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RESEARCH ARTICLE

Stress before training alters memory retrieval of a non-declarative memory in *Lymnaea*

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ABSTRACT

Stress alters both memory formation and its retrieval. Here, we show that a combination of stressors before an associative learning event alters memory retrieval of a non-declarative memory in an invertebrate model system. Previously, two combinations of stressors were purported to prevent long-term memory (LTM) formation in 'smart' *Lymnaea* and this inability to form LTM was considered to be a cost of being smart. Here, we show that is not the case. The specific combinations of stressors used here cause emotional memory formation. Previously, it was shown that propranolol, a synthetic beta-blocker, altered emotional memory in *Lymnaea.* We show here that when propranolol but not saline is injected into smart snails before they perceive the combination of stressors, these snails form LTM. We then show that the injection of propranolol but not saline before a memory activation session allowed the memory to be recalled. That is, LTM formed but was not retrievable unless propranolol was injected pre-retrieval. Thus, the smart snails formed LTM in the face of the stressors but could not retrieve it.

KEY WORDS: Memory recall, Propranolol, Long-term memory, Emotional memory

INTRODUCTION

In *Lymnaea*, as well as in other species, including *Homo sapiens*, both the timing of when stress is perceived and the nature of the perceived stressor are critical factors in determining how memory formation and its recall are altered. Broadly speaking, stress in close proximity to the initial learning event alters not only the learning process itself but also the processes of memory consolidation and memory retrieval. This is especially true for emotional stimuli (Roozendaal, 2002; Roozendaal et al., 2003, 2006a,b). Indeed, in humans, the perception of stressful stimuli before a learning event is able to alter the ability of memory recall at later dates (Sandi and Pinelo-Nava, 2007; Wolf, 2017). Here, we explored in *Lymnaea* how stressors that lead to an enhanced emotional memory in average cognitive ability snails, may not prevent memory formation in the 'smart' snails, as was previously concluded (Hughes et al., 2017). Instead, those stressors in smart snails lead to an inability to retrieve a formed memory (i.e. a memory retrieval block). That is, a non-declarative memory formed but could not be retrieved because of a stressful state. We believe this is the first instance of this to be demonstrated in an invertebrate model system. Thus, a cost of being smart is not an inability to form memory but rather the inability to retrieve it.

A scene that plays out at the end of each academic year involving stress and memory is the 'I don't know the correct answer during the test; but as soon as I hand in the paper, I remember the answer'. The question becomes: why is there an obstruction of the memory retrieval process? It has been demonstrated repeatedly that stress exposure, whether occurring before the 'fact' is learned and committed to memory (possibly by cramming the night before the exam) or before the attempt to retrieve the memory (i.e. the exam in a large gym-like room), may significantly impair the retrieval of the information that has been successfully committed to memory (Buchanan et al., 2006; De Quervain et al., 1998, 2000; Roozendaal, 2003; Sandi and Pinelo-Nava, 2007; Smeets et al., 2008; Wolf, 2017; Wolf and Kluge, 2017). In the scenario just presented, it is the retrieval of a formed declarative memory that is being blocked. Here, we hypothesize that it is also possible to block retrieval of a non-declarative memory by stressors that are perceived before the learning event in an invertebrate model system.

Lymnaea stagnalis is the first invertebrate species in which significant naturally occurring variability in the ability to form long-term memory (LTM, i.e. cognitive ability) has been identified among naturally occurring populations at both the behavioural and neuronal levels (Orr et al., 2009; Dalesman et al., 2011). The classification of *L. stagnalis* strains as smart, average or below average is operationally defined. Thus, a smart strain forms LTM with a single 0.5 h training session (Orr et al., 2009), an average strain requires two 0.5 h sessions and a below average strain requires four 45 min sessions to produce LTM (Rothwell and Lukowiak, 2019).

The specific cognitive ability of each strain is heritable (i.e. off spring of smart snails grown in the lab are smart) but can be altered by local environmental conditions (Dalesman et al., 2011; Rothwell et al., 2018). At the nervous system level, the smart snails differ from average snails in the excitability of an identified neuron, RPeD1 (Braun et al., 2012), which is a necessary site of LTM formation (Scheibenstock et al., 2002).

