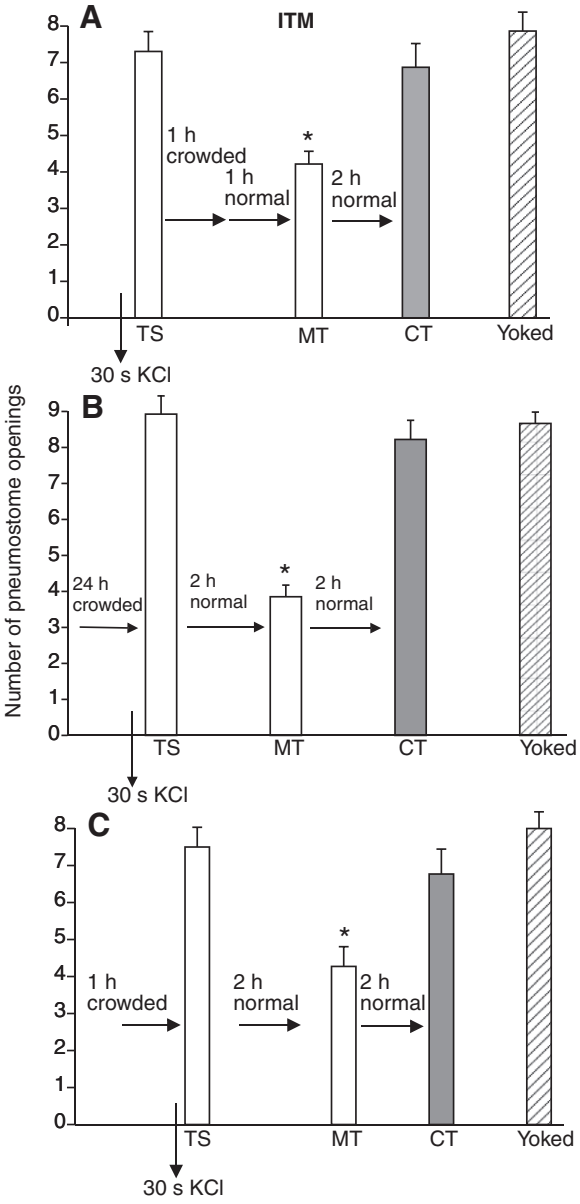

Fig. 7. Crowding does not block intermediate-term memory (ITM). (A) A naïve cohort of snails (N=46; 23 operantly conditioned and 23 yoked control snails) was subjected to 1 h of crowding immediately after the memory augmentation procedure and then placed into an uncrowded aquarium for an additional 1 h period. The snails were then tested for memory (MT). That is, memory was tested 2 h after training. A Kruskal–Wallis (non-parametric ANOVA) was performed on these data (KW=27.726; P<0.01) followed by a Dunn's Multiple Comparison test. In the cohort that received operant conditioning training, ITM is present as MT is significantly less than TS (*P<0.01). These snails were then challenged with a change-of-context session (CT) 2 h later. Memory is not present as CT is not significantly different from TS (P>0.05). The snails that received the yoked control procedure (Yoked) do not exhibit ITM (Yoked is not significantly different from TS; P>0.05). (B) Another cohort of snails (N=27 operantly conditioned and 27 yoked controls) was crowded for 24 h before the memory augmentation procedure. Following training they were all placed into an uncrowded aquarium for 2 h before the memory test (MT or Yoked). Data were subjected to a repeated-measures ANOVA ($F_{53,3}$=31.651; P<0.01) followed by a Tukey–Kramer comparison test. Snails that had been operantly conditioned exhibit ITM when tested (MT; *P<0.01). The yoked control snails do not exhibit ITM (Yoked not significantly different from TS; P>0.05). Snails that had received operant conditioning training were also challenged 2 h after MT with a change-of-context test (CT). In CT, the number of attempted openings was not statistically different than TS (P>0.05), indicating that snails were not sick. (C) As in B except snails were only crowded for 1 h before the operant conditioning training or the yoked control procedure. A Kruskal–Wallis (non-parametric ANOVA) was performed on these data (KW=43.536; P<0.01) followed by a Dunn's Multiple Comparison test. As in B, ITM is present in the operantly conditioned snails (MT is significantly different from TS; *P<0.01) but is not present in the yoked control (Yoked is not significantly different from TS; P>0.05). Also as in B, when the context was changed, snails behaved as though they were naïve (i.e. CT is not significantly different from TS; P>0.05). Values are means ± s.e.m.

was present. When challenged with a change-of-context test 2 h later, they behaved as naïve snails. Finally, the yoked control snails showed no evidence of memory. Thus, with 24 h of crowding before training, ITM was still formed. In a second experiment (Fig. 7C), a cohort of snails was subjected to crowded conditions for 1 h only prior to the memory augmentation procedure. Following TS1, snails were placed into uncrowded conditions for 2 h before testing for memory. We found memory to be present. Moreover, when challenged with the change-of-context test they behaved as naïve snails. Finally, the response in MT of the yoked control snails was not significantly different from TS. Thus, we concluded that crowding either before or after TS does not block ITM, suggesting that the effect crowding has on LTM formation is due to an alteration of the transcription process.

Aerial breathing behaviour and memory formation in CPW

In the next series of experiments, we determined whether snails had to experience crowding with other live snails (i.e. 'rubbing shoulders') in order for LTM formation to be blocked or whether there was a chemical signal, e.g. in the water released by snails in crowded conditions, that would be sufficient to block LTM. A 30-min breathing observation (Pre-obs) was performed as in Fig. 1. These snails were then placed in an aquarium containing CPW for 24 h but were maintained in uncrowded conditions (i.e. two snails per 100 ml of PW). A 30-min post-observation was then performed; no significant changes in TBT of the snails was demonstrated (Fig. 8A). Thus, unlike crowding, CPW did not alter breathing behaviour.

We then placed snails in CPW for 1 h prior to TS1 (1 h OC water) (Fig. 8B). Following the memory augmentation procedure they were maintained in uncrowded conditions for 24 h before MT. Results reveal that LTM was present. When challenged with a change-of-context test 2 h later, they behaved as naïve snails. Finally, the yoked control snails showed no evidence of memory. In a similar manner, snails were placed for 1 h in CPW immediately after TS1 (Fig. 8C; 1 h OC water). LTM was observed 24 h later. When challenged with the change-of-context test 2 h later, snails behaved as they did in TS. Moreover, the yoked control snails did not exhibit LTM.

Having demonstrated that CPW is not sufficient to block LTM formation, we asked whether crowding with empty clean snail shells would block LTM. We placed a cohort of naïve snails with clean, empty snail shells for 24 h before the memory augmentation procedure. We found that this crowding was insufficient to block LTM formation (Fig. 9A) either before or after training (data not shown). Finally, we tested whether we could use a combination of clean shells of deceased snails (same density used as for live snails), to produce crowding, together with CPW, to alter either aerial respiratory behaviour or block LTM formation. We found that the combination of shells and CPW was insufficient to block the

