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RESEARCH ARTICLE

Sequential exposure to a combination of stressors blocks memory reconsolidation in *Lymnaea*

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ABSTRACT

Stress alters the formation of long-term memory (LTM) in *Lymnaea*. When snails are exposed to more than one stressor, however, how the memory is altered becomes complicated. Here, we investigated how multiple stressors applied in a specific pattern affect an aspect of memory not often studied in regards to stress – reconsolidation. We hypothesized that the application of a sequence of stressors would block the reconsolidation process. Reconsolidation occurs following activation of a previously formed memory. Sequential crowding and handling were used as the stressors to block reconsolidation. When the two stressors were sequentially presented immediately following memory activation, reconsolidation was blocked. However, if the sequential presentation of the stressors was delayed for 1 h after memory activation, reconsolidation was not blocked. That is, LTM was observed. Finally, presentation of either stressor alone did not block reconsolidation. Thus, stressors can block reconsolidation, which may be preferable to pharmacological manipulations.

KEY WORDS: *Lymnaea stagnalis*, Long-term memory, Multiple stressors, Crowding

INTRODUCTION

Numerous studies have shown in humans and rodents, as well as in our model system, *Lymnaea*, that stress plays important and complex roles in learning and memory (Lukowiak et al., 2014). Stressors can either impair or enhance learning and memory. The specific effect of a stressor on memory depends on many factors including the specific type of stressor, the time at which the stressor is experienced, the nature of the behaviour, the arousal state of the subject, and characteristics of the subject such as sex and age (Shors, 2004; Kim and Diamond, 2002). We have used our simpler *Lymnaea* model to overcome many of these complications when studying the effects of stress on memory (Lukowiak et al., 2008, 2010, 2014). In reviewing the literature, it is apparent that there is not an abundance of studies describing the effects of stress on one aspect of memory, the reconsolidation process (Akirav and Maroun, 2013). Memory reconsolidation is the process by which a memory is destabilized by its retrieval and becomes modifiable (Misanin et al., 1968; Nader et al., 2000a). The reason stressors have not been used in reconsolidation studies might be that other interventions work better than stress or that inappropriate stressors have been utilized that do not interfere with reconsolidation.

Memories are dynamic; they can be strengthened, weakened or even modified (i.e. changed or altered) after they have been formed (Nader et al., 2000a,b; Dudai, 2006; Lukowiak et al., 2007; Reichelt and Lee, 2013). One modifying mechanism is the reconsolidation process. For example, in *Lymnaea*, following activation of a memory, placing snails into a different context during the reconsolidation period results in the reconsolidated memory being updated so that snails have a memory for the new context (i.e. memory infidelity), even though they received no training in that context (Lukowiak et al., 2007). This is similar to the idea of the implantation of a false memory (e.g. 'Bugs Bunny at Disneyland') following activation of a previously consolidated memory in humans (Loftus, 2003; Schater, 1999).

An idea that has attracted much attention is the notion that it might be possible to use an understanding of the neuronal basis of the memory reconsolidation to treat a number of 'memory disorders' such as phobias, post-traumatic stress disorder (PTSD) and substance abuse (Dębiec, 2012; Agren, 2014). It was hypothesized that administration of propranolol (a β-adrenoceptor antagonist) after memory retrieval would disrupt the reconsolidation process and could thus effectively treat PTSD, etc. This treatment has worked in animal models (e.g. contextual fear; Abrari et al., 2008; Dębiec and Ledoux, 2004) and in some cases in humans suffering from PTSD (Pitman et al., 2002; Pitman and Delahanty, 2005; Brunet et al., 2008, 2011; Soeter and Kindt, 2011; Kindt and Soeter, 2013). However, more recent, larger studies have not found a significant effect of propranolol administration after trauma (Sharp et al., 2010; Parsons and Ressler, 2013). Thus, new strategies are needed in the hope of alleviating such 'memory disorders'.

A possible avenue of research would be to explore the possibility that behavioural rather than pharmacological procedures can be used to block reconsolidation. In performing a series of experiments examining how repeated presentations of a stressor altered memory formation, we found we could block reconsolidation in *Lymnaea* by behavioural means. In *Lymnaea*, ecologically relevant stressors alter long-term memory (LTM) formation (Lukowiak et al., 2010, 2014). Depending on the specific stressor used, LTM formation can either be enhanced or suppressed. For example, predator detection enhances LTM formation. That is, snails exposed to predator scent form LTM (i.e. a memory lasting at least 24 h) with a single 0.5 h training session, whereas, in the absence of the predator, the memory only persists for a few hours. In addition, snails exposed to the predator scent and trained with a procedure that normally only results in LTM persisting for 24 h exhibit enhanced memory that persists for 8 days (Orr et al., 2007, 2008, 2009a,b; Orr and Lukowiak, 2008). In contrast, two other environmentally relevant stressors, crowding and a low concentration of calcium in pond water, block LTM formation even though snails do exhibit learning (de Caigny and Lukowiak, 2008a; Dalesman and Lukowiak, 2011a,b, 2012; Dalesman et al., 2011b). That is, snails exposed to these stressors and trained with procedures that in control snails result in LTM do not exhibit LTM even though they learn. While these two stressors block LTM formation, they do so via different sensory pathways (Dalesman et al., 2011a,b,c). Thus, the sensory pathway that is necessary to detect hypoxic pond water and low environmental calcium pond water (osphradial nerve input) is not the