In previous work, we demonstrated strain-specific differences in the effects of emotional stressors (e.g. food deprivation coupled with smelling an inaccessible food substance) on memory formation and reconsolidation (Hughes et al., 2016, 2017). Those stressors had significantly different effects on memory formation depending on whether the average or smart snail phenotype was used. Based on those data, it was hypothesized that a cost of being smart was less resilience to stress, such that certain combinations of stressors blocked the ability to form LTM in smart but not in average snails (Hughes et al., 2017). Those results were thought to be consistent with the Yerkes–Dotson/Hebb law (Hebb, 1955; Ito et al., 2015), with the hypothesis that smart snails are more easily stressed than are average snails. We (Hughes et al., 2016) further showed that only combinations of stressors that created an 'emotional memory' were

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susceptible to disruption by the synthetic beta-blocker propranolol following reactivation (i.e. memory reconsolidation). More recently, it was shown that propranolol could also disrupt the consolidation of emotional memory in average *L. stagnalis* (Shymansky et al., 2018). In mammalian systems it has been shown that injection of propranolol reversed the blocking effect that certain stressors had on memory retrieval (De Quervain et al., 2007a,b, 2017). Thus, it seemed appropriate to ask whether propranolol would have an effect on memory formation or retrieval in smart snails subjected to the combination of stressors that cause emotional memories and block memory formation in smart snails.

We hypothesized that stressors leading to emotional memories in smart snails do not block LTM formation but instead obstruct the ability to retrieve the formed memory. That is, memory is formed but cannot be recalled and the injection of propranolol before the memory test session enables the memory to become retrievable.

MATERIALS AND METHODS

Snails

The strain of *Lymnaea stagnalis* used in this study was obtained from Whitesand Lake (WSL; 51°46′12.45″N, 103°21′14.16″W), approximately 250 km east of Saskatoon, SK, Canada. This strain of snails has been shown to possess both the smart and predator-experienced (to a crayfish predator) phenotypes. Snails were collected there and then kept in home aquaria in the Lukowiak laboratory at the University of Calgary for approximately 2 weeks before being used for experimentation. The aquaria contained oxygenated artificial pond water (PW: 0.25 g l^{-1} Instant Ocean, Spectrum Brands, Madison, WI, USA) supplemented with 0.34 g l^{-1} $CaSO_4$ (Sigma-Aldrich, St Louis, MO, USA) at a room temperature of 20°C on a light:dark cycle of 16 h:8 h, which approximates summer hours. Romaine lettuce was provided *ad libitum*. A total of 196 naive WSL smart snails were used in this study. It is important to note that a snail was only used in an experiment once.

Operant conditioning

Each snail was labelled 24 h prior to the training session. Snails were placed in a 1 l beaker filled with 500 ml of PW made hypoxic (<0.1 ml O_2 l^{-1}) by bubbling N_2 gas through the water for 20 min prior to a training (TS) or memory test (MT) session. Animals were allowed to acclimate for 10 min in the beaker prior to the initiation of the TS or MT session. Here, we used only a single 0.5 h TS and then tested for memory (MT) at specified times (e.g. 24 or 72 h after the TS). During the 0.5 h TS or MT session, a tactile stimulus (‘poke’) was applied to the edge of the pneumostome each time a snail attempted to open it. This causes the pneumostome to close and does not harm the snail. The number of pokes was recorded for each snail. This same procedure was performed for all TS and MT sessions.

We operationally define LTM in these experiments, using a smart snail strain, as significantly fewer attempted pneumostome openings performed during the 0.5 h MT memory test than during the single 0.5 h TS (Lukowiak et al., 1996; Hughes et al., 2017). In Figs 3 and 5, snails were tested twice for memory (i.e. MT1 and MT-sal or MT-prop). Thus, in these snails they were stimulated three times: TS, MT1 and MT-sal or MT-prop. MT1 occurred before an injection of saline or propranolol and MT-sal or MT-prop occurred after the injection of saline or propranolol. In all other figures, snails received a TS and a single MT at the specified time after the TS.

Stressful stimuli

We used two combinations of stressors that cause formation of an emotional LTM in average snails: (1) crayfish effluent (CE) plus immersion for 30 s in a 25 mmol l^{-1} potassium chloride (KCl) bath (KCl+CE); and (2) 3 days food deprivation (FD) combined with a carrot odour (CO; FD+CO; Shymansky et al., 2018). The stressors were applied just prior to the single 0.5 h TS. The stressors were not present in the MTs. All TSs and MTs were performed in hypoxic PW.

KCl+CE

KCