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Qualitatively different memory states in *Lymnaea* as shown by differential responses to propranolol ☆

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ABSTRACT

Mixed results with the synthetic β-adrenergic receptor blocker, propranolol, have been reported in human populations with regards to its therapeutic efficacy for PTSD treatments targeting the memory reconsolidation process. Stress alters the ability to form and maintain memory, but whether the causal neuronal mechanisms underling memory formation in PTSD are similar to normal memory is not clear. Here, we use *Lymnaea* to study the effects of combinations of stressors on the quality of the formed memory state. We show reactivation dependent pharmacologic disruption of reconsolidation using propranolol in *Lymnaea*; specifically, we show that only certain memories created under conditions of a combination of stressors are susceptible to disruption. Our data suggest that phenotypically similar memories may be molecularly diverse, depending on the conditions under which they are formed. Applied to human PTSD, this could account for the mixed results in the literature on disrupting reconsolidation with propranolol.

1. Introduction

In humans, emotional memories formed under conditions of high stress can be intrusive, long lasting, and can lead to the development of disorders such as post-traumatic stress disorder (PTSD; Breslau, 2009). It is well known that stress alters the ability to form and maintain memory (Hebb, 1955); however, the molecular mechanisms through which this occurs have yet to be fully elucidated. It is unclear as to if or how the neural mechanisms causal for the consolidation of memory formed under certain conditions of high stress (i.e.: a PTSD memory) differ from the processes underlying the consolidation of memories created in less stressful circumstances.

It was initially thought that once consolidated, memory was static and unchanging; but we know memory is a dynamic process. The occurrence of a reconsolidation phase was demonstrated first in 1968 (Misanin, Miller, & Lewis, 1968) and since has been demonstrated across species (e.g. rodents, Kim et al., 2010; Nader, Schafe, & Le Doux, 2000; Tronson & Taylor, 2007), including our model system, *Lymnaea* (Sangha, Scheibenstock, & Lukowiak, 2003; Sangha, Scheibenstock, Morrow, & Lukowiak, 2003). Thus, when memory is recalled, it enters a transient labile phase followed by a new stabilization process. During reconsolidation, memory can be enhanced, impaired, or updated with new information (Lukowiak, Fras, Smyth, Wong, & Hittel, 2007; Agren, 2014). In both rodent models and humans, it has been demonstrated that propranolol, a synthetic β-adrenergic receptor blocker, can block the reconsolidation process (Debiec & Ledoux, 2004; Kindt, Soeter, & Vervliet, 2009; Przybyslawski, Roullet, & Sara, 1999). However, despite initial enthusiasm, these results have not reliably translated to treatment of PTSD patients in the clinic (Wood et al., 2015). Debate still exists in the literature as to whether the administration of propranolol with the goal of blocking reconsolidation represents a potentially viable clinical treatment.

Certain memories are more susceptible to propranolol disruption. In humans, propranolol has a more significant amnesic effect on memories created under highly charged conditions than neutral conditions (Schwabe, Nader, Wolf, Beaudry, & Pruessner, 2012). Here we ask whether it is possible, using a combination of stressors, to create a memory in *Lymnaea* that is susceptible to disruption by propranolol. We hypothesize that there are qualitatively different forms of memory in *Lymnaea* as a result of experiencing different combinations of stressors around the time of memory formation. Further, we hypothesize that one of these different 'memory states' may be susceptible to propranolol disruption.

☆ These survival circuits include, at a minimum, circuits involved in defense, maintenance of energy and nutritional supplies, fluid balance, thermoregulation, and reproduction.

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Reconsolidation has been demonstrated in evolutionarily diverse systems; thus, there is an expectation that the molecular events that underlie reconsolidation are conserved across species (Sangha, Scheibenstock, & Lukowiak, 2003; Sangha, Scheibenstock, Morrow, et al., 2003). *Lymnaea* is an excellent model for studying learning and memory and how stress alters those memories (Lukowiak & Dalesman, 2012, chap. 23, 2014). For example, in addition to demonstrating the phenomenon of reconsolidation (Sangha, Scheibenstock, & Lukowiak, 2003) and how reconsolidation can be blocked by ablating the soma of a single neuron or applying sequential exposure to a combination of stressors (Dodd & Lukowiak, 2015), it has been shown that memory recall is context specific (Haney & Lukowiak, 2001); behavioural extinction occurs (Sangha, Scheibenstock, & Lukowiak, 2003; Sangha, Scheibenstock, Morrow, et al., 2003), forgetting is an active process (Sangha et al., 2005) and it is possible to implant a false memory into the snail following memory activation (Lukowiak et al., 2007).

Lymnaea are bi-modal breathers. That is, they can satisfy their respiratory requirements through both cutaneous and aerial respiration (Lukowiak, Ringseis, Spencer, Wildering, & Syed, 1996). Using an operant conditioning procedure, we can decrease the occurrence of aerial respiration while leaving cutaneous respiration intact, thus our training procedure is not harmful to the animal. Using our standard training procedure, two 0.5 h training sessions spaced 1 h apart will produce a LTM that persists for at least 24 h. In contrast, a single 0.5 h training session under standard conditions is only sufficient to produce an intermediate term memory (ITM) that persists for only 3 h. In addition, ITM has been shown to be dependent on new protein synthesis while LTM is dependent on both new protein synthesis and altered gene activity (Sangha, Scheibenstock, & Lukowiak, 2003; Sangha, Scheibenstock, Morrow, et al., 2003). In our hands, certain stressors are said to enhance memory formation. That is, if the stressor is presented to the snail before or during training, the single 0.5 h training session becomes capable of causing LTM formation (Lukowiak et al., 2014). For example, when the thermal stressor (Teskey, Lukowiak, Riaz, Dalesman, & Lukowiak, 2012) is applied, a single 0.5 h training session is sufficient to elicit a memory persisting for 24 h. A number of other stressors (e.g. predator detection or an application of KCl) cause a similar enhancement of memory formation (Martens et al., 2007; Orr & Lukowiak, 2008). Thus, a stressor is said to enhance memory formation if it causes the training that would normally only result in ITM to result in LTM. This is significant because at the molecular level ITM is only dependent on new protein synthesis whilst LTM is dependent on both new protein synthesis and altered gene activity (Sangha, Scheibenstock, & Lukowiak, 2003; Sangha, Scheibenstock, Morrow, et al., 2003). In *Lymnaea*, it is unknown how the memory enhancement causes this change. Here, we place snails in different stressful environments that enhance memory formation and ask whether the synthetic β-adrenergic receptor blocker, propranolol, will disrupt the memory reconsolidation process in all these stressful environments.

2. Materials and methods

2.1. Snails

Lymnaea were bred from a laboratory strain maintained at the University of Calgary Biology Department, originating from animals collected in the 1950s from a polder near Utrecht, The Netherlands. Snails were maintained at room temperature (~20 °C) in home aquaria containing oxygenated artificial pond water (0.25 g/L Instant Ocean, Spectrum Brands, Madison, WI, USA; 0.34 g/L $CaSO_4$, Sigma-Aldrich, St-Louis, MO, USA). Washed Romaine lettuce was fed to the snails *ad libitum*.

2.2. Drug exposure

(±)-Propranolol hydrochloride ⩾99% (TLC) powder was obtained from Sigma-Aldrich (St. Louis, MO, USA). Before injection, snails were anesthetized by placing them in an ice bath for 15 min. Drug-treated snails were injected into their foot with 0.1 mL of 50 μM propranolol in *Lymnaea* saline. Often snails withdraw into their shell; however, because *Lymnaea* do not possess an operculum it is possible to still inject drug into them through the foot. We chose this concentration of propranolol based on pilot studies in our lab. Snails were placed in eumoxic home aquaria for 1 h after injection, immediately before memory reactivation. Administration of propranolol 1 h before reactivation is consistent with human studies (Schwabe, Nader, & Pruessner, 2013). Control group snails were anesthetized according to the same procedure as the drug-treated snails before injection of 0.1 mL of *Lymnaea* saline. After injection, snails were placed in eumoxic home aquaria for 1 h, immediately before memory reactivation.

2.3. Aerial respiratory behavior

Lymnaea are bimodal breathers. In eumoxic conditions (6 mL O_2/L) they obtain oxygen though cutaneous respiration; however, in hypoxic conditions with low dissolved oxygen (<0.1 mL O_2/L), they switch to aerial respiration using their respiratory orifice called the pneumostome. To see whether propranolol affected homeostatic breathing behavior, we measured total breathing time (TBT) and number of breaths (TBN) in pond water for propranolol injected snails and saline injected snails. We found no significant difference in breathing behavior between the two groups (TBT: 308 ± 20.2 vs. 296 ± 18.7 s; TBN: 9.9 ± 1.9 vs. 8.7 ± 1.8 s; $t = 1.108$; $df = 6$ $p < 0.05$; $t = 0.1936$; df 6; $p < 0.05$ respectively).

2.4. Standard operant conditioning procedure

Snails were labeled individually and placed in a 1L beaker containing 500 mL of artificial pond water made hypoxic by bubbling N_2 gas through the water for 20 min prior to each operant conditioning session. They were allowed to acclimatize to their conditions for 10 min. Immediately before each session, snails were gently pushed under the water surface. During the session, each time a snail attempted to open its pneumostome for gas exchange, a sharped wood applicator was used to gently poke the edge of the snail's pneumostome. This causes the pneumostome to close without causing the snail to retract completely into its shell. The number of pokes was recorded. Between sessions, snails were returned to their home, eumoxic aquaria. This same procedure was performed for the training sessions, memory tests, and memory reactivation sessions.

Using the standard operant conditioning procedure, two 0.5 h training sessions spaced one hour apart are required to form a 24-h long-term memory (LTM, Lukowiak, Nimet, Krygier, & Syed, 2000). We operationally define LTM as significantly fewer attempted pneumostome openings during the second training session (TS2) and the 24-h memory test (MT) compared to the first training session (TS1). Additionally, our definition of LTM posits that the number of attempted pneumostome openings in MT cannot be significantly greater than the number in TS2. For snails to meet criteria for LTM in sessions after the initial 24 h MT, the number of attempted pneumostome openings must be significantly less than TS1, but not significantly different from the previous training session. We choose here in our control experiment to use a training procedure consisting of two 0.5 h training sessions separated by a 1 h interval on Day 1 and then to repeat this sequence on Day 2. Thus the snail receives four 0.5 h training sessions over the course of two days. This results in a LTM that persists for at least 5 days.

Memory tests were performed 24 h following the last training session and reactivation sessions were done 24 h after the first memory test. The procedure for a memory test and a reactivation session is the same as for a training session. During the 30-min session in hypoxic water, a sharped wood applicator is used to poke the edge of the snail's pneumostome each time a snail attempts to open it for gas exchange. The number of pokes are recorded and compared between sessions.

2.5. Stressors

Stressors applied before and/or during training can alter memory in *Lymnaea* (Lukowiak et al., 2014). Individually, or in combination, stressors can block or enhance memory formation. We say that a stressor enhances memory if, when it is applied, snails are able to form LTM following a single 0.5 h training session as opposed to normally requiring two standard training sessions. The following stressors were used in the current study:

2.6. Food deprivation + carrot odour

For *Lymnaea* to live long and prosper, they must have access to an adequate supply of food. Restrictions can lead to stunting of growth and reproduction. Therefore, food deprivation (FD) acts as an environmentally relevant stressor (Ito et al., 2015). That said, we have shown previously that 5-day food deprived snails form LTM normally when given the standard training procedure (two 0.5 h training sessions separated by an hour) (Haney & Lukowiak, 2001). As in our previous papers, we food deprived snails by completely removing lettuce from their home aquaria. Before food deprivation, snails for at least three months (since hatching from their egg capsule) have had *ad libitum* access to lettuce. It is possible to expose snails to a carrot odour (CO) without allowing them to feed on carrot. This can be done by setting up an apparatus which bubbles eumoxic air through carrots that have been blended and placed in a sealed flask, while simultaneously diverting air, smelling of carrot, from the sealed flask and into a beaker containing pond water and the snails. We choose to use carrot because we: (1) know that carrot juice or CO will reliably elicit a feeding response from snails even if they have never encountered carrot previously (Sugai et al., 2006); and (2) have successfully used it before (in operant conditioning experiments (Haney & Lukowiak, 2001). We predict that when a food source is detected in a food deprived snail, it acts a stressor if the snail cannot access the food source (Haney & Lukowiak, 2001; Ito et al., 2015; Lukowiak et al., 2014). For example, Haney and Lukowiak (2001) demonstrated that food deprived snails show neither learning nor memory formation if they are trained whilst smelling a food source and it did not matter if they had previously experienced the odour as an obtainable food source. Snails respond to carrot with an increased feeding response even though they had never before experienced carrot as a food source (Sugai et al., 2006). Here, we demonstrate for the first time that 3 or 5-day food deprived snails exposed to half an hour of carrot scent immediately before training in hypoxic pond water exhibit enhanced memory.

2.7. Crayfish effluent (CE)

Crayfish are a natural predator of *Lymnaea*. They are housed in a 70L aquarium in our lab, maintained on a diet of lettuce and snails. We term the water in the crayfish tank crayfish effluent (CE) (Orr, El-Bekai, Lui, Watson, & Lukowiak, 2007). Previously, we have demonstrated that exposure to CE during training causes significant enhancement of LTM formation (Orr & Lukowiak, 2008, 2010; Sunada, Horikoshi, Lukowiak, & Lukowiak, 2011; Lukowiak et al., 2014).

2.8. Potassium chloride (KCl)

KCl exposure is noxious to *Lymnaea*. Previously, we have shown that a 30 s exposure to 25 mM KCl immediately prior to a training session causes significant enhancement of memory formation (Martens et al., 2007).

2.9. Cold block procedure

A 1-L beaker filled with 0.5 L of eumoxic water was pre-chilled and maintained at 4 °C. We cooled snails immediately (i.e. within 30 s) for 1 h after reactivation of memory, as this procedure blocks the reconsolidation process and does not adversely affect the health of the snails (Sangha, Scheibenstock, & Lukowiak, 2003; Sangha, Scheibenstock, Morrow, et al., 2003).

2.10. Learning grade distributions

Snail learning grade distributions were calculated for snails exposed to KCl, CE, KCl + CE and FD + CO (Fig. 7). Snails were given either a pass or fail grade based on their individual performance. Grades were calculated as follows: a 20% or greater reduction in the number of attempted pneumostome openings from TS to MT1 was considered a pass and anything lesser was considered a fail.

2.11. Statistical analyses

To determine whether the experimental manipulation had an effect when compared to a control group and whether the number of pokes delivered was significantly altered as a result of operant conditioning or other procedures (i.e. cooling, etc.), we performed both repeated-measures one-way and two-way ANOVA's, testing both a between-group factor and a within-group factor. If the ANOVA was significant ($p < 0.05$), a post hoc Tukey's *t*-test was performed to show which sessions (i.e. within-group) and which groups (i.e. between-group) were significantly different. We used a paired *t*-test when we only compared a single training session with a single memory test session. Differences were considered to be significant if $p < 0.05$.

3. Results

We first trained a naive cohort of snails (Fig. 1A), with two 0.5 h training sessions on Day 1 followed 24 h later by a second series of two 0.5 h training sessions. As can be seen, this procedure results in a long-term memory (LTM) that persists for at least 5 days. That is, learning was demonstrated as TS2 was significantly less than TS1 and LTM was shown as MT (5 days after TS4) was significantly less than TS1 and not significantly greater than TS4 (Lukowiak et al., 1996). That this is a *bona fide* example of associative learning and memory has previously been demonstrated many times using, for example, yoked control procedures. We next used a similar training procedure on another naive cohort of snails but injected propranolol just before we reactivated the memory (Fig. 1B). Propranolol did not block memory recall (RM). That is, the number of attempted openings in the reactivation session (RM) was not significantly different than the previous memory test (TS4), but it was significantly different than the first training session (TS1). Thus, immediately following the propranolol injection, snails still met the criteria for LTM. We then asked whether the injection of propranolol altered reconsolidation. As can be seen, the number of attempted pneumostome openings in the memory test (MT) after the propranolol injection was not significantly different than the memory reactivation session (RM), but was significantly differ-

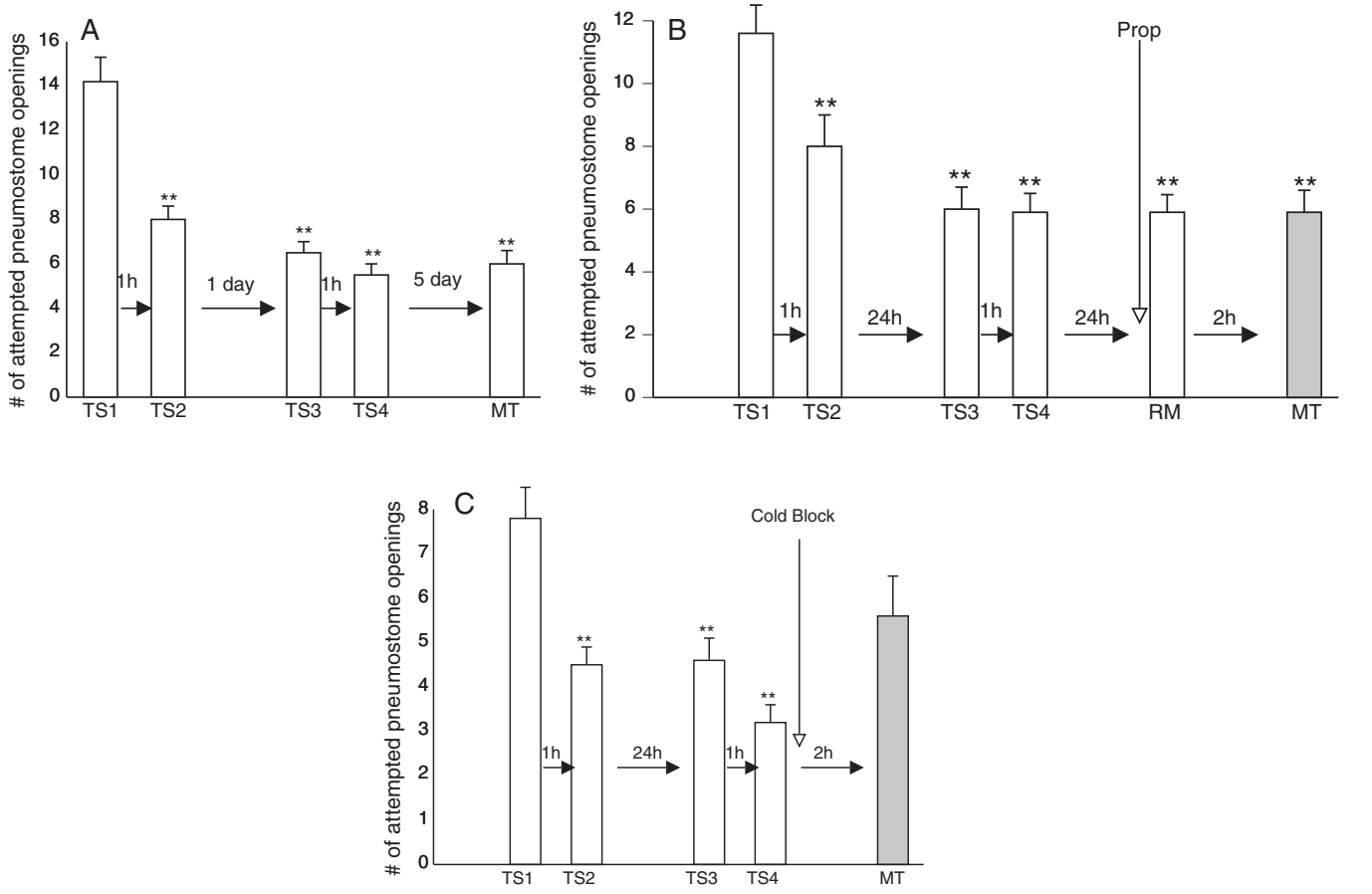

Fig. 1. Reconsolidation is blocked using a cold block but not propranolol when memory is formed using standard training. Snails received two 0.5 h training sessions (TS1 and TS2) separated by 1 h, in pond water, on Day 1. Snails received another two 0.5 h training sessions (TS3 and TS4) in pond water, separated by 1 h, 24 h later. We term this the standard training procedure. (A) A cohort of snails (n = 25) were tested for LTM 5 days after the standard training procedure, an ANOVA showed that LTM was present in the memory test (MT) (ANOVA; $F_{2.862,68.68}$, $p < 0.0001$). (B) Propranolol did not block reconsolidation of memory formed by standard training. Following standard training, 23 h later, snails (n = 19) were injected with propranolol. Memory was reactivated 1 h later (RM). Snails were memory tested 24 h after reactivation, an ANOVA showed that LTM was present (ANOVA; $F_{3.027,54.49} = 10.47$, $p < 0.0001$). That is, propranolol did not disrupt reconsolidation. (C) A cold block successfully disrupted reconsolidation of memory formed by standard training. Following standard training, snails were placed in 4 °C water for 1 h and then tested for LTM 2 h later (MT); an ANOVA showed that no LTM was present (ANOVA; $F_{2.514,40.23} = 21.18$, $p < 0.0001$). That is, cold block disrupted reconsolidation. Food deprivation for 5 days and carrot scent, when applied as individual stressors, do not enhance LTM formation. **Significant difference from the number of pneumostome openings in TS1 **$p < 0.01$.

ent than in TS1. Thus, these snails still exhibited LTM, meaning that neither the memory recall process nor the reconsolidation process was affected by the propranolol injection.

As a positive control to demonstrate that using the above training procedure reconsolidation could be blocked we employed the cold block procedure (see methods, Fig. 1C). If snails are maintained for 1 h in 4 °C eumoxic pond water immediately after reactivation of memory, reconsolidation is blocked (Sangha, Scheibenstock, & Lukowiak, 2003; Sangha, Scheibenstock, Morrow, et al., 2003). As can be seen, when the cold block was applied to the snails immediately after TS4, reconsolidation was blocked. That is, the number of attempted pneumostome openings in MT was significantly larger than in TS4 and was not significantly less than in TS1. Thus, we could block the reconsolidation process with the cold block technique.

In humans, data suggests that the reconsolidation of emotional memories formed under conditions of high stress is more susceptible to disruption by propranolol than neutral memories (Schwabe et al., 2012). This suggested to us that the causal neuronal mechanisms underlying memory formation differ based on the stressors applied around the time of memory formation. We were thus curious as to whether snails operantly trained around the time of experiencing different combinations of stressors formed memory that could be disrupted by propranolol.

3.1. Food deprivation + Carrot odour

Food deprivation (FD) acts as an environmentally relevant stressor in classical food aversion conditioning (Ito, Kojima, Lukowiak, & Sakakibara, 2013; Ito et al., 2015). However, when we food-deprived snails for 3–5 days, the snails did not have enhanced memory formation following operant conditioning of aerial respiration (Fig. 2A). These food-deprived snails received a single 0.5 h training session and when we tested for memory 24 h later, memory was not observed. These data are consistent with our previous findings (Haney & Lukowiak, 2001) showing that food deprivation by itself is not sufficient to cause either enhancement or blockage of memory formation.

We next performed a second experiment to determine if snails that smelled a food substance (carrot odour, CO) but couldn't access it to eat would have memory enhancement (Fig. 2B). Therefore, a naive cohort of fed snails was exposed to CO before training. The smell of unattainable food was also not sufficient to cause memory enhancement. That is, the single 0.5 h training session following CO was not sufficient to cause LTM.

We hypothesized that we could create a qualitatively different, more highly stressed state in snails by food depriving them, and then allowing them to smell, but not obtain, a food substance before training. That is, food deprived snails were exposed to CO

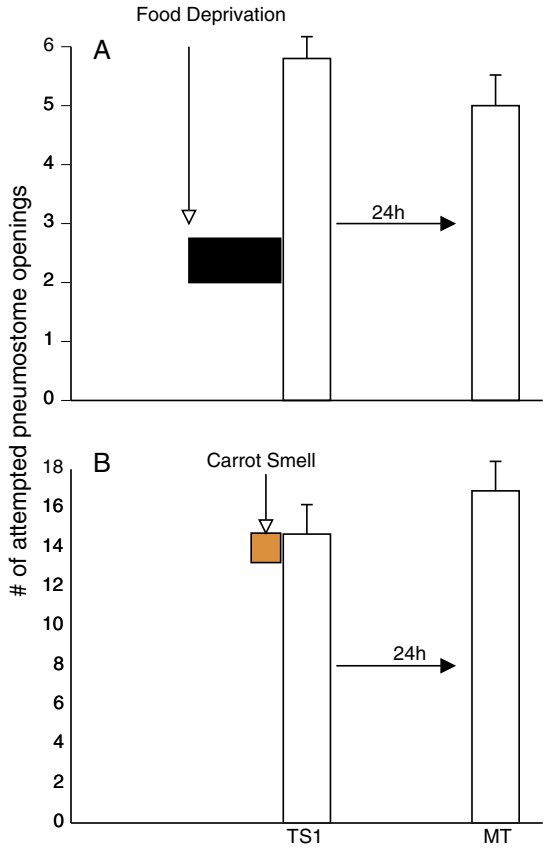

Fig. 2. Neither food deprivation nor smell of food in fed snails caused enhancement of LTM formation. (A) A single 0.5 h training session (TS) in pond water was given to a cohort of naïve snails (n = 11) that were food deprived for 3 days. Snails were tested for LTM 24 h later (MT). A paired *t*-test indicated that no memory was present ($p > 0.05$). (B) A separate cohort of naïve snails (n = 10) were exposed to carrot scent for 30mins immediately prior to a 0.5 h training session (TS) in pond water. Snails were tested for LTM 24 h later (MT). A paired *t*-test indicated that no memory was present ($p > 0.05$).

before training. Food deprivation plus the smell of unattainable food conferred on these snails enhanced memory forming ability (Fig. 3A). That is, the single 0.5 h training session now resulted in LTM formation that persisted for at least 48 h. It needs to be pointed out here that combining the two stressors also result in a 24 h memory, but we thought it was important to show that the combination of these two stressors result in LTM that persists for at least 48 h.

Having shown that the combination of food deprivation with CO resulted in snails obtaining enhanced memory forming capabilities, we next asked whether propranolol would block reconsolidation in these snails. A cohort of naive snails was food deprived for 5 days and then subjected to a 0.5 h exposure of CO immediately before training (Fig. 3B). One group of this cohort (n = 13) was subsequently injected with propranolol 1 h before memory reactivation while the other group (n = 6) received a saline injection 1 h before reactivation of the memory. We then tested in both groups whether memory was present 24 h later. We first observed that neither the propranolol nor the saline injection altered the ability of snails to access their already formed memory (RM). However, when we tested the group that received the propranolol injection for memory 24 h after RM we found that memory was not present (MT2). That is, the number of attempted openings in MT2 was significantly greater than MT1 and RM but was not statistically different than TS. On the other hand, memory (MT2) was present in the group that received the saline injection. Thus, the memory formed as a result of combining food deprivation with CO resulted in a memory that was susceptible to disruption by propranolol.

3.2. KCl + CE

Crayfish are a natural predator of *Lymnaea* and exposure of *Lymnaea* to crayfish effluent (CE) causes enhancement of memory formation (Orr & Lukowiak, 2008). Detection of CE likely indicates to *Lymnaea* that a predator is nearby, thus heightening awareness to external stimuli (Orr, Hittel, Lukowiak, Han, & Lukowiak, 2009; Orr et al., 2007). Here we confirmed those earlier findings by showing in a naive cohort of snails that CE caused enhancement of memory formation (Fig. 4A). That is, a single 0.5 h training session in CE is sufficient to cause enhancement of memory formation, and here results in a LTM that persists for at least 48 h. We had also previously shown that subjecting snails to a 30 s bath in 25 mM potassium chloride (KCl) resulted in snails with enhanced memory forming abilities (Martens et al., 2007). We repeated those experiments here and show (Fig. 4B) that the KCl stressor caused snails to have enhanced memory forming capabilities. That is, subjecting snails to the KCl bath is sufficient to cause an enhancement of memory formation that persists for at least 48 h. Thus, with either the CE or KCl stressors, a single 0.5 h training session was sufficient to cause enhancement of memory formation.

Since both training snails in CE and training snails following the KCl bath resulted in snails acquiring enhanced memory forming capability we asked (Fig. 5) whether the memory formed as a result of either or both of these stressors was susceptible to propranolol disruption. Training snails in CE resulted in LTM (MT1). Snails were then injected with propranolol 23 h after the MT1, 1 h before we reactivated the memory (RM). Memory was present in RM, thus propranolol did not block access to the memory. However, the propranolol injection did not disrupt the reconsolidation process as memory was still observed 24 h later (MT2). Thus, even though CE causes enhancement of memory formation, this enhanced memory is not susceptible to the propranolol block of reconsolidation.

In a similar manner we then determined whether the memory formed following the KCl bath was susceptible to propranolol disruption. Here we found, as with the snails trained in CE, that propranolol did not disrupt memory in the snails (Fig. 5B). Thus, propranolol injection was not a memory disrupter in snails trained either in CE or trained following the KCl bath.

Through indicating to *Lymnaea* that a predator is nearby, exposure to CE likely only warns snails of possible life-threatening danger. KCl is a noxious stimulus to snails; upon exposure, snails completely withdraw into their shells. Snails show the full body withdrawal response when facing imminent predation by a crayfish (ie: the snail is in the crayfish's grasp). We have observed this behavior in the lab. In an attempt to create a memory to mimic a close encounter with a predator, we exposed snails to 25 mM KCl for 30 s, immediately followed by a half hour training session in hypoxic CE. Thus, we combine a predator threat stimulus with an extremely noxious stimulus that elicits the whole animal withdrawal response that is a response of last resort for the snail. We know from our previous work that when a combination of stressors is presented it is difficult to predict what the effect on memory formation will be (Dalesman, Sunada, Teskey, & Lukowiak, 2013; Lukowiak et al., 2014). When we exposed a naive cohort of snails to a combination of KCl + CE these snails continued to exhibit enhanced memory forming abilities (Fig. 6A). Thus, the memory phenotype resulting from exposure to these two stressors and a single 0.5 h training session continues to be one of enhancement.

We next determined whether training snails with this combination of stressors would result in memory susceptible to propranolol disruption (Fig. 6B). As we did with food deprivation and

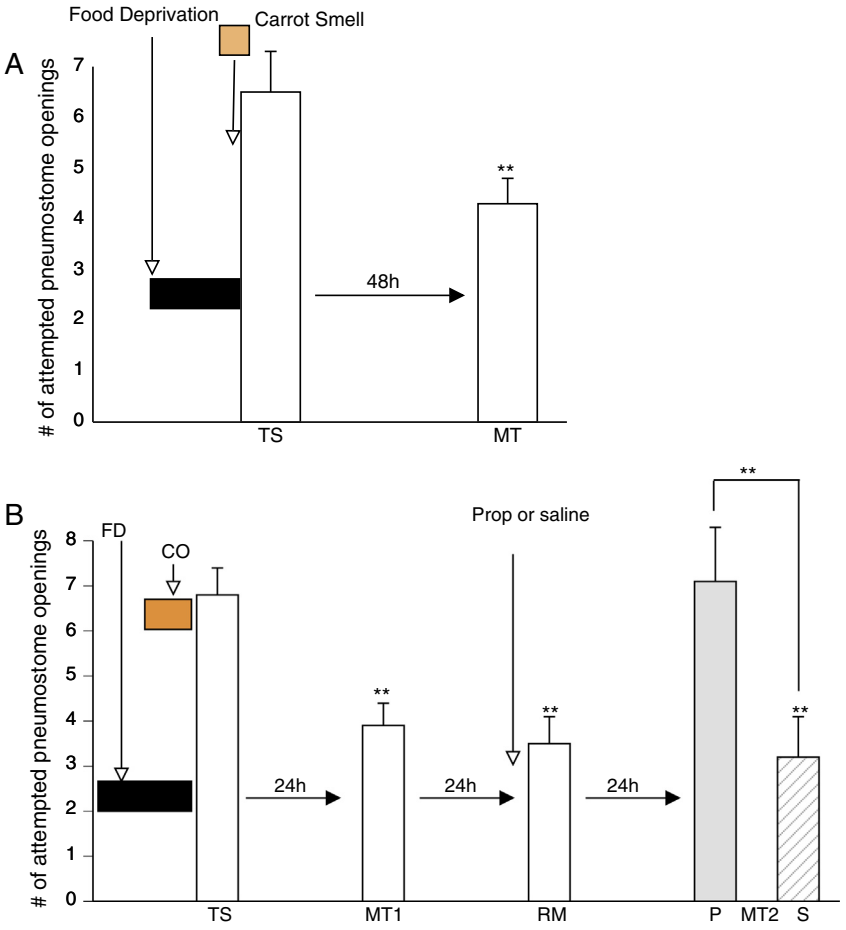

Fig. 3. The combination of 5-day food deprivation and carrot smell before training creates a memory that lasts at least 48 h and can be disrupted by propranolol. (A) Snails (n = 8) were food deprived for 5 days and exposed to 30 min of carrot scent immediately prior to a 0.5 h training session (TS) in pond water. Snails were tested for LTM 48 h later (MT). A paired *t*-test showed that memory was present ($p < 0.05$). (B) The same procedure as in (A) was performed, except MT1 was performed 24 h after the TS. Snails (n = 19) were then injected either with propranolol (0.1 mL of 50 μM propranolol in *Lymnaea* saline) or saline (0.1 mL of *Lymnaea* saline) 23 h after MT1. Memory was reactivated (RM) 1 h after injection. Snails were then tested for LTM 24 h after memory reactivation (MT2). A One-way ANOVA indicated that no memory was present in MT2 in the propranolol injected group (ANOVA; $F_{1.608, 19.30} = 5.272$, $p < 0.05$). That is, propranolol disrupted reconsolidation. A one-way ANOVA performed on the saline injected group indicated that memory was present in MT2 (ANOVA; $F_{3, 36} = 6.781$, $p = 0.001$). That is, successful reconsolidation occurred. Additionally, a two-way ANOVA and a post hoc Tukey's *t*-test indicated that the propranolol injected group had a significantly greater number of attempted pneumostome openings than the saline injected group in MT2 (ANOVA; $F_{3, 125} = 11.49$, $p < 0.0001$). A memory reactivation session is necessary for propranolol to block memory reconsolidation. *Significant difference from the number of pneumostome openings in TS1 **$p < 0.01$.

CO, we exposed a naive cohort of snails to KCl + CE. One group of this cohort (n = 17) was subsequently injected with propranolol 1 h before we reactivated the memory (RM) while the other group (n = 14) received a saline injection 1 h before RM. Memory was then tested (MT2) 24 h later. Again, we noticed that in this cohort neither the propranolol nor the saline injection altered the ability of snails to access their already formed memory (i.e. RM). However, in the group that received the propranolol injection, memory was not present in MT2 while it was for the group receiving the saline injection. Thus, the memory formed as a result of combining KCl + CE resulted in a memory that was susceptible to disruption by propranolol.

3.3. Learning grade distributions

We next asked the question whether there was any qualitative difference in the memory phenotype between those memories susceptible to propranolol disruption and those memories that propranolol did not disrupt. Previously, (Lukowiak et al., 2003; Orr et al., 2009; Rosenegger, Roth, & Lukowiak, 2004) a method used to look at differences in memory formation between snails is to examine individual data for each trained snail and to assign a 'mark' to each snail based on its ability to form a memory. Marks are then compared, for example, between various treatments or strains of snails. For example, some strains of snails form better or worse memory than others. Here we took a slightly different approach and rather than giving a specific 'mark' (e.g. A, B, C, or F) we used a 'pass vs fail' system (See Material and Methods). As can be seen in Fig. 7, the% of snails that received a passing grade following the single 0.5 h TS and MT 24 h later was similar in the four groups (KCl, CE, KCl + CE, and FD + CO). We did not plot the pass rate for the snails in the FD only and CO only groups as there was no memory formed following the single 0.5 h training session. We conclude based on these data that there are not quantitative differences in the memory formed under each of the four separate groups. However, in only two of the groups (CE + KCl and FD + CO) did propranolol impact memory. Thus, there would appear to be qualitative differences between the memory phenotypes.

3.4. Reconsolidation blockade by propranolol is reactivation dependent

Finally, reconsolidation theory posits that consolidated memory is stable, and only enters an active, labile, state upon reactivation. Only during this labile state can previously consolidated memory

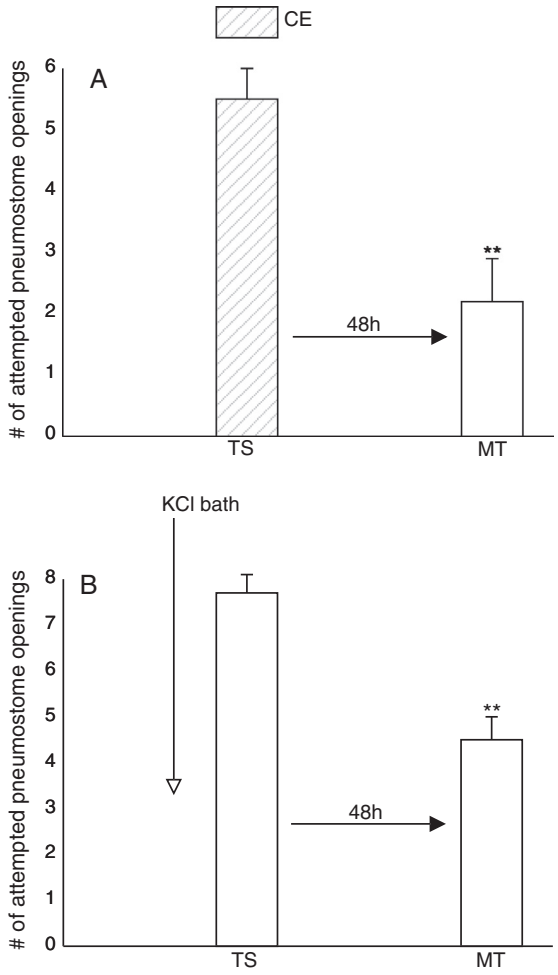

Fig. 4. Crayfish Effluent (CE) and KCl, when applied as individual stressors, enhance LTM formation. (A) Snails (n = 7) were given a 0.5 h training session (TS) in CE. A memory test (MT) was performed 48 h later; a paired *t*-test indicated that memory was present ($p < 0.05$). (B) Snails (n = 10) were exposed to 25 mM KCl for 30 s immediately prior to a 0.5 h training session (TS) in pond water. A memory test (MT) was done 48 h later; a paired *t*-test indicated that memory was present. Significant difference from the number of pneumostome openings in TS1 $^{**}p < 0.01$).

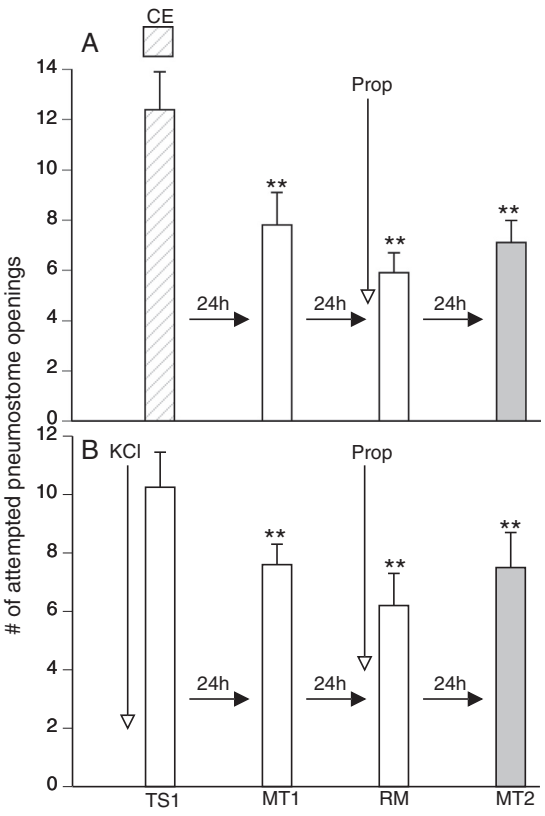

Fig. 5. Enhanced memory formed as a result of the application of the individual stressors CE and KCl is not susceptible to propranolol induced disruption of reconsolidation. (A) Snails (n = 9) were given a 0.5 h training session (TS) in CE. A memory test (MT1) was performed 24 h later. Propranolol (0.1 mL of 50 μM propranolol in *Lymnaea* saline) was injected into snails 23 h later, followed by a memory reactivation session (RM) 1 h after injection. Another memory test (MT2) was performed 24 h after injection; an ANOVA indicated that memory was present (ANOVA; $F_{3,24} = 8.194$, $p < 0.001$). That is, propranolol did not block reconsolidation. (B) Another cohort of snails (n = 20) were exposed to 25 mM KCl for 30 s immediately prior to a 0.5 h training session (TS) in pond water. A memory test (MT) was performed 24 h later. Propranolol (0.1 mL of 50 μM propranolol in *Lymnaea* saline) was injected into snails 23 h after the MT, followed by a memory reactivation session (RM) 1 h after injection. Another memory test (MT2) was done 24 h after memory reactivation; an ANOVA showed that memory was present (ANOVA; $F_{2.517,47.83} = 8.660$, $p < 0.001$). That is, propranolol did not block reconsolidation. Significant difference from the number of pneumostome openings in TS1 $^{**}p < 0.01$.

be altered (Lukowiak et al., 2007; Dodd & Lukowiak, 2015). We thus performed a series of experiments using the training procedures that result in a memory susceptible to propranolol disruption but where we did not reactivate the memory following the propranolol injection. These experiments are shown in Fig. 8A and B. When we tested for memory 24 h later (MT2) we found that memory was present. Thus, the propranolol injection did not block the reconsolidation process because the memory had not been activated 1 h after the injection of the drug (i.e. the reconsolidation process was not necessary as the memory was not reactivated). We can conclude that propranolol in these snails is only capable of blocking reconsolidation if the memory is put into an active state.

4. Discussion

We have demonstrated memory reactivation dependent pharmacological (propranolol) disruption of the reconsolidation process in *Lymnaea*. However, the propranolol block of reconsolidation only occurred if memory was formed around the time that certain combinations of stressors were applied. Thus, it appears as though the causal neuronal mechanisms that underlie memory formation and/or stability vary depending on the stressors applied at the time of memory formation. The memories may appear phenotypically similar even though they are molecularly distinct. The memory phenotype as measured by a 'pass-fail' analysis of performance was not different between all cohorts of snails exhibiting enhanced memory performance, even though these memories were differentially susceptible to disruption by propranolol. Finally, even in the memories that were sensitive to disruption by propranolol, propranolol only blocked reconsolidation when memory was in an active state. All these data are consistent with the hypothesis that while the behavioural phenotype of memory may be similar, the causal neuronal mechanisms underlying memory depend on the conditions of stress surrounding the time of memory formation. Only a subset of memories are susceptible to propranolol block of reconsolidation.

We define stress here as any condition that seriously alters the physiological or psychological homeostasis of an organism (Kim & Diamond, 2002). The so-called Yerkes-Dodson law describes the effect of stress on learning and memory, stating that at different stress levels the ability to form memory changes. In their 1908

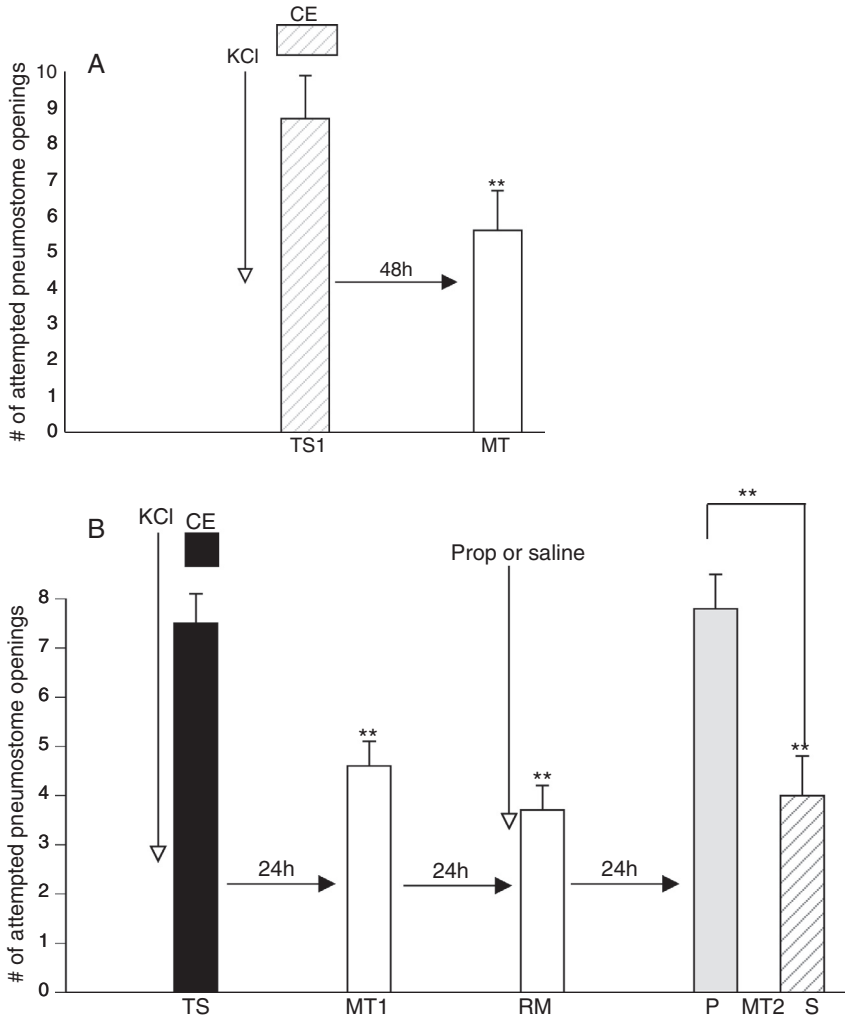

Fig. 6. Combining the stressors CE and KCl creates a memory that lasts at least 48 h and is susceptible to disruption by propranolol. (A) Snails (n = 10) were exposed to 25 mM KCl for 30 s immediately prior to a 0.5 h training session (TS) in CE. Snails were tested for memory 48 h later (MT). A paired *t*-test showed that memory was present ($p < 0.05$). (B) The same procedure was followed as in (A) except the snails were tested for memory 24 h later (MT1). Snails (n = 31) were then injected either with propranolol (0.1 mL of 50 μM propranolol in *Lymnaea* saline) or saline (0.1 mL of *Lymnaea* saline) 23 h after MT1. Memory was reactivated (RM) 1 h after injection. Snails were then tested for LTM 24 h after memory reactivation (MT2). A one-way ANOVA showed that no memory was present in MT2 in the propranolol injected group (ANOVA; $F_{2.676, 42.82} = 9.899$, $p < 0.0001$). That is, propranolol disrupted reconsolidation. A one-way ANOVA performed on the saline injected group indicated that memory was present in MT2 (ANOVA; $F_{2.701, 35.12} = 10.73$, $p < 0.0001$). That is, reconsolidation was not disrupted. Additionally, a two-way ANOVA and a post hoc Tukey's *t*-test indicated that the propranolol injected group had a significantly greater number of attempted pneumostome openings than the saline injected group in MT2 (ANOVA; $F_{3, 209} = 18.59$, $p < 0.0001$). A memory reactivation session is necessary for propranolol to block memory reconsolidation. Significant difference from the number of pneumostome openings in TS1 $^{**}p < 0.01$.

paper (Yerkes and Dodson, 1908), the relationship between stimulus strength and rapidity of learning in rodents was studied. Their basic finding was 'an easily acquired habit may be readily formed under strong stimulation, whereas a difficult habit may be acquired only under relatively weak stimulation.' Typically, however, in textbooks this 'law' is shown as an inverted U function. However, this inverted U function is actually a figure adapted from Donald Hebb's 1955 presidential address to the American Psychological Association (Hebb, 1955; see also Diamond, Campbell, Park, Halonen, & Zoladz, 2007; Ito et al., 2015). Hebb hypothesized that with too little or too much stress, learning is not optimal, hence the inverted U curve. Perhaps there is a molecular correlate to Hebb's 1955 hypothesis. That is, when a memory is formed under a combination of stressors, the causal neuronal mechanisms underlying memory are fundamentally different from memory created under conditions involving individual stressors, even though the memory phenotype (e.g. % of snails achieving a pass mark) is similar.

We do not know why certain stressors result in a memory that is propranolol sensitive while others do not. Damasio (2010) has written "in simple organisms capable of behavior but without a mind process, emotions can be alive and well…". That is, changes in memory formation as a result of subjecting an animal to certain stressors or combinations of them can result in the creation of an emotional state, which may significantly alter the memory. In humans, as an emotion develops in response to environmental stimuli, certain styles of mental processing are promptly instituted. The aggregate of all of the responses to environmental stimuli that result in certain styles of mental processing constitutes an "emotional state" (Damasio, 2010). That this may occur in *Lymnaea*, and affect memory formation, is evidenced by the fact that stressors (i.e. crowding and a low calcium pond water environment) that individually only block LTM formation, when combined, block associative learning and all forms of memory (i.e. STM, ITM and LTM; Dalesman et al., 2013). Damasio acknowledges that in simple animals capable of emotion, feelings (perceptions of what happens in body and mind when emoting) may not necessarily follow. Thus, a possible explanation for why individual stressors create memories that are not susceptible to propranolol while

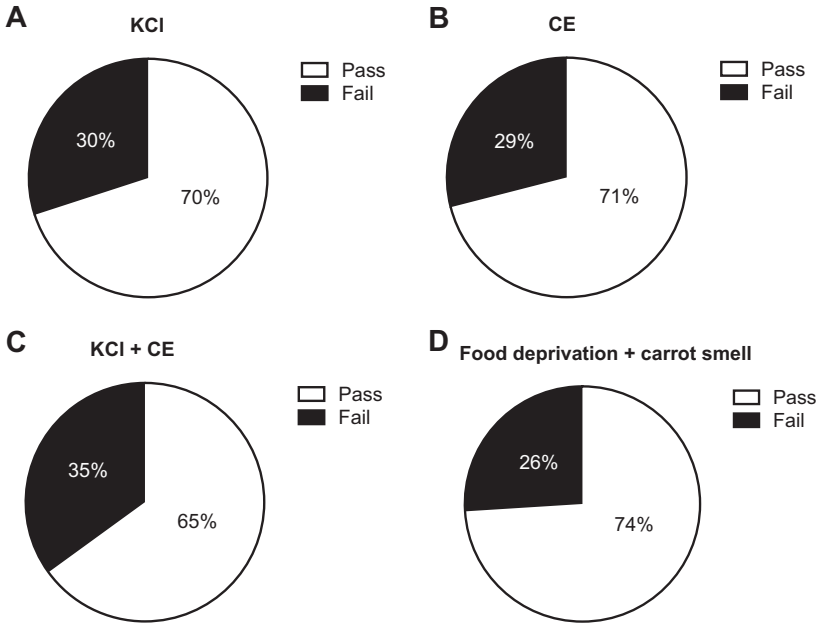

Fig. 7. Snail learning grade distributions. Snails were given either a pass or a fail grade based on their individual performance. Grades were calculated as follows: a 20% or greater reduction in the number of attempted pneumostome openings from TS to MT1 was considered a pass and anything lesser was considered a fail. Grades were calculated for snails exposed to (A) KCl (n = 10), (B) CE (n = 7), (C) KCl + CE (n = 31) and (D) Food deprivation + carrot scent (n = 19).

memories formed under a combination of stressors are, may be that a certain combination of stressors create a qualitatively different state resulting in what could be considered an 'emotional' memory. This makes sense, as in human studies, propranolol has a more significant disruptive effect on memories created under emotionally charged conditions than on neutral conditions (Schwabe et al., 2012). However, this is merely speculation and further research will need to be conducted to determine if these finding extend to *Lymnaea*.

In our experiments we use a savings test to reactivate the memory. That is, the memory recall session is no different than another training session in that each time the snail attempts to open its pneumostome it receives the tactile stimulus. This 'extra training session' should actually make the memory stronger. If anything using the savings test should make the possibility of blocking reconsolidation harder as there is in reality another training session. Thus, if a procedure (e.g. cold block, injection of propranolol) is performed that results in there being no memory it is acting on a stronger memory.

What is different about the memories that are susceptible to propranolol disruption of reconsolidation compared to those that are not in *Lymnaea*? The behavioural phenotype of the memory also did not appear to differ as to whether the LTM resulted from an applied single stressor, a combination of two stressors or a greater number of training sessions. Single stressor-induced memory enhancement (i.e. CE or KCl) alone is not sufficient to bring about the necessary causal changes in neuronal activities that are susceptible to propranolol blockade of reconsolidation. Individually CE and KCl caused enhancement of memory formation (i.e. LTM was seen 48 h after the single training session); but in neither case were the activated memories susceptible to propranolol blockade of reconsolidation. Only when these two stressors were combined did we obtain a memory that was susceptible to propranolol block of reconsolidation. These data are consistent with the Cahill, Pham, and Setlow (2000) findings, using a rodent model, that the role of the adrenergic system in memory reconsolidation is likely modulatory. They discovered that the likelihood of detecting memory impairment with adrenergic blockade was related to the degree to which the training situation activated the endogenous adrenergic system. Rats trained in a highly stressful environment produced stronger memories than rats trained for the same task in a neutral environment. Additionally, the memories in the rats that were created in the stressful environment were much more strongly impaired by propranolol after reactivation than the memories in the rats trained in the neutral environment. This pattern of results is what is expected from manipulation of a memory modulatory system. Our results, consistent with the Cahill et al. study, suggest that molecular mechanisms of reconsolidation in addition to the modulation of memory by stress are highly conserved between species.

Central to reconsolidation theory is the notion that recalled or reactivated previously consolidated memories enter a transient labile phase followed by a new stabilization process (Agren, 2014). It is only during this process that memory can be enhanced, impaired, or updated with new information. Our two-propranolol sensitive memories (KCl + CE and food deprivation + exposure to carrot odour) were disrupted only when memory was reactivated (Fig. 8A and B). In the absence of a reactivation session in the KCl + CE and food deprivation + carrot odour experiments, memory was not disrupted by propranolol. Thus, our data are consistent with what is expected for successful propranolol disruption according to reconsolidation theory. Consistent with this notion are data in *Lymnaea* where the implantation of a 'false' memory can only occur in the 1 h period following activation of a previously formed memory (Lukowiak et al., 2007).

We showed that only memories formed under certain conditions are susceptible to propranolol disruption upon reactivation. This may account for the inconsistencies in results published in the literature documenting propranolol's use in a clinical setting to disrupt the reconsolidation of traumatic memories (Lonergan, Olivera-Figueroa, Pitman, & Brunet, 2013; Wood et al., 2015). Certain memories in humans are more susceptible than others to propranolol disruption. Propranolol has a more significant amnesic effect on memories created under emotionally charged conditions than neutral conditions (Schwabe et al., 2012). Perhaps clinical studies focused on disruption of PTSD memories with propranolol

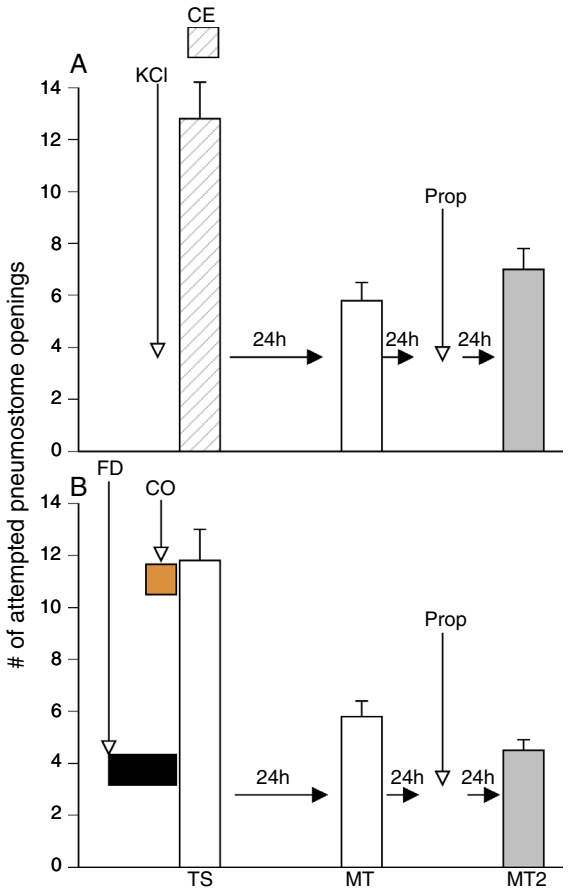

Fig. 8. The memory must be activated in order for reconsolidation to be blocked. (A) The same procedure was followed as in Fig. 6B except no memory reactivation session was given. In this cohort of snails (n = 9) an ANOVA indicated that memory was present in MT2 (ANOVA; $F_{1.442, 11.54} = 5.634$, $p < 0.05$). That is, propranolol did not disrupt memory. (B) The same procedure was followed as in Fig. 3B except no memory reactivation session was given. In this cohort of snails (n = 9) an ANOVA showed that memory was present in MT2 (ANOVA; $F_{1.527, 12.22} = 12.25$, $p < 0.01$). That is, propranolol did not disrupt memory. Significant difference from the number of pneumostome openings in TS1 $^{**}p < 0.01$.

should not assume homogeneity with respect to potential for therapeutic efficacy of propranolol treatment in all patients diagnosed with PTSD. Our data suggest that the precise conditions under which a memory is formed may profoundly impact its ability to be disrupted by propranolol. If greater effort was employed in clinical studies to characterize exact conditions under which targeted traumatic memories were formed, subgroups responsive to propranolol treatment may be identified. A recent meta-analysis of human clinical trials on propranolol's effects on the reconsolidation of long-term emotional memory concluded that propranolol shows promise in reducing subsequent memory of recalled emotional material, but not neutral material, in adults (Lonergan et al., 2013).

Our data further show that the memories susceptible to reconsolidation disruption by propranolol are not based on the 'quantity' of memory (e.g. its duration or% of animals achieving a passing grade). The combination of stressors that produce a memory that is propranolol sensitive causes memory to be made or maintained in a different form from the 'more usual' forms of memory. We do not understand what the difference is or how it comes about. That will be the focus of future research.

The neural circuit that controls aerial respiratory behavior in *Lymnaea*, as well as storing memory for the behavior, has been characterized. The RPeD1 neuron is the necessary site for memory formation (Scheibenstock, Krygier, Haque, Syed, & Lukowiak, 2002); reconsolidation (Sangha, Scheibenstock, & Lukowiak, 2003); extinction (Sangha, Scheibenstock, Morrow, et al., 2003), and forgetting (Sangha et al., 2005). Memory, as well as exposure to stressors, alters the 'state' of RPeD1. For example, the Lukowiak lab has shown (Braun et al., 2012) that RPeD1 is in a 'primed state' in naive 'smart' snails compared to that of 'average' snails. Future experiments will explore whether there are differences in the state of RPeD1 in snails that have differing susceptibilities of propranolol disruption of LTM.

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