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same as that used to sense crowding (Dalesman and Lukowiak, 2011a; Dalesman et al., 2011a,c; Karnik et al., 2012). Our current working hypothesis is that it is the mucous that snails detect that signals overcrowding. Currently, we are unable to predict ahead of time whether a specific stressor will enhance or block LTM formation. As such, empirical evidence regarding the effect of a stressor is required in order to characterize the effect that a specific stressor will have on LTM formation potential. Finally, in a similar manner, we are unable to predict what will happen to memory formation when a combination of stressors is used, even though we know the effect of each individual stressor on LTM formation (Dalesman et al., 2013).

Here, we used a combination of stressors – crowding and handling – to examine the effects on LTM formation. Sequential exposure of *Lymnaea stagnalis* (Linnaeus 1758) to handling (i.e. snails handled multiple times) and crowding blocked reconsolidation when they were applied immediately after a memory retrieval session. If, however, we allowed a 1 h interval between reactivating the memory and the application of the sequential stressors, reconsolidation was not blocked. Finally, application of handling or crowding singly after memory activation did not block reconsolidation.

RESULTS

In the initial experiments shown in Fig. 1, either the crowding (Fig. 1A) or the handling (Fig. 1B) stimulus was presented to naive

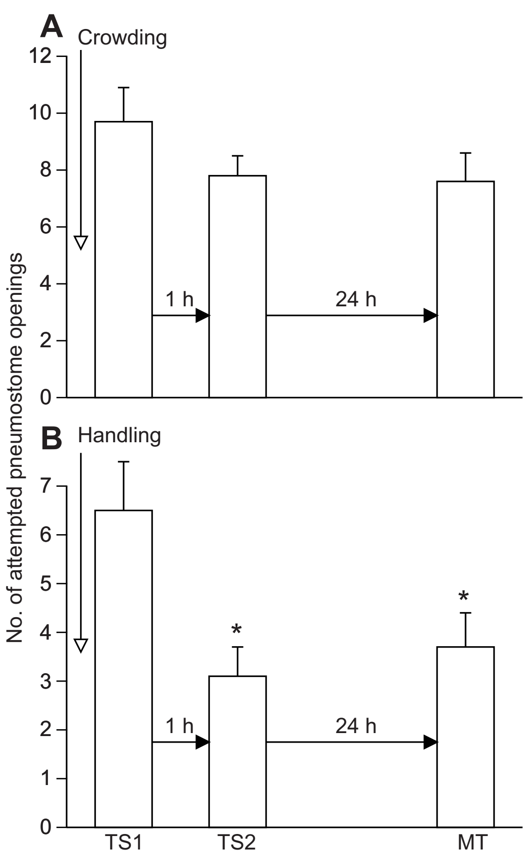

Fig. 1. Experimental protocol for sequential exposure to handling and crowding. (A) A cohort of naive snails (*N*=9) was crowded before operant conditioning training. Snails received two, 0.5 h training sessions (TS1 and TS2) separated by a 1 h interval. Twenty-four hours later, they were tested (memory test, MT) for long-term memory (LTM) formation. (B) As in A (*N*=8), only in this case the handling procedure preceded the operant conditioning training. Values are means±s.e.m. *Significant difference from the number of pneumostome openings in TS1 (P<0.05).

snails before the operant conditioning training procedure to determine the effect the respective stimuli had on both intermediate-term memory (ITM) and LTM formation. Memory was operationally defined as a significant reduction in the number of attempted pneumostome openings in the training session (TS) or memory test session (MT) compared with the first training session. Additionally, the number of attempted pneumostome openings in the MT must not be significantly greater than the number of attempts in TS2 (Lukowiak et al., 1996, 1998, 2000).

Naive snails were crowded for 1 h prior to training. Immediately following the crowding stimulus, snails received two, 0.5 h training sessions (TS1 and TS2, respectively), separated by 1 h. Following the second training session, snails were returned to their home eumoxic aquarium. Snails were tested for LTM 24 h later (MT). We found that crowding blocked both ITM and LTM (ANOVA; $F_{2,16}$=1.354, P<0.05). That is, the number of attempted pneumostome openings in TS2 (i.e. ITM) was not statistically smaller than that in TS1. Likewise, the number of attempted openings in MT was not significantly different from that in TS1. Thus, crowding before training in this cohort results in the blockade of memory formation.