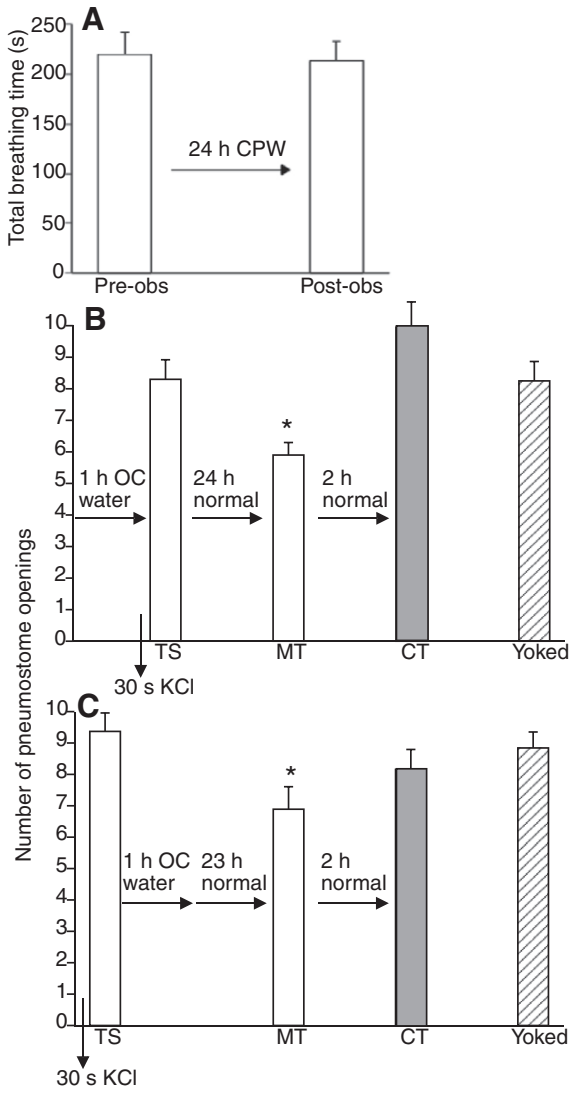

Fig. 8. Aerial respiratory behaviour and LTM formation in CPW. (A) Total breathing time (TBT) was unaffected by placing snails in crowded pond water (CPW) for 24 h. TBT was first calculated for a cohort of naïve snails (Pre-obs; N=20). The snails were then placed in CPW for 24 h after which TBT was then calculated (Post-obs). A paired t-test was performed on these data. There is no difference in TBT between the Pre-obs and the Post-obs sessions (t=0.4442; P>0.05). (B) A 1 h exposure to CPW (OC in figure) immediately before the memory augmentation procedure does not block LTM formation. The data were subjected to a repeated-measures ANOVA ($F_{18,3}$=7.299; P<0.01) followed by a Tukey–Kramer comparison test. The operantly conditioned snails (N=10) exhibit LTM (MT is significantly less than TS; *P<0.01) while yoked control snails (N=10) do not exhibit LTM (Yoked is not significantly different from TS; P>0.05). Additionally, the operantly conditioned snails behaved as naïve snails to the change-of-context challenge (CT is not significantly different from TS; P>0.05). (C) A 1 h exposure to CPW immediately after operant conditioning training (memory augmentation procedure) does not block LTM formation. A naïve cohort of 20 snails (N=20; 10 operantly conditioned and 10 yoked controls) received their respective training procedures and was then immediately placed into CPW for 1 h. Following the 1 h exposure to CPW, snails were moved to an uncrowded aquarium for 23 h. The data were subjected to a repeated-measures ANOVA ($F_{19,3}$=5.384; P<0.01) followed by a Tukey–Kramer comparison test. When tested for LTM (MT), snails that received operant conditioning training exhibited LTM. That is, MT is significantly less than TS (*P<0.05). Yoked control snails do not exhibit LTM (i.e. Yoked is not significantly different from TS; P>0.05). The operantly conditioned snails were also challenged with a change-of-context test (CT) 2 h after TS. In CT, snails behaved as naïve snails. That is, CT is not significantly different from TS (P>0.05). Values are means ± s.e.m.

formation of LTM (Fig. 9B). Thus, we conclude that exposure to CPW alone or exposure to a combination of shells and CPW is not sufficient to alter aerial respiratory behaviour or LTM formation.

DISCUSSION

Crowding for as little as 1 h immediately before or after either the traditional or the memory augmentation training procedures is sufficient to block LTM but not ITM formation. We also found that crowding for up to 23 h did not prevent snails from recalling an already-formed LTM. As LTM requires both altered gene activity and new protein synthesis, whereas ITM requires only new protein synthesis (Lukowiak et al., 2000), we hypothesise that crowding interferes with the necessary genomic activity to produce LTM in neurons, such as RPeD1, that are necessary for LTM formation (Scheibenstock et al., 2002).

The data showing that LTM, but not ITM, is blocked show that the effects of crowding are specific to memory formation and are not the result of general malaise. If crowding induced a general depression of behaviour, this would have been observed in the change-of-context challenge and in the blockage of ITM. However, in all cases with the change-of-context controls, snails behaved as they did in the initial TS. We believe that the blockage of LTM formation by crowding is relatively specific. It remains to be determined whether crowding alters any other easily observable behaviours (e.g. feeding or locomotion) or whether crowding blocks LTM formed following either appetitive or aversive food conditioning (Azami et al., 2006; Sugai et al., 2007).

Crowding of snails at densities equal to or greater than those we used in the present study (Colton, 1908; Crabb, 1924; Forbes and Crampton, 1942; Noland and Carriker, 1946) negatively affected the extent and rate of growth, reproductive success and development of recently hatched *Lymnaea*. However, these previous studies employed chronic crowding, whereas acute crowding (1 h to a maximum of 24 h) was investigated in the present study. Whether chronic crowding (i.e. days to weeks) would have any different effect(s) on aerial respiration and/or memory formation remains to be determined.

Crowding has been used as a stressor in studies investigating behaviours such as memory formation, immune system functioning, and longevity in both vertebrates and invertebrates (Gems et al., 1998; Vanitallie, 2002; Bowman et al., 2003; Roman et al., 2004). For example, Gems et al. showed that crowding in *Caenorhabditis elegans* arrests development (Gems et al., 1998). Mice have also been observed to become more susceptible to nematode infection when crowded due to stress-mediated immuno-depression (Abu-Madi and Lewis, 1997). However, our present study is the first we know of to use crowding as a stressor to block LTM formation in a model system where it may be relatively easy to demonstrate how this stressor acts at the single cell level, as the molecular processes that cause LTM formation occur within RPeD1 (Scheibenstock et al., 2002).

