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Propranolol disrupts consolidation of emotional memory in *Lymnaea*

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ABSTRACT

The therapeutic efficacy of the synthetic β-adrenergic receptor blocker, propranolol, for the treatment of post-traumatic stress disorder (PTSD) is currently being debated. Mixed results have been published regarding propranolol's ability to disrupt the consolidation and reconsolidation of memories. Here, we use the invertebrate model *Lymnaea* to study propranolol's ability to disrupt consolidation of memories formed under varying various types of stress which cause differing degrees of emotional memory. We show that when propranolol is administered immediately following operant conditioning, only the consolidation process of memories enhanced by individual stressors (i.e. a non-emotional memory) is susceptible to disruption. However, when propranolol is administered prior to training, only memories enhanced by a combination of stressors leading to an emotional memory are susceptible to disruption. These data suggest that the time of propranolol administration, as well as the type of memory formed play a key role in propranolol's ability to obstruct memory consolidation.

1. Introduction

Emotional memories created under highly stressful conditions can be invasive and result in the development of disorders such as post-traumatic stress disorder (PTSD; Breslau, 2009). The ability of stress to alter the ways in which memory is formed and maintained is well known (Hebb, 1955). Similarly, it has been repeatedly demonstrated that emotion enhances memory encoding and facilitates its later recall (Mueller & Cahill, 2010). However, it is not clear how the mechanism (s) underlying the consolidation of memories created during highly stressful, emotional situations differs from the mechanism(s) underlying the consolidation of memories created under 'typical' (i.e. normal, 'non-stressful') circumstances. During exposure to trauma, release of endogenous stress hormones results in over-consolidation of the traumatic memory (Pitman & Orr, 1990). As a result, this memory may later be reactivated much too easily by contextual cues, causing strong conditioned emotional responses (Pitman, 1989).

One method under investigation to decrease the impact of an emotional memory leading to PTSD involves the use of the synthetic β-adrenergic receptor blocker, propranolol, to disrupt the consolidation and reconsolidation of memory. Consolidation occurs when memories that initially exist in a fragile state are strengthened over time (Nader, Schafe, & Le Doux, 2000; Sangha, Scheibenstock, & Lukowiak, 2003a; Sangha, Scheibenstock, McComb, & Lukowiak, 2003b). Reconsolidation, on the other hand, occurs when recalled memories enter a transient labile phase and undergo a new stabilization process before once again returning to a stable state (Sangha et al., 2003a). Propranolol is a synthetic molecule that crosses the mammalian blood–brain barrier and exerts central inhibitory effects on protein synthesis and peripheral effects on the noradrenergic system (Przybyslawski, Roullet, & Sara, 1999). Protein synthesis is required for both the consolidation of short-term memories that are in the fragile state into long-term memories (LTM) as well as the reconsolidation of memory (Nader et al., 2000; Sangha et al., 2003a). The use of propranolol as a treatment for PTSD has been tested in human populations (Lonergan, Olivera-Figueroa, Pitman, & Brunet, 2013) as well as in animal model systems such as the rodent (Cahill, Pham, & Setlow, 2000; Debiec & Ledoux, 2004; Przybyslawski et al., 1999) and the snail (Hughes, Shymansky, Sunada, & Lukowiak, 2016). Mixed results have been reported with regards to propranolol's therapeutic efficacy in human populations (Lonergan et al., 2013).

In studies investigating PTSD, propranolol is commonly used to disrupt the reconsolidation of emotional memories (Debiec & Ledoux, 2004; Hughes et al., 2016; Kindt, Soeter, & Vervliet, 2009; Przybyslawski et al., 1999). The Lukowiak lab has previously shown that only memories created under certain stressful conditions, which create an 'emotional memory', are susceptible to disruption by propranolol following reactivation (i.e. reconsolidation, Hughes et al., 2016). These results are similar to what is seen in humans, where some memories are more susceptible to disruption by propranolol than others. Human studies report that propranolol's amnesic effect is more sizable on memories created under highly emotional conditions compared to neutral conditions (Lonergan et al., 2013; Schwabe, Nader, Wolf, Beaudry, & Pruessner, 2012).

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A second method used to lessen the effect of an emotional memory is to interfere with the initial consolidation process of is memory formation, resulting in a degraded memory trace (Lonergan et al., 2013). Studies have found that when propranolol is administered prior to viewing a series of emotionally upsetting images, the heightened recall of these images is prevented (Cahill, Prins, Weber, & McGaugh, 1994). However, much like with reconsolidation, debate still exists as to whether using propranolol to disrupt the consolidation of memory is a potential clinical treatment. For example, a recent mouse study found that propranolol affected memory consolidation in non-aversive tasks such as object recognition and object location, but had no effect on moderately aversive (Morris water maze) and highly aversive (passive avoidance, conditioned taste aversion) tasks (Villain et al., 2016). A meta-analysis in healthy human participants, on the other hand, found that when compared with placebo, propranolol given prior to memory consolidation resulted in a reduction of subsequent recall for negatively valenced stories, pictures and word lists (Lonergan et al., 2013). If propranolol can be shown to reliably disrupt the consolidation of human PTSD memory, it could prove valuable in clinical settings, such as the emergency department, to reduce the likelihood of developing PTSD after a traumatic event. Thus, further investigation is warranted.

Lymnaea is a valuable neurobiological model for investigating learning and memory because it allows one to easily study the heterogeneity of memories (Lukowiak & Dalesman, 2012). For example, at the single neuron level a memory lasting only 3 h can be seen to be different than a memory that persists for 24 h even though at the behavioural level they do not appear to be different in regards to the percentage decrease from their initial level of responsiveness on the first training session (Braun & Lukowiak, 2011). *Lymnaea* are bi-modal breathers; they are capable of respiration through both cutaneous and aerial means (Lukowiak, Ringseis, Spencer, Wildering, & Syed, 1996). Aerial respiration can be operantly conditioned (a form of associative learning), resulting in a decrease of this behavior. Our standard training procedure (two 0.5 h training sessions separated by 1 h) results in a LTM that persists for at least 24 h; while a single 0.5 h training session is only sufficient to produce an intermediate-term memory (ITM) that persists for 3 h. Whereas ITM is dependent on new protein synthesis, LTM is dependent on both altered gene activity and new protein synthesis (Sangha et al., 2003b).

Some stressors are capable of enhancing memory formation such that if presented prior to or during training, a single 0.5 h training session becomes sufficient for LTM formation (Lukowiak, Sunada, Teskey, Lukowiak, & Dalesman, 2014; Martens, De Caigny, et al., 2007; Martens, Amarell, et al., 2007). That is, some stressors can enhance memory formation such that a 0.5 h training session that does not normally cause LTM now results in LTM. These stressors include thermal stress, predator detection or application of potassium chloride (KCl) (Teskey, Lukowiak, Riaz, Dalesman, & Lukowiak, 2012; Martens, De Caigny, et al., 2007; Martens, Amarell, et al., 2007; Orr & Lukowiak, 2008). Hughes et al. (2016) hypothesized that in *Lymnaea* certain combinations of stressors caused a different form of memory to be made, which they termed an emotional memory. Importantly for our present study, reconsolidation of this emotional memory was blocked by an injection of propranolol; whereas with 'normal' memories propranolol did not block reconsolidation.

The question may arise in some as to whether an invertebrate such as *Lymnaea* can have an emotion or an emotional memory. Emotion in invertebrates remains poorly understood, even though Darwin (1872) in his book on emotion suggested that invertebrates possessed emotions. Many species of invertebrates (e.g. crayfish, ants, bees) display physiological and behavioural changes similar to what is considered to be an emotion in a vertebrate. When we use the word emotion here, we are not suggesting that snails and other invertebrates have feelings. Simple animals that have the capacity for emotion may not necessarily be capable of 'feeling' (perceiving what happens in the body and mind when emoting; Damasio, 2010). Thus, simple organisms can have emotions without experiencing feelings (Damasio, 2010; LeDoux, 2012). Several recently published studies (e.g. Fossat, Bacqué-Cazenave, De Deurwaerdère, Delbecque, & Cattaert, 2014; Perry, Baciadonna, & Chittka, 2016) suggest that invertebrates do in fact exhibit not only negative affect but also positive emotion-like states.

In this study, we explore propranolol's efficacy in disrupting the memory consolidation process following exposure to different stressors or combinations of stressors which may lead to an emotional memory. We hypothesize that propranolol will only disrupt the consolidation of memories created under conditions that lead to an emotional memory. Further, we hypothesize that propranolol injected prior to or immediately following a memory training procedure will impede recall of memories created under conditions that lead to an emotional memory.

2. Methods

2.1. Snails

The *Lymnaea* used in this study were bred from a laboratory strain maintained at the University of Calgary Biology Department. These animals were originally collected in the 1950s from a polder near Utrecht, The Netherlands. Snails were kept 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) at a room temperature of 20 °C. Romaine lettuce was provided ad libitum. A total of 180 naïve snails were used in the study. It is important to note that a snail was only used in a single experiment.

2.2. Drug exposure

(±)-Propranolol hydrochloride (TLC) powder was obtained from Sigma-Aldrich (St. Louis, MO, USA). The concentration of propranolol was chosen based on pilot studies previously done in the Lukowiak lab, and is consistent with the published literature (Hughes et al., 2016). Immediately prior to injection, snails were placed in an ice bath for 5 min in order to anesthetize them. Propranolol-treated snails were injected into their foot with 0.1 mL of 50 μM propranolol dissolved in *Lymnaea* saline and saline treated snails (vehicle controls) were injected with of 0.1 mL *Lymnaea* saline. Injections were either performed prior to or following the training session (TS). If injections were done prior to TS, snails were returned to their eumoxic (6 mL O_2/L) home aquaria for 1 h after injection to recover before undergoing a 0.5 h training session. If injections were performed following TS, snails were simply placed back into their eumoxic home aquaria and remained there until the memory test (MT) 24 h later. Injection of propranolol at the concentration used here has previously been demonstrated to not affect homeostatic breathing behavior in *Lymnaea* (Hughes et al., 2016). Finally, it has previously been shown (e.g. Hughes et al., 2016; Sunada et al., 2017) that the injection of saline before or after training does not alter (i.e. neither enhancing or obstructing) memory formation.

2.3. Aerial respiratory behavior

In eumoxic conditions (6 mL O_2/L) *Lymnaea* primarily acquire oxygen by means of cutaneous respiration. In hypoxic conditions, on the other hand, with low dissolved concentration of oxygen (< 0.1 mL O_2/L), *Lymnaea* shift to aerial respiration and use their lung which is connected to the atmosphere via a structure called the pneumostome.

2.4. Standard operant conditioning procedure

Each snail was labelled 24 h prior to the training session. Snails were placed in a 1L beaker filled with 500 mL of artificial pond water made hypoxic by bubbling N_2 gas through the water for 20 min prior to a training session. Animals were allowed to acclimatize for 10 min in the beaker prior to the initiation of the training session. During the

0.5 h training session, a tactile stimulus ('poke') was applied to the edge of the pneumostome each time a snail attempted to open it. This results in the closing of the pneumostome without harming the snail and without causing the snail to retract completely into its shell. The number of pokes was recorded for each snail. This same procedure was performed for all training sessions and memory tests.

Normally, using the standard operant conditioning procedure, two 0.5 h training sessions spaced one hour apart are required to form a 24 h LTM in this strain of snails (Lukowiak, Nimet, Krygier, & Syed, 2000; Lukowiak et al., 1996). We operationally define LTM as being present if MT is significantly less than TS1 and not significantly greater than TS2 (Lukowiak et al., 1996). When using an enhancing stressor presented prior to or during training, a single 0.5 h training session is used. We operationally define LTM in these experiments as significantly fewer attempted pneumostome openings performed during the 24 h memory test compared to the single training session (Dalesman & Lukowiak, 2012).

2.5. Stressors

When applied individually or in combination, stressors in *Lymnaea* can block or enhance memory formation (Lukowiak et al., 2014). Here we use two stressors that cause an enhancement of LTM formation, crayfish effluent (CE) and potassium chloride (KCl), either individually or in combination. KCl and CE were applied only once for a given memory training procedure, prior to or during a training session, respectively. Thus, stressors were not used in the memory test session.

Crayfish effluent (CE): Crayfish are a natural predator of *Lymnaea*. In our lab, they are kept in 70 L aquaria, where they are provided lettuce and snails ad libitum. Crayfish effluent (CE) refers to the water from the crayfish tanks (Orr, El-Bekai, Lui, Watson, & Lukowiak, 2007). In the past, the Lukowiak lab has shown that exposing snails to CE during a training session results in significant enhancement of memory formation. That is, after just one training session in CE, snails had LTM 24 h later (Orr & Lukowiak, 2008; Sunada, Horikoshi, Lukowiak, & Sakakibara, 2010; Lukowiak et al., 2014).

Potassium chloride (KCl): KCl exposure is noxious to *Lymnaea*. Previously, the Lukowiak lab has demonstrated that a 30 s exposure to 25 mM KCl immediately before a training session results in significant enhancement of memory formation. That is, after just one training session immediately following 30 s in 25 mM KCl, snails had LTM 24 h later (Martens, De Caigny, et al., 2007; Martens, Amarell, et al., 2007).

When combined, these stressors were administered in the same manner as individually. Snails were first exposed to 30 s of KCl, followed by a training session in CE immediately after. When applied individually, these stressors create 'non-emotional stress', whereas the combination of these stressors creates an 'emotional stress' (Hughes et al., 2016). Snails are first exposed to a noxious stimulus then placed into an environment where they can sense that a predator is nearby. Thus, this combination of nociception and fear places the animals in a state of emotional arousal.

2.6. Numbers of snails used in each experiment

The total number of snails used in the experiments reported here (Figs. 1–8) was 180. The number of snails used in each experiment is noted both in the Results section and the Figure Legends. It is important to note two important facts: (1) Each snail was initially naïve, it had not previously been used in any experiment and all snails were initially in the same state; and, (2) Each snail was only used once in a specific experiment. For example, if the snail was in an experiment that utilized two training sessions and a memory test session the snail was in two training sessions and a memory test session.

In Figs. 1–4 the number of snails used varied between 9 and 13. We attempted to use similar numbers in each of these experiments; however, occasionally a snail died or in the first training session did not attempt to perform a pneumostome opening. Thus, that snail could not be used. In Fig. 5, 15 snails were used in A and 11 in B. In Fig. 6, 10 snails were used. In Fig. 7A 31 snails were used while 11 were used in B. Finally, in Fig. 8, 17 snails were used. We used more snails in 7A because we put more of our resources into testing that hypothesis. We had hoped to use a similar number of snails in Fig. 7B and 8. However, we had a die-off in our snail colony and we had just enough snails to perform the experiments shown in Fig. 7B and 8. All snails used were healthy and there are no significant differences in the number of attempted pneumostome openings in the initial training sessions (TS) (see below).

4. Statistical analyses

Statistical tests were done using Prism 6 software for the Mac OS 10.12 system. We performed a 2-Way ANOVA followed by a Tukey's *post hoc* test in Figs. 1–4, 5 and 7. In addition, paired t-tests were performed in order to determine whether memory was present 24 h following a single 0.5 h training session on snails shown in Figs. 6 and 8. That is, if the number of pokes in MT was significantly lower than in TS, memory was said to be present. In Fig. 1 for us to conclude that LTM formed (see Lukowiak et al., 1996) the number of attempted openings in TS had to be significantly less than TS1 and not significantly greater than TS2.

5. Results

Normally this strain of *Lymnaea* require two 0.5 h training sessions separated by 1 h to form LTM (i.e. a significant decrease in the number of attempted pneumostome openings 24 h after training; Lukowiak et al., 2000; Shymansky et al., 2017). Thus, we first trained a cohort of naïve snails (N = 12) with two 0.5 h training sessions separated by 1 h. However, 1 h before training began we injected these snails with saline in order to test whether a possible stress associated with the injection process obstructs normal memory formation. We then used a similar training procedure on another naive cohort of snails (N = 11) but injected propranolol instead of saline prior to giving the animals two 0.5 h training sessions separated by 1 h. A 2-way ANOVA followed by a Tukey's *post hoc* test was performed on the data presented in Fig. 1. The comparison of saline vs propranolol ($F_{(1,60)} = 0.4252$; $p = .5168$) shows that the two cohorts did not differ. The analysis of training and memory test sessions $F_{(2,60)} = 14.95$; $p < .0001$ showed that there was a training effect. When multiple *post hoc* comparisons were made in each cohort it was found that TS1 and TS2 were significantly different ($p < .001$) and MT was significantly less than TS 1 ($p < .001$) but MT was not significantly greater than TS2. Thus, the criteria for LTM formation was met in both cohorts. We conclude that neither an injection of saline nor propranolol disrupted normal LTM formation.

We next gave two separate naïve cohorts of snails (N = 10) a single 0.5 h training session 1 h following a saline injection or a propranolol injection (Fig. 2A and B, respectively) to test whether either injection was sufficient to enhance memory formation. A 2-way ANOVA followed by a Tukey's *post hoc* test was performed on these data. This analysis showed that there was not an interaction ($F_{(1,36)} = 0.0672$; $p = .7969$) between the variables (i.e. the sessions (TS and MT) and treatment (saline vs propranolol) The comparison of the saline injected cohort (2A) vs the propranolol injected cohort (2B) showed there was no difference the number of attempted pneumostome openings in the training (TS) sessions and the memory-test sessions (MT) between the cohorts ($F_{(1,36)} = 0.1317$; $p = .7188$). Moreover, a comparison in the two cohorts showed there were no significant differences between the TS and MT in each group; meaning that LTM formation did not occur ($F_{(1,36)} = 0.9704$; $p = .3311$). Thus, there was no difference in the number of attempted opening in snails receiving saline or propranolol before training and neither the saline nor propranolol injection before the snails received the single 0.5 h training procedure resulted in

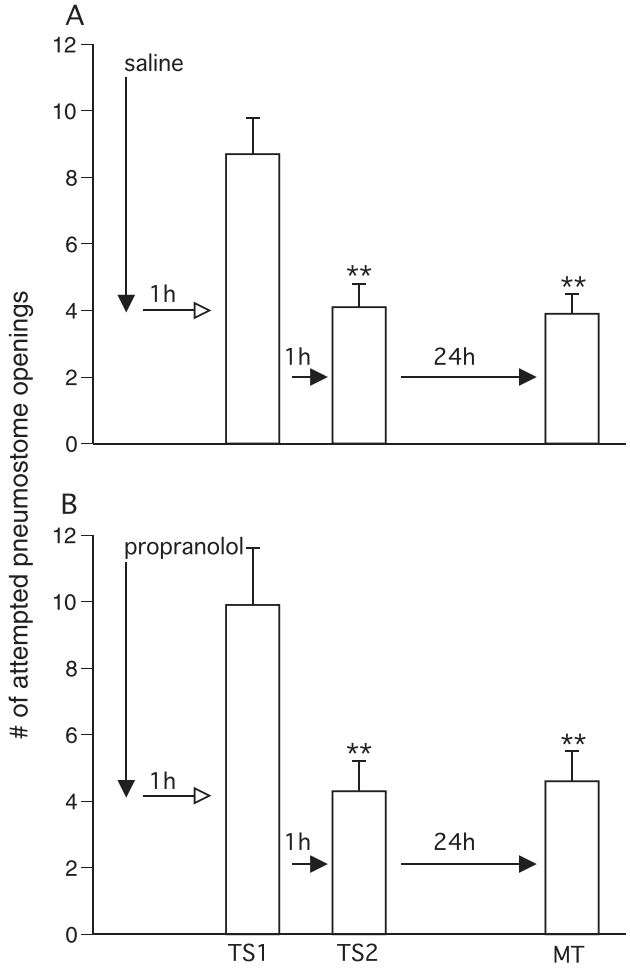

Fig. 1. Saline and propranolol injections do not disrupt LTM formation. (A) Two 0.5 h training sessions (TS1 and TS2) were given to a cohort of naïve snails (n = 12) 1 h following a saline injection. Snails were tested for LTM 24 h later (MT). A 2-Way ANOVA indicated that memory was present 24 h later. That is, the stress of an injection did not disrupt normal memory formation. (B) Two 0.5 h training sessions (TS1 and TS2) were given to a cohort of naïve snails (n = 11) 1 h following a propranolol injection. Snails were tested for LTM 24 h later (MT). A 2-Way ANOVA indicated that memory was present 24 h later. That is, propranolol did not disrupt normal memory formation. ** represents $p < .01$.

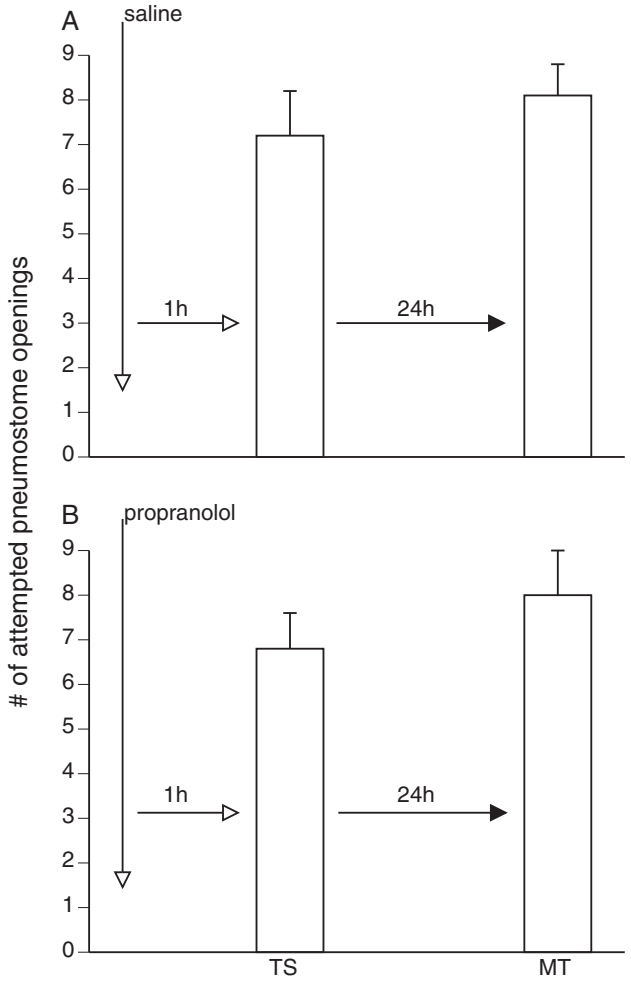

Fig. 2. Saline and propranolol injections do not enhance memory formation when administered prior to training. (A) A 0.5 h training session (TS) was given to a cohort of naïve snails (n = 10) 1 h following a saline injection. Snails were tested for LTM 24 h later (MT). (B) A 0.5 h training session (TS) was given to a cohort of naïve snails (n = 10) 1 h following a propranolol injection. Snails were tested for LTM 24 h later (MT).

enhanced memory forming ability.

Administering a saline or propranolol injection to naïve cohorts of snails (Fig. 3A and B respectively; N = 9 in each) following a 0.5 h training session did not result in enhanced memory, as no memory was present 24 h later. A 2-way ANOVA followed by a Tukey's *post hoc* test was performed on these data. This analysis showed that there was not an interaction ($F_{(1,32)} = 0.03584$; $p = .8518$) between the variables (i.e. the sessions (TS and MT) and treatment (saline vs propranolol). A comparison of the saline vs propranolol injected cohorts revealed that there was a significant difference in the TS before either injection ($F_{(1,32)} = 9.082$; $p = .005$). The number of attempted pneumostome openings in the group that received the propranolol injection after the training session was smaller than the cohort that received the saline injection. However, there was no difference in the number of attempted openings in the memory test session between the two cohorts. Finally, in both groups there was no difference between the TS and MT sessions ($F_{(1,32)} = 1.499$; $p = .2298$). Thus, neither a saline nor propranolol injection after the training session enhanced memory formation.

To test whether the control injection process (i.e. saline) disrupts the consolidation process of a memory enhanced by a stressor, we separately trained two cohorts of naïve snails exposed to either the KCl bath or CE (Fig. 4A and B, respectively). One cohort (N = 11) received a saline injection 1 h prior to the KCl bath and TS and the other cohort (N = 13) received the injection following the TS in CE. A 2-Way ANOVA followed by the Tukey's *post hoc* test was performed on these data. These analyses showed that there was not an interaction ($F_{(1,44)} = 0.2133$; $p = .6465$) either between the variables of each cohort (i.e. the sessions (TS and MT) or the treatment (before vs after saline and stressor). The analysis further showed that there were no differences in the training sessions or the memory test session between the two cohorts ($F_{(1,44)} = 1.920$; $p = .1729$). However, in each cohort the memory test session was significantly smaller than the training session ($F_{(1,44)} = 21.33$; $p < .0001$). We conclude that a saline injection administered prior to (Fig. 4A) or following (Fig. 4B) a single 0.5 h TS in the presence of these stressors does not disrupt LTM consolidation.

Next, we tested whether a propranolol injection following training in the presence of a single stressor disrupts the memory consolidation process. A naïve cohort of snails (Fig. 5A, n = 15) was trained in CE and immediately following the single TS was injected with 0.1 mL of 50 μM propranolol. Memory was then assessed 24 h later. A second naïve cohort of snails (Fig. 5B, n = 11) was exposed to 25 mM KCl for 30 s immediately prior to training in pond water. The animals were then injected with 0.1 mL of 50 μM propranolol following the TS and tested for memory 24 h later. A 2-Way ANOVA followed by the Tukey's *post*

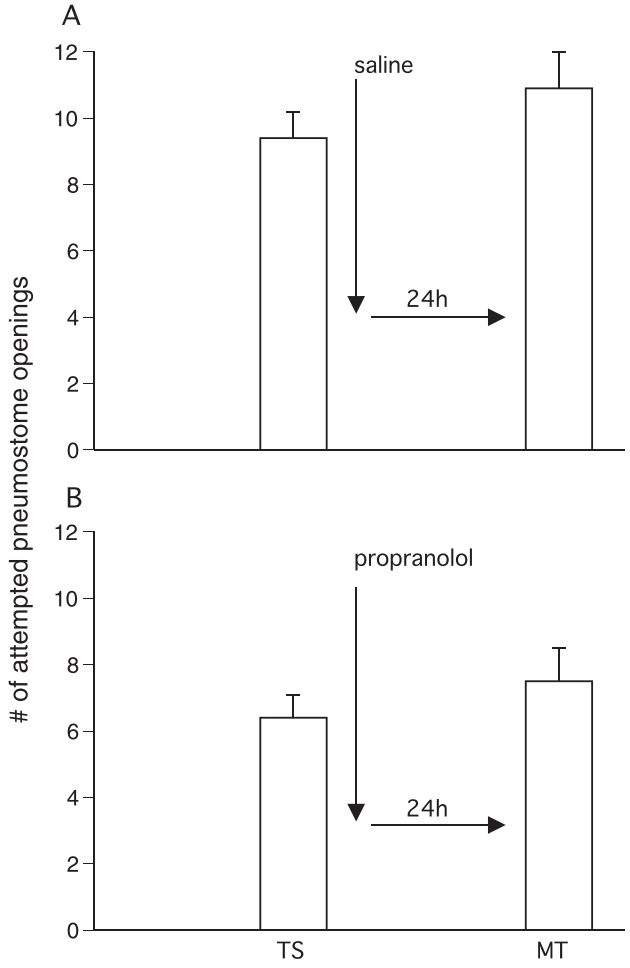

Fig. 3. Injection of saline and propranolol do not enhance memory formation when administered following training. (A) A saline injection was given to a cohort of naïve snails (n = 9) immediately following a 0.5 h training session (TS). Snails were tested for LTM 24 h later (MT). (B) A propranolol injection was given to a cohort of naïve snails (n = 9) immediately following a 0.5 h training session (TS). Snails were tested for LTM 24 h later (MT).

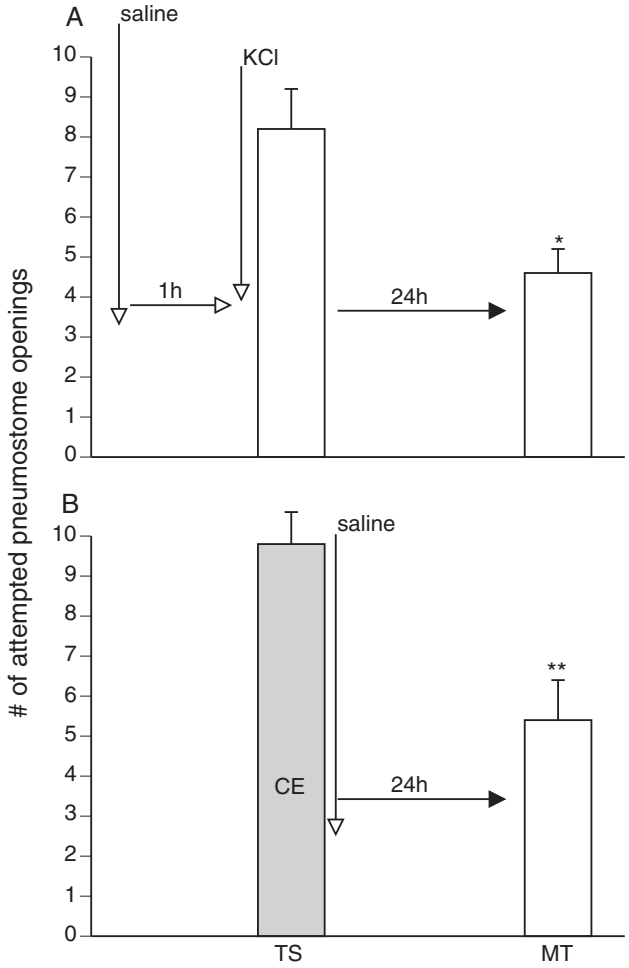

Fig. 4. Saline injections do not disrupt consolidation of a stressor-enhanced memory when administered either prior to or following training. (A) A saline injection was given to a cohort of naïve snails (n = 11) 1 h prior to a 30 s exposure to KCl and a 0.5 h training session (TS). Snails were tested for LTM 24 h later (MT). (B) A saline injection was given to a cohort of naïve snails (n = 13) immediately following a 0.5 h training session (TS) in CE. Snails were tested for LTM 24 h later (MT). * represents $p < .05$; ** represents $p < .01$.

hoc test was performed on these data. These analyses showed that there was not an interaction ($F_{(1,48)} = 0.8044$; $p = .3742$) between the variables (i.e. the sessions (TS and MT) and treatment (CE vs KCl bath). Further, the analyses showed that there was a difference in the number of attempted pneumostome openings between the two groups ($F_{(1,48)} = 4.922$; $p = .0313$). However, the Tukey's *post hoc* test showed no significant difference between the two TSs and the two MTs. Finally, in each cohort the memory test session was not significantly smaller than the training session ($F_{(1,48)} = 0.3766$; $p = .5423$). Again, the Tukey's *post hoc* tests showed no difference between TS and MT in both cohorts. We conclude that the propranolol injection after the training session in the presence of a stressor obstructs memory formation.

Previously it was shown that the combination of the two stressors (KCl bath + training in CE) leads to an emotional memory (Hughes et al., 2016). We therefore asked whether in the face of these two encountered stressors propranolol injection following training would obstruct LTM formation (Fig. 6). When propranolol was administered following the TS in this cohort of snails (n = 10), the number of pneumostome openings was significantly lower in the MT as compared to the TS (i.e. memory was present 24 h later; Paired *t*-test. $t = 4.819$, $p = .0009$). Thus, the administration of propranolol immediately after training did not disrupt the consolidation of this emotional memory caused by the combination of CE and KCl.

Studies done in humans normally administer propranolol prior to any memory training (Cahill et al., 1994). Thus, we injected propranolol before we trained naïve cohorts of snails in the presence of single stressors. A naïve cohort of snails (n = 31; Fig. 7A) was trained in CE 1 h after the animals were injected with propranolol. A second naïve cohort of snails (n = 11; Fig. 7B) was trained in pond water following the KCl bath. A 2-Way ANOVA followed by the Tukey's *post hoc* test was performed on these data. The analyses showed that there was not an interaction ($F_{(1,80)} = 0.1963$; $p = .6589$) between the variables (i.e. the sessions (TS and MT) and treatment (CE vs KCl bath). Nor was there any difference in the number of attempted pneumostome openings in either TS or MT between the two groups ($F_{(1,80)} = 0.6012$; $p = .4404$). However, in each cohort the memory test session was significantly smaller than the training session ($F_{(1,80)} = 9.620$; $p = .0027$). Thus, pre-treatment with propranolol does not significantly interfere with the memory consolidation process in snails facing a single stressor.

Finally, the two stressors, the KCl bath and CE were applied to a naïve cohort of snails 1 h following a propranolol injection. The naïve snails (Fig. 8, n = 17) were first injected with propranolol and 1 h later exposed to KCl and then trained in CE. The 24 h memory test revealed that propranolol successfully disrupted the consolidation process as LTM was not present. That is, the number of pneumostome openings was not significantly different between TS and MT (Paired *t*-test, $t = 0.6594$, $p = .5190$).

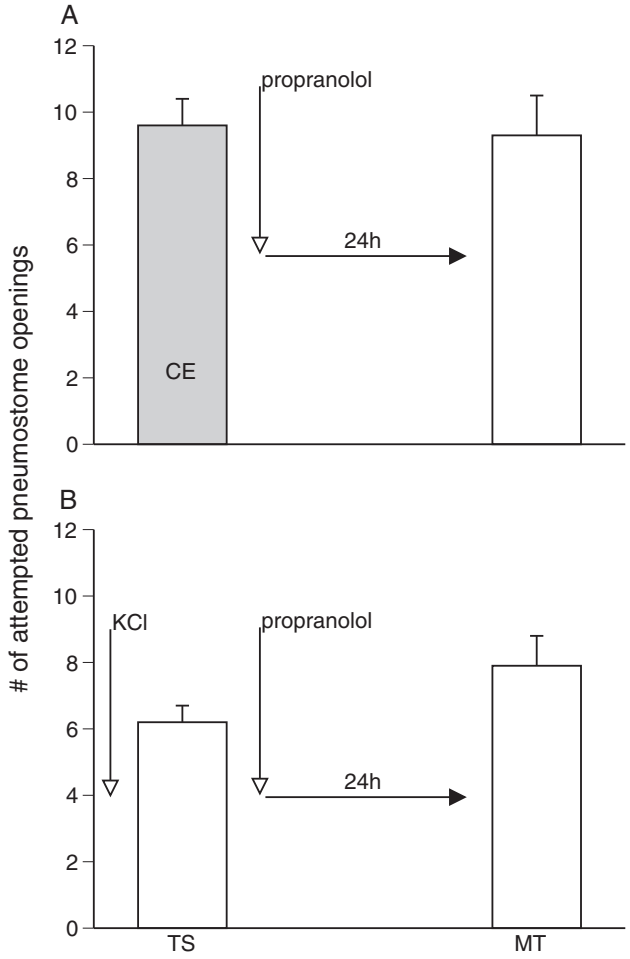

Fig. 5. Propranolol disrupted the consolidation process of memories enhanced by CE or KCl. (A) A single 0.5 h training session (TS) in CE was given to a cohort of naïve snails (n = 15). Snails were then injected with propranolol and tested for LTM 24 h later (MT). (B) A separate cohort of naïve snails (n = 11) was exposed to KCl for 30 s immediately prior to a 0.5 h training session (TS) in pond water. Snails were then injected with propranolol and tested for LTM 24 h later (MT).

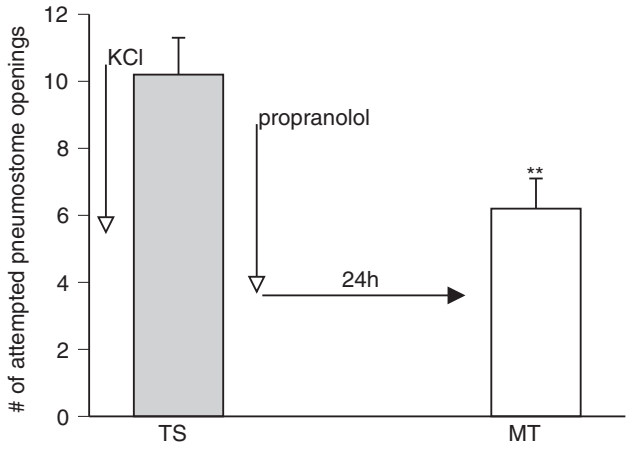

Fig. 6. Propranolol does not disrupt memory formation when both CE and KCl are experienced combined. A separate cohort of naïve snails (n = 10) was exposed to KCl for 30 s immediately prior to a 0.5 h training session (TS) in CE. Snails were then injected with propranolol and tested for LTM 24 h later (MT). A paired *t*-test (t = 4.819, p = .0009) indicated that memory was present (** represents p < .01).

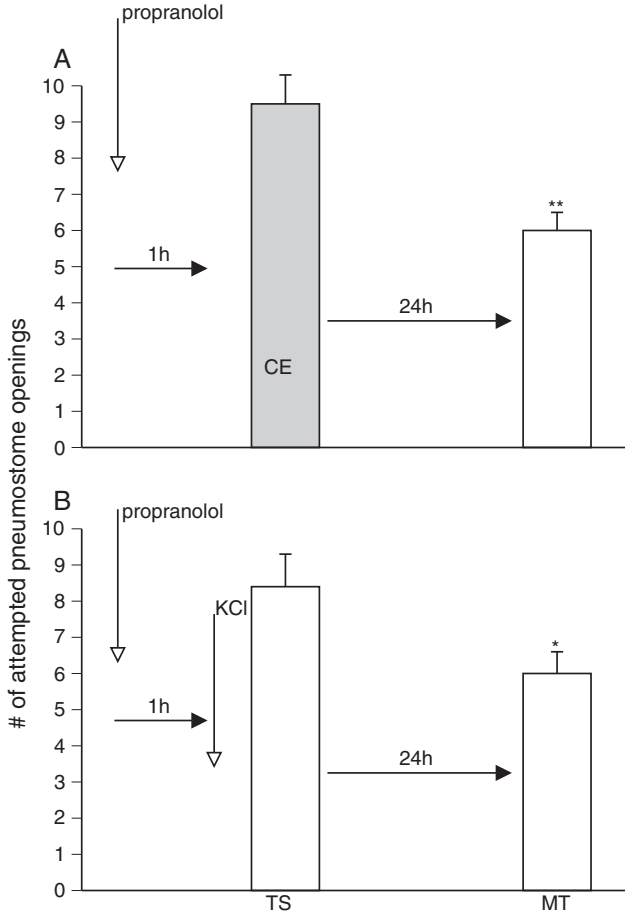

Fig. 7. When injected prior to training propranolol does not disrupt the consolidation process of memories enhanced each stressor individually. (A) A cohort of naïve snails was injected with propranolol and 1 h later, a single 0.5 h training session (TS) was given in CE (n = 31). Snails were tested for LTM 24 h later (MT). (B) A separate cohort of naïve snails (n = 11) was injected with propranolol 1 h prior to exposure to 30 s of KCl. A 0.5 h training session (TS) was then given in pond water and snails were tested for LTM 24 h later (MT). * represents p < .05; *** represents p < .001.

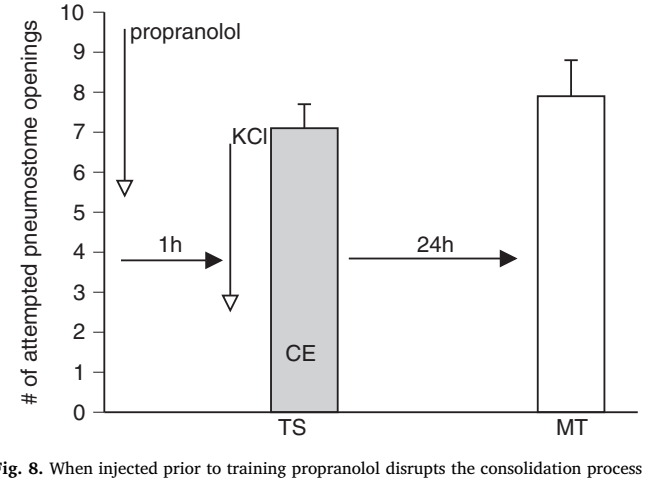

Fig. 8. When injected prior to training propranolol disrupts the consolidation process in snails experiencing the combination of CE and KCl. A cohort of naïve snails (n = 17) was injected with propranolol 1 h prior to exposure to 30 s of KCl and a 0.5 h training session (TS) in CE. Snails were tested for LTM 24 h later (MT). A paired *t*-test indicated that no memory was present (p = .5190).

6. Discussion

We show here that memories formed when encountering different stressors result in what we termed emotional vs. non-emotional memory can be differentially disrupted by propranolol at different time points. These data show: (1) Although the behavioural phenotype of memory may appear similar (e.g. a similar decrement in the number of attempted pneumostome openings in MT), the causal neuronal mechanisms underlying memory depend on the conditions of stress that were present around the time of memory formation; and, (2) Propranolol can disrupt the consolidation of different memories at different times of administration.

Not too long ago, the notion that an invertebrate, such as a snail, could possess an emotional memory was rejected. This occurred because many, as LeDoux (2012) pointed out, believe that emotion is something that is 'human' mostly because it can be linked to our own subjective feelings. In that paper, LeDoux suggested that 'card carrying' comparative neurobiologists (e.g. the Lukowiak lab) who study in model systems 'survival circuits and functions' (which he posits mediate emotional behavior) would have no problem accepting that an invertebrate could have an emotional memory. Damasio (2010) also posits that invertebrates could exhibit emotion. In the past few years a number of high-profile reports using an invertebrate model system have now used the word emotion in their title (e.g. Perry et al., 2016; "Unexpected rewards induce dopamine-dependent positive emotion–like state changes in bumblebees"). We direct interested readers who wish to further pursue this topic to two excellent reviews (Anderson & Adolphs, 2014; Baciadonna & Perry, 2017). However, it should be remembered that Darwin posited that invertebrates have emotion. In his 1872 book The Expression of the Emotions in Man and Animals, Darwin states "Even insects express anger, terror, jealousy and love, by their stridulation". Darwin asserted that this insect behavior is an expression of emotions homologous to anger or terror states in humans. This view is also congruent with the present Oxford English Dictionary definition of emotion as: An agitation of mind or instinctive feeling (e.g. fear) deriving from one's circumstances (i.e. experienced environment).

The Yerkes-Dodson/Hebb law, which defines the effect of stress on learning and memory, states that the ability to form memory differs with the perception of stress (Yerkes & Dodson, 1908; Hebb, 1955; Ito, Yamagishi, Sakakibara, Fugito, & Lukowiak, 2015). Here, we define stress as a condition that alters the physiological or psychological homeostasis of an organism (Kim & Diamond, 2002). Thus, both the 'degree' and the 'type' of stress have an important impact on memory formation. The perception of the stressor by the organism, which is probably the most important parameter in determining the stressor's effect on memory formation, is dependent on both the 'type' and 'level' of the stressor. As seen in the inverted U-shaped curve, which is a derivative of Hebb's (1955) curve, both low and high levels of perceived stress are not conducive to memory formation. Instead, learning and memory formation are optimal with moderate stress. Thus, the causal neuronal mechanism underlying memory formation differs with both the type and level of stress encountered when the memory is formed. For example, we previously found (Hughes et al., 2016; Kita et al., 2011) that phenotypically similar memories may be molecularly diverse, depending on the stressful conditions under which they are formed. This is consistent with our finding that memories created under a single stressor are different than memories created under a combination of stressors (Dalesman, Sunada, Teskey, & Lukowiak, 2013). Moreover, combinations of certain stressors lead to emotional memories that are sensitive to propranolol (Hughes et al., 2016). Based on these findings, we speculated here that propranolol would work in a similar manner and only disrupt the consolidation process of memories created under highly stressful conditions that lead to the formation of emotional memories.

We demonstrated previously and here that neither the saline nor the propranolol injection alters the consolidation of memory when applied before or after training in the absence of externally applied stressors (i.e. what we refer to as typical memory). This may indicate that the molecular pathway that propranolol blocks is only recruited in memory consolidation under only certain conditions of stress, and it is not recruited under neutral conditions. Through investigating the effects of propranolol on reconsolidation, Hughes et al. (2016) determined that quantitatively similar memories (i.e. similar reduction in attempted pneumostome openings from the TS to the MT) may be molecularly distinct. The findings from our current study build on the Hughes et al. (2016) study through investigating the effects of propranolol on the initial consolidation process. Certain memories that are created under conditions of externally applied stress are susceptible to propranolol disruption (Figs. 5 and 8), but 'typical' memories created under the absence of externally applied stress are not susceptible to propranolol disruption (e.g. Fig. 1B). This finding suggests that although memories created under stress versus no stress appear quantitatively similar, they are probably molecularly distinct at the time of consolidation in addition to the time of reconsolidation.

We found here (Fig. 5) that the injection of propranolol immediately after training in snails stressed by the KCl bath or CE blocked the memory consolidation process. Thus, the consolidation process initiated by the two stressors experienced individually is susceptible to disruption by propranolol. However, when the combination of the KCl bath + CE, which results in an emotional memory (Hughes et al., 2016) is experienced by the snails (Fig. 6) the consolidation process is not blocked by propranolol.

As Hughes et al. (2016) demonstrated the combination of the KCl bath + CE stressors creates an emotional memory in the snail. A possible reason why the administration of propranolol following training did not disrupt the consolidation of this emotional memory is because the memory consolidation had already been initiated before the time of injection. When stressors are combined and a state of high emotional arousal is reached, the consolidation process may be initiated earlier or even sped up. This phenomenon may be similar to 'flashbulb memories'. Studies have shown that a strong emotional charge allows the hippocampus to switch from a cognitive mode to a flashbulb memory mode, resulting from a rapid storage of emotional memories (Ceccom, Halley, Daumas, & Lassalle, 2014). *Lymnaea* are capable of one-trial learning (Alexander, Audesirk, & Audesirk, 1984; Martens, De Caigny, et al., 2007; Martens, Amarell, et al., 2007; Sugai et al., 2007) and it is plausible that with very stressful stimuli the processes underlying memory formation are initiated with the first paired trial. Thus, the causal processes underlying LTM formation would not be susceptible to propranolol intervention following the conclusion of the 0.5 h TS. Propranolol was capable of blocking LTM formation when these same stressors were combined, but in that case the injection had to be made before training commenced.

We demonstrated (Fig. 7) that when propranolol was injected 1 h prior to training memory consolidation was not disrupted when the individual stressors CE and KCl were used. Propranolol injection prior to training only disrupted the consolidation of memories created under the combination of stressors that leads to an emotional memory being formed (CE + KCl; Fig. 8). This finding was in line with our prediction. Consistent with what we previously hypothesized, certain combinations of stressors create a qualitatively different memory state that results in an emotional memory (Hughes et al., 2016). This is similar to what has been reported in several human studies, where propranolol was shown to have a more disruptive effect on the consolidation of memories created under emotionally charged conditions than under neutral conditions (Lonergan et al., 2013). Most human studies have replicated the paradigm of Cahill and colleagues (Cahill et al., 1994), where participants watch a series of slides complemented by either an emotionally disturbing or a neutral verbal narrative. In those human studies the researchers administer propranolol or placebo 1–1.5 h prior to the time when subjects view the slides (Lonergan et al., 2013).

It is clear based on the data presented here and previously (Hughes

et al., 2016) that propranolol injection can have significant disruptive effects on emotional memory formation in *Lymnaea*. These findings prompt an important question: Are there beta-adrenergic or other types of receptors in *Lymnaea* that propranolol could interact with to bring about the effects we describe? Adamo (2008) has pointed out molluscs are unique in that they use both norepinephrine (i.e. noradrenalin, NA) and octopamine (OA) as neurotransmitters/neuromodulators. It has also been suggested that the octopaminergic system in invertebrates is homologous to the vertebrate adrenergic system (Massarsky, Trudeau, & Moon, 2011). Consistent with that suggestion is the finding, that the molluscan OA receptor and the human -beta adrenergic receptor share 40% identity; thus, it is not too difficult to see that propranolol could act at an OA receptor (Gerhardt et al., 1997). In molluscs both NA and OA can act as stress hormones by affecting tissue energy reserves and muscle tissue to prepare the organism to respond (Massarsky et al., 2011; Roeder, 1999). More to the point, Lacoste, De Cian, Cueff, and Poulet (2001) showed that NA is released into the circulatory system of molluscs in response to stress; providing direct evidence for the existence of an adrenergic response in molluscs to stress. A similar finding was more recently reported by Fabbri and Capuzzo (2010). Franzellitti, Buratti, Valbonesi, Capuzzo, and Fabbri (2011) then showed the effects of propranolol in the mollusk, Mytilus, subjected to stress. Exposure to propranolol blocked the cAMP-mediated signaling pathway elicited by the stressor. It needs to be remembered in light of that finding that a cAMP-mediated signaling pathway plays a key role in LTM formation in *Lymnaea* (Otsuka et al., 2013; Sadamoto et al., 2003). At the electrophysiological level studies in *Lymnaea* directly show that NA altered the activity of pedal ganglion neurons (Anokhin, Orlov, & Osipovskii, 1973). Moreover, in that same study the application of propranolol blocked the response to NA and to the response elicited by tactile stimulation of the foot. More recently Samarova et al. (2005) further showed in *Lymnaea* that propranolol reversibly blocked synaptic potentials elicited by both photic and tactile stimuli in Lymnaea. Thus, there is strong experimental evidence that propranolol acts in the molluscan nervous system in a similar manner as it does in mammalian systems.

As mentioned in the Introduction it is thought that in mammalian preparations propranolol may block protein synthesis (Przybyslawski et al., 1999). However, our data (e.g. Fig. 1) do not indicate that propranolol acts in this manner in *Lymnaea*. If propranolol blocked new protein synthesis we would expect that its injection, as in Fig. 1B, would prevent LTM formation as LTM is dependent on altered gene activity and new protein synthesis (Sangha et al., 2003b).

Together our data show that 'typical' memory (i.e. formed in the absence of stressors) is a type of memory that propranolol is not capable of disrupting regardless of the time of its application. Other 'types' of memories, dependent on the application of different stressors, are susceptible to propranolol injection and the disruption is dependent on the time of propranolol administration. Further investigation is necessary to explain this dependency on the time of administration.

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Conflicts of interest

None.

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