We next performed a similar experiment on another cohort of naive snails. However, these snails were exposed to the repeated handling stimulus. Here, we found that both ITM and LTM were formed ($F_{2,30}$=5.278, P<0.05). That is, the number of attempted openings in TS2 was significantly less than that in TS1. Moreover, the number of attempted openings in MT was significantly less than that in TS1 and was not significantly greater than that in TS2. Thus, the repeated handling of snails is not sufficient by itself to block formation of either ITM or LTM.

Sequential crowding blocks memory reconsolidation

The experimental protocol used in the experiments reported here is shown in Fig. 2. The details of how snails were crowded and handled are reported in the Materials and methods section. In order to be able to investigate whether sequential handling and crowding (handling and crowding combined) blocked memory reconsolidation, we employed the following training procedure. Snails underwent a total of five, 0.5 h training/memory testing sessions in hypoxic pond water over the course of 3 days. The first two training sessions (TS1 and TS2) were separated by a 1 h interval. The next two sessions (TS3 and TS4) each occurred with an interval of 24 h. We designated the 5th session as a memory test session (MT) to enable us to determine whether reconsolidation occurred. The training and memory test sessions were similar in that each time the snail attempted to open its pneumostome, a tactile stimulus was presented to the pneumostome area to prevent opening. To determine whether the sequential handling–crowding stressor blocked reconsolidation, we presented this sequential stressor immediately after the TS4 session.

To demonstrate that the training procedure shown in Fig. 2 resulted in LTM in MT, the sequential stressor handling and crowding procedure was not presented to the snails after TS4 (Fig. 3A). The data are unambiguous. First, 24 h after TS2 (i.e. TS3), the number of attempted openings was significantly lower than that in TS1 and not significantly greater than that in TS2 (ANOVA; N=20, $F_{4,64}$=6.599, P<0.05). Thus, LTM was present. The same holds for the data obtained 24 h later (i.e. TS4). Finally, 4 h after the activated TS4 memory, LTM was demonstrated in MT. The number of attempted pneumostome openings in MT was significantly less than in TS1 and not significantly greater than that in TS2, TS3 or TS4.

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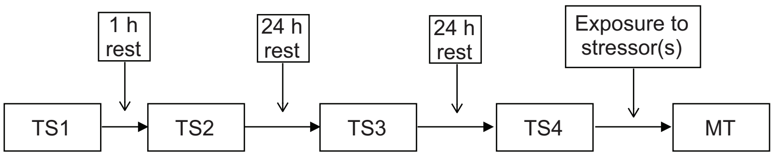

Fig. 2. Experimental protocol for sequential exposure to crowding. Snails underwent a total of five, 0.5 h training sessions in hypoxic pond water, resulting in the formation of LTM. The initial two training sessions were separated by 1 h and subsequent training sessions were separated by 24 h. Between the fourth and fifth sessions, snails were exposed to the assigned stressor.

Using the same training procedure, we asked whether the interposition of the sequential handling and crowding procedure would block reconsolidation. Thus, following TS4, snails were immediately subjected to the sequential crowding and handling sequence (Fig. 3B). As can be seen, following the sequential handling and crowding procedure, LTM was not observed. That is, the number of attempted pneumostome openings in MT was not significantly lower than that in TS1 and was significantly greater than the number of attempted openings in each of the previous training sessions (i.e. TS2, TS3 and TS4; ANOVA $F_{4,76}=13.559$, $P<0.05$, Fig. 3B). Thus, memory reconsolidation was blocked by the sequential stressor, as LTM was not observed.

In the previous experiment, LTM was tested 4 h after TS4 and the interposition of the sequential stressors. Thus, it was possible that the reconsolidation process may not have been completed in that period of time. We therefore trained snails in the exact same manner, except that LTM was tested 24 h after TS4 (Fig. 4A). Again, we found that reconsolidation was blocked, as LTM was not present. Thus, snails immediately subjected to the sequential handling and crowding procedure following memory activation did not exhibit LTM, showing that reconsolidation was blocked.

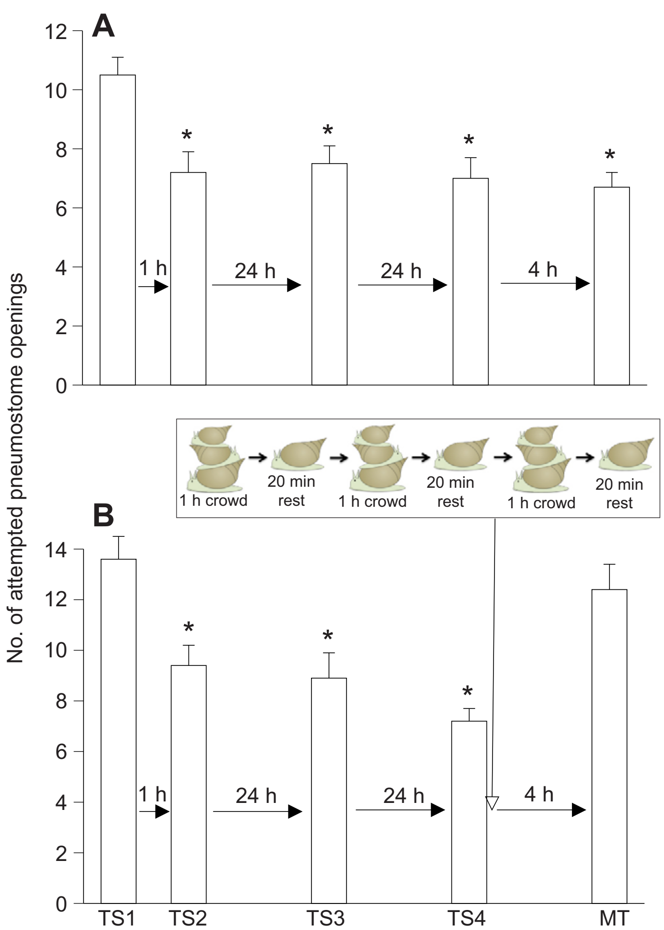

Fig. 3. Sequential handling and crowding blocks reconsolidation. (A) In this naive cohort of snails (N=20), the training procedure results in LTM. (B) A new cohort of naive snails (N=20) received the training as above. However, in this cohort, the sequential handling and crowding combined stressor was given to the snails immediately after TS4. An ANOVA ($F_{4,76}$=13.559, P<0.05) showed that, as above, both ITM (TS2) and LTM (TS3 and TS4) were observed; however, LTM was not observed in MT. Values are means±s.e.m. *Significant difference from TS1 (P<0.05).

In the above experiments, the sequential handling and crowding procedure was applied to the snails immediately following the TS4 session and it blocked the reconsolidation process. Previously in *Lymnaea*, Sangha et al. (2003a,b,c) showed that procedures that blocked the consolidation and reconsolidation processes had to be applied immediately after the memory was re-activated; if a delay of 1 h or more occurred, the reconsolidation process was not blocked (i.e. LTM was seen). We therefore wished to see whether delaying the presentation of the sequential handling and crowding procedure for 1 h after memory was reactivated (i.e. TS4) would fail to block reconsolidation. These data are shown in Fig. 4B. If we waited 1 h after TS4, the sequential handling and crowding procedure did not block reconsolidation (ANOVA; N=21, $F_{3.558,64.052}$=10.035, P<0.05). Thus, delaying the presentation of this sequential stressor allowed the reconsolidation process to occur.

Prolonged crowding does not block reconsolidation

We next sought to investigate whether reconsolidation was blocked with just one of the stressors. We initially exposed snails to just crowding. In this experiment, following TS4, snails were immediately crowded for 1 h (Fig. 5A). As can be seen, LTM was present (ANOVA; N=17, $F_{4,64}$=15.234, P<0.05). Thus, the reconsolidation process was not blocked with a single 1 h exposure to crowding.

It was possible, however, that a longer period of crowding as experienced in the sequential handling–crowding procedure could block the reconsolidation process. Thus, we trained another naive cohort of snails (Fig. 5B) in a similar manner but now, following TS4, snails were subjected to a 3 h ‘prolonged’ crowding procedure. The total time of crowding was equivalent to that experienced in the sequential crowding–handling procedure. However, as with just 1 h of crowding, LTM was observed (ANOVA; N=19, $F_{4,72}$=15.444, P<0.05). Thus, crowding as an isolated stressor was not sufficient to block the reconsolidation process.

Handling snails does not block their ability to recall memory

As the sequential crowding procedure required snails to be frequently handled, we investigated whether such handling of snails was sufficient to block reconsolidation. Handling was investigated by repeating sequential handling with a reduced crowding density (eight snails in 100 ml of pond water; Fig. 5C). This altered protocol alleviated the severity of the crowding stress while allowing handling frequency to remain unchanged. We subjected snails to this ‘handling’ procedure immediately following TS4. As is apparent, such a handling procedure did not block the reconsolidation process as snails expressed LTM (ANOVA; N=20, $F_{4,76}$=7.242, P<0.05).

DISCUSSION

We tested the hypothesis that a combination of stressors (i.e. crowding and handling) applied sequentially to snails immediately

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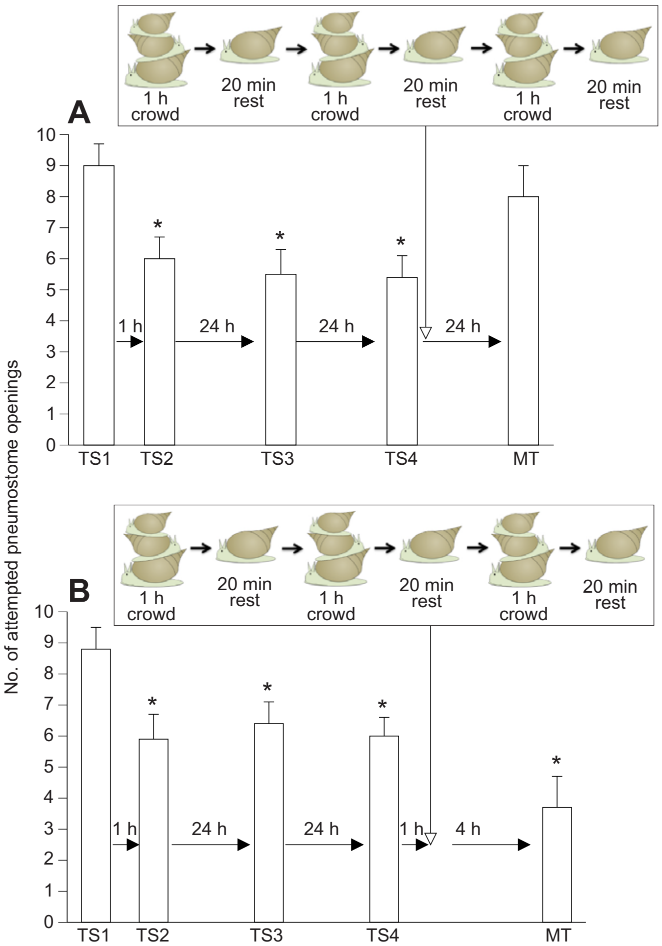

Fig. 4. LTM is not observed 24 h later if stressor is applied immediately after training session. (A) As in Fig. 3, but the presence of LTM was tested 24 h after the sequential stressor was given (N=21). An ANOVA showed that LTM was not present in MT but was present in TS3 and TS4 ($F_{4,80}$=6.317, P<0.05). (B) As above, but here the sequential stressor was given 1 h after TS4. Values are means±s.e.m. *Significant difference from TS1 (P<0.05).

following memory activation, is sufficient to block reconsolidation. We report here that the sequential handling and crowding procedure blocked reconsolidation, but only if the procedure was applied immediately after memory activation. Further, neither handling nor crowding by itself was sufficient to block reconsolidation. This finding is important in that it shows that a behavioural procedure is sufficient to block the reconsolidation process.

In *Lymnaea*, it is known that various stressors modify LTM formation (Lukowiak et al., 2014). Some stressors enhance LTM formation [e.g. predator detection (CE); Orr et al., 2009a,b; thermal stress; Teskey et al., 2012]. That is, following a 60 min exposure to 30°C pond water, a single 0.5 h training session results in LTM formation that persists for up to 2 days. However, as pointed out above, other stressors (e.g. low environmental calcium or crowding; Dalesman et al., 2011a,b,c; de Caigny and Lukowiak, 2008a; Knezevic et al., 2011) block LTM formation. In our *Lymnaea* studies, as well as in other animals, the typical experiment only assesses the effect of a single stressor on memory formation or its recall. However, in 'real life', animals, including humans, are faced with multiple sources of stress, both sequentially and in combination. We have become interested in how learning and memory in *Lymnaea* would respond to multiple stressors. For example, can we predict how different stressors will interact to alter learning and memory based on their individual effects? To address this question, we have combined exposure to different stressors, where we know their individual effects on memory. It appears that the interaction of stressors on memory demonstrates emergent properties. For example, CE enhances LTM formation whereas crowding suppresses LTM formation. When *Lymnaea* were crowded and then exposed to CE, we found that crowding effectively 'trumped' the effects of CE, blocking the memory-enhancing effects of predator detection (de Caigny and Lukowiak, 2008b). However, when we combined low calcium pond water, which blocks LTM formation, with CE we found that CE allowed LTM to form, although a more persistent LTM was not observed. Thus, in that situation the effects of each stressor appear to cancel each other out (Dalesman and Lukowiak, 2011b). We have also shown that social isolation of snails alters how they respond to a stressor and, as mentioned above, low environmental calcium blocks LTM formation. However, in socially isolated snails, the low calcium environment no longer blocks LTM formation (Dalesman and Lukowiak, 2011a). Finally, when we combined two stressors (low calcium and crowding) that on their own block LTM formation but not learning, we found that all memory processes [short-term memory (STM) persisting for 10 min; ITM and LTM] were blocked (Dalesman et al., 2013). Thus, stressors applied in combination have powerful effects on all aspects of memory formation in *Lymnaea*. Interestingly, the notion that stressors in *Lymnaea* could alter the reconsolidation process had not previously been examined.

Reconsolidation demonstrated that a LTM once formed could again be made labile and susceptible to disruption upon its reactivation (Misanin et al., 1968; Eisenberg et al., 2003; Nader et al., 2000a,b; Sara, 2000). Typically, amnestic agents (e.g. protein synthesis blockers, transcriptional blockers, beta blockers) are applied either just before or after memory reactivation and these agents result in the loss of memory; that is, reconsolidation is blocked (Schwabe et al., 2012; Reichelt and Lee, 2013; Kindt and Soeter, 2013). If, however, the amnestic agent is not present, new information can be incorporated into the pre-existing memory trace, which can lead to memory infidelity (Loftus, 2003; Schater, 1999; Dudai, 2006). In *Lymnaea*, reconsolidation has been demonstrated with amnestic agents as diverse as cooling, protein synthesis blockers and removal of the soma of a single neurone that is necessary for LTM formation (Scheibenstock et al., 2002; Sangha et al., 2003c). In addition, because the reconsolidation process is present in *Lymnaea*, it allowed the implantation of a false memory (Lukowiak et al., 2007).

Given that reconsolidation occurs, it was thought that an understanding of how it occurs at the neuronal level would be of great use to treat PTSD and other 'memory problems' (Parsons and Ressler, 2013). However, because the use of the typical protein synthesis inhibitors is not feasible for the ethical treatment of humans, researchers examined other possible pharmacological agents. One such agent was the beta-blocker propranolol, as it is probable that the adrenergic system is involved in at least certain aspects of the stressful event and also because propranolol may inhibit noradrenergic-stimulated CREB phosphorylation, a necessary pathway involved in memory consolidation (Thonberg et al., 2002; Sadamoto et al., 2003; Azami et al., 2006). Systemic administration of propranolol in conjunction with memory reactivation disrupts reconsolidation of LTM in auditory and contextual fear and avoidance in rodents (Abrari et al., 2008; Dębiec and Ledoux, 2004; Dębiec et al., 2011; Przybyslawski et al., 1999). In some human patients, propranolol has been successfully used to treat PTSD in conjunction with memory activation (Brunet et al., 2008; Orr et al., 2006; Pitman et al., 2002; Pitman and Delahanty, 2005). However, subsequent larger trials have

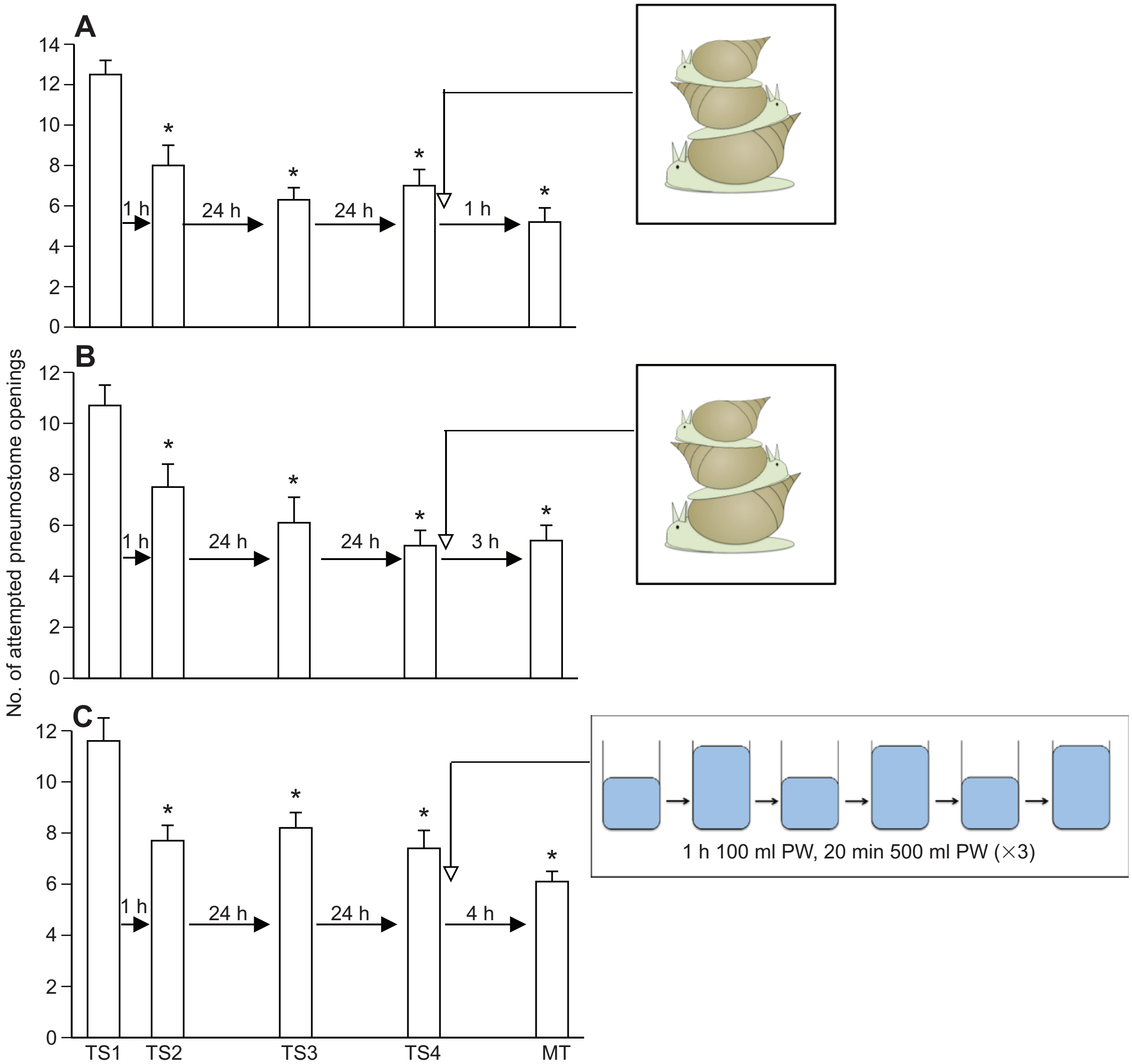

Fig. 5. Reconsolidation is not blocked by the presentation of crowding or handling alone. (A) Snails (*N*=17) received similar training to that in Fig. 3; however, following TS4 they were crowded for 1 h. (B) As in A (*N*=19), only now snails were crowded for 3 h immediately after TS4. (C) As above, except the sequential handling stimulus was presented to the snails after TS4 (*N*=20). Values are means±s.e.m. *Significant difference from TS1 ($P<0.05$). PW, pond water.

not found a significant effect of propranolol administration after trauma (Sharp et al., 2010; Parsons and Ressler, 2013).

We chose here a non-pharmacological approach, stressors in combination, to disrupt reconsolidation. However, sparse evidence indicates a critical role of stress in modulating reconsolidation. One study (Maroun and Akirav, 2008) has shown that stress might have an inhibitory effect on the reconsolidation process. In humans, Schwabe and Wolf (2010) showed that a cold stressor test (immersing a hand in cue-water for 3 min) following activation of a memory impaired some, but not all, LTM. Interestingly, emotional memories were not affected. However, another study (Coccoz et al., 2011) using a similar stressor found that this stressor, when applied after memory re-activation, strengthened the memory. Similar findings have been obtained in a number of other studies (Fukushima et al., 2014).

Our results are unambiguous: only the presentation of the sequential crowding procedure was sufficient to block reconsolidation (Figs 3–5). Moreover, the sequential crowding procedure only blocked reconsolidation when it was administered to the snails immediately after memory reactivation. Delaying the presentation of the sequential crowding procedure for as little as 1 h following memory reactivation did not block reconsolidation. This finding is consistent with previous data concerning the reconsolidation process in *Lymnaea*. Delaying the various agents or procedures that successfully blocked reconsolidation for 1 h after memory activation did not block reconsolidation (Sangha et al., 2003c). Thus, there is in *Lymnaea* only a limited temporal opportunity to employ stress to block reconsolidation.

Our data show that, individually, each stressor could not block reconsolidation. In fact, on its own, handling did not block the initial consolidation process. This raises an interesting point alluded to above (i.e. emergent properties of combined stressors): when combined with another stressor, a stimulus may take on properties (blocking memory processes) that on its own it does not possess. Based on our behavioural data, it is likely that environmental stimuli that result in greater stress than is needed to block the initial consolidation process may be required to block reconsolidation. These data are consistent with the general consensus that reconsolidation does not recapitulate all the aspects of the consolidation process (Nader and Hardt, 2009; Alberini, 2011). That is, the molecular processes in neurons underlying reconsolidation are more resistant to the modifying effects of stress. Our findings may help to explain why some stressors have not previously been effective in blocking reconsolidation.

Our study is the first to investigate the effects of a repeated sequential presentation of multiple stressors on memory reconsolidation using *Lymnaea*. As we know that reconsolidation is dependent on molecular processes occurring in an identified neurone, RPeD1 (Sangha et al., 2003c; Braun et al., 2012), we may be able to obtain neuronal correlates of how the sequential handling–crowding procedure blocks reconsolidation and thus cause memory to be forgotten.

MATERIALS AND METHODS

Lymnaea stagnalis

Originally collected near Utrecht, The Netherlands in the 1950s, *Lymnaea* have been bred in the laboratory for close to 300 generations (Orr et al., 2007, 2009a,b). Laboratory-bred adult snails were used for experimentation and were raised at the Biological Sciences snail facility at the University of Calgary. The snails were stored in aquariums containing approximately 5 l of eumoxic (i.e. normal O_2 levels; P_{O_2}>9975 Pa), artificial pond water (distilled water with 0.26 g l^{-1} Instant Ocean, Spectrum Brands, Madison, WI, USA, and 80 mg l^{-1} of calcium sulphate dehydrate; Dalesman and Lukowiak, 2010). Each aquarium contained 10 snails and snails were fed the leafy portion of Romaine lettuce. Water from the home aquarium was changed weekly. Air was bubbled continuously through the water in the aquariums, which were kept at room temperature (~20°C).

Operant conditioning of aerial respiratory behaviour and the classification of LTM and ITM

Snails are bimodal breathers where cutaneous respiration normally predominates (Lukowiak et al., 1996). The other mode of respiration, aerial respiration (Syed et al., 1990, 1992), is increased by placing snails in hypoxic pond water. To perform aerial respiration, snails come to the surface and open their pneumostome, their breathing tube, allowing atmospheric air to come into contact with the lung (Lukowiak et al., 2003). Hypoxic pond water (P_{O_2}<931 Pa) was produced by bubbling nitrogen gas vigorously through 500 ml of pond water in a 1 l beaker for 20 min. After 20 min, the flow rate of nitrogen gas was reduced to a level where bubbling did not disturb the snails. Snails were then introduced to the hypoxic water, where they acclimatized for 10 min. The hypoxic water caused an increase in aerial respiratory behaviour. Following the 10 min acclimatization period, the snails were subjected to a 0.5 h operant conditioning session known as a training session (TS). A training session consisted of applying a gentle tactile stimulus to the pneumostome as it attempted to open. The stimulus was forceful enough to ensure that the pneumostome did not open but not so forceful that the snail underwent a full body withdrawal. The number of attempted pneumostome openings over the course of the 0.5 h training session was recorded. Following the first training session (TS1), snails were returned to eumoxic water (P_{O_2}>9975 Pa) in their home aquarium. A second training session (TS2) occurred 1 h after the first training session. A subsequent training session (TS3) occurred 24 h later. Finally 24 h after TS3, memory was reactivated (TS4) and then at a specified interval a second memory reactivation occurred (MT).

Memory was operationally defined as a significant reduction in the number of attempted pneumostome openings in the session, compared with the first training session. Additionally, the number of attempted pneumostome openings in the memory test sessions (MT) must not be significantly greater than the number of attempts in the second (TS2) or, if more than two training sessions (TS3 and TS4), the last training sessions (Lukowiak et al., 1998, 2000).

Crowding

Previously, it was shown (de Caigny and Lukowiak, 2008a) that LTM formation was blocked following crowding (20 snails in 100 ml of pond water) for 1 h before training. Following crowding, snails were placed in hypoxic water for 10 min to acclimatize before the 0.5 h memory test.

Handling

To investigate how the repeated handling stressor altered LTM formation, snails were given a total of five training/memory test sessions. Handling was investigated by repeating sequential handling with a reduced crowding density (eight snails in 100 ml of pond water) between each training/ memory test session.

Experimental protocol for sequential handling and crowding

To investigate the effects of sequential crowding on LTM, naive snails underwent a total of five training sessions (Fig. 2). The initial two training sessions were separated by 1 h and the subsequent three training sessions were each separated by 24 h. In order to determine whether sequential exposure to handling and crowding alters LTM, snails were sequentially handled and crowded immediately following the initial memory activation session (TS4). Sequential handling and crowding was performed by introducing snails to crowded conditions (20 snails in 100 ml of pond water) for 1 h and then transferring snails to a beaker containing 500 ml of eumoxic pond water for 20 min. This crowd and rest sequence was repeated for a total of three cycles. During the final rest period, snails were placed in eumoxic pond water for 10 min and then into hypoxic water for 10 min to acclimatize for a 0.5 h memory session.

Statistics

The number of attempted pneumostome openings in each training session was compared using repeated measures analysis of variance (RM-ANOVA). Data were tested for equal variance using Mauchly's test for sphericity or Greenhouse–Geisser *P*-values if sphericity could not be assumed. Tukey's *post hoc* test was performed for pairwise comparisons. Significance was considered to be at least *P*<0.05. Statistical analysis was performed using Prism 6.

Competing interests

The authors declare no competing or financial interests.

Author contributions

K.L. and S.X.D. conceptualized and performed the experiments, analyzed the data and wrote the manuscript.

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