TBT significantly decreases following crowding. Intuitively, this appears to be an inappropriate response. However, it is not. When exposed to an environmental stressor such as hypoxia or prey detection, *Lymnaea* respond first with compensatory and then adjustive changes in metabolism, respiration and heart rate (Hochachka et al., 1996; Taylor et al., 2003; Orr et al., 2007).

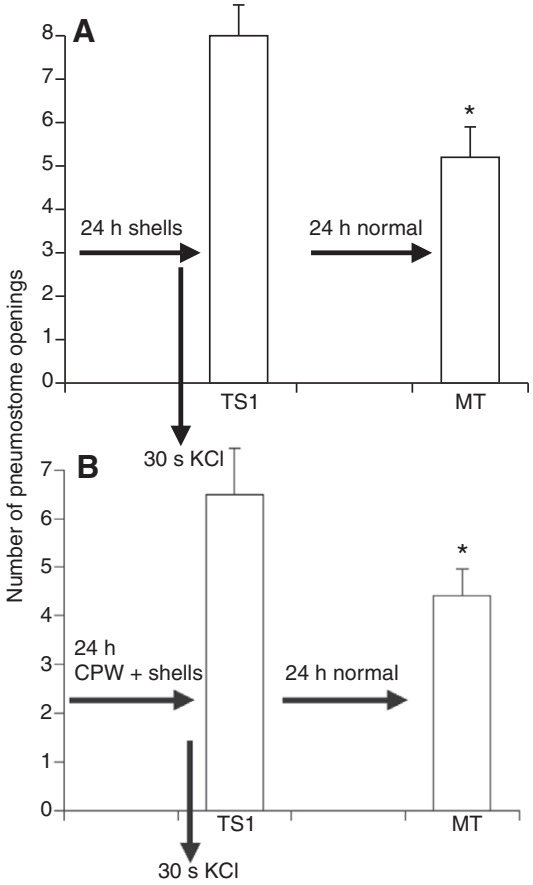

Fig. 9. The overcrowding effect is not caused by the physical lack of space. (A) When snails (N=18) were exposed to crowding with clean shells for 24 h prior to the memory augmentation procedure, LTM forms (*P<0.05). This was determined with the use of a Wilcoxon matched-pairs signed ranks test, which showed that the number of pneumostome openings significantly decreases in the MT compared with the TS. (B) As in A, except that in addition to clean shells we added crowded pond water (CPW) as in Fig. 8. The data were subjected to a paired t-test (t=2.391; *P<0.02) and show that LTM is formed despite the snails being exposed to CPW and clean snail shells.

Therefore, a paradox exists between compensatory and adjustive changes. Compensatory responses involve changes in ventilation and cardiac output (e.g. they increase) and in turn tend to minimize the fall in blood oxygen. These compensatory responses represent an attempt by the organism to continue to meet cellular metabolic demands. However, if the compensatory changes fail to stem the drop in oxygen, *Lymnaea* switch to an adjustive strategy, i.e. TBT is depressed (Taylor et al., 2003). This adjustive response represents an attempt to minimize oxygen requirements, thereby avoiding hypoxic impairment (Hochachka et al., 1996). We hypothesize that since there is a metabolic cost of LTM formation (i.e. altered gene activity and new protein synthesis), a consequence of *Lymnaea* assuming the adjustive strategy as a result of crowding is that LTM formation will be suppressed. That is, we suggest that the blocking of LTM formation is a side effect of crowding on overall genomic activity. Whether or not crowding at similar densities as we used here with non-conspecifics (e.g. other species of snails or with non-predatory vertebrates) would have the same effect on LTM formation remains to be determined. However, it seems unlikely based on our findings with the clean shell data.

A different environmental stressor, predator detection, alters both aerial respiratory behaviour and LTM formation (Orr et al., 2007; Orr and Lukowiak, 2008). However, upon predator detection, aerial respiratory behaviour increases and LTM is significantly enhanced. To date, in *Lymnaea*, crowding has been the only stressor we have found that blocks LTM formation by itself. If we had used only the memory augmentation procedure to produce associative learning and the subsequent formation of LTM, we could possibly have interpreted our findings to indicate that the reason LTM formation was blocked was that there was too much stress. That is, the memory augmentation procedure uses a stressor (KCl bath) to enhance memory formation and, coupled with an additional stress (crowding), LTM would be blocked. As Martens et al. demonstrated, too much stress blocks LTM formation (Martens et al., 2007a). However, we also showed that crowding blocks LTM using the traditional training procedure, which does not utilize a stressor (e.g. KCl bath) to enhance memory formation. Thus, we conclude that the effect of crowding either immediately before or after operant conditioning for a period as short as 1 h is sufficient to block LTM formation.

As previous data demonstrate, stress can modify LTM formation either by enhancing it or, as in our case, suppressing it (e.g. Yerkes and Dodson, 1908; Shors, 2006). For example, using an acute heat shock as a stressor, Beck and Rankin showed that LTM formation could be blocked in *C. elegans* (Beck and Rankin, 1995). Likewise, with an acute stress (e.g. inescapable exposure to a cat), spatial memory in rats was impaired (Sandi, 2004). By contrast, acute stress improved eye blink conditioning in both animals (Shors, 2006) and healthy humans (Duncko et al., 2007). Therefore, many factors, including the type of stress, the response of the organism to the stress, the nature of the task and the previous history of the organism, determine whether acute stress will suppress or enhance memory formation. Chronic stress has also been demonstrated to impair hippocampal-dependent spatial memory in rats without affecting their motor skills (Kleen et al., 2006). Equally, LTM can be enhanced through fearful stressors (Blank et al., 2002; Nijholt et al., 2004; Orr and Lukowiak, 2008). Thus, conflicting data exist regarding the effect that stress has on LTM formation. The effect that stress will have on the formation and/or recall of LTM is dependent on a myriad number of factors, including the age and gender of the organism, its previous history dealing with stress and whether or not the stress is in any way controllable by the animal. It is thus problematic to predict ahead of the actual data what effect a particular stressor will have on LTM. This is one of the main reasons why it has been so difficult to understand how stress alters memory formation.

For crowding to block LTM formation it must occur either immediately before or after the training procedure and it can be as short as 1 h. These data are consistent with the notion that memory modification is time dependent, i.e. it does not occur instantaneously (McGaugh, 2000; Nielson and Powless, 2007). We have not yet determined either the minimal duration of crowding or the minimal gap between training and crowding necessary to block LTM formation. Previously, we have found that to block LTM formation successfully, cooling to 4°C must occur within 15 min of training and must be at least 1 h duration (Sangha et al., 2003b). We hypothesize that the ability of crowding to block LTM formation will have similar time parameters; however, these experiments are still to be performed. Recent data show that, in humans, arousal after learning is capable of enhancing memory formation even when the arousal stimuli are delayed by up to 30 min (Nielson and Powless, 2007). Whether delaying crowding by 30 min following training will affect the ability of crowding to block LTM formation remains to be determined. We do know, however, that crowding does not block

ITM formation, which is dependent on new protein synthesis. The fact that ITM formation is resistant to blockade by crowding also reinforces our notion that the effects of crowding are specific to LTM formation and not the result of some epiphenomenon.

We initially hypothesized that, as for some other stressors (Diamond et al., 1996; Kirschbaum et al., 1996; de Quervain et al., 1998; Diamond et al., 1999; Payne et al., 2002; Roozendaal, 2002; Woodson et al., 2003), crowding would impair memory recall. However, this was not the case, because even if we crowded snails for 23 h before giving them a memory test, LTM was still apparent. As LTM was still present in the memory recall experiments, we concluded that the ability of crowding to block memory formation was therefore not the result of a deficit in a general metabolic process that caused snails to perform aerial respiration to a greater degree and thus mask LTM.

Earlier researchers (Crabb, 1929) hypothesized that the reduced growth of snails in crowded conditions was due to a 'factor' in the water that the snails were maintained in. Voronezhskaya et al. also found that 'chemicals' released into the water by snails in crowded conditions delayed embryonic development of *Lymnaea* (Voronezhskaya et al., 2004). Our initial working assumption was that water from the crowded snail aquarium would be sufficient to block LTM formation. However, our attempts to block LTM formation with just water taken from a crowded aquarium (CPW), or a combination of CPW and empty snail shells allow us to conclude that in order to block LTM formation snails must experience crowding with other live snails. It is possible that substances released by other live snails in the mucus that snails secrete to move may contain the substance(s) sensed by snails that causes LTM to be blocked. Further experimentation will be needed to test this hypothesis.

In conclusion, our data reveal that stress associated with crowding blocks LTM formation and that there is a critical period persisting for 1 h following training when crowding blocks LTM formation. However, crowding during this period does not block ITM formation. Thus, we hypothesize that this stressor acts on genomic activity to prevent the molecular processes necessary for LTM from being initiated or brought to completion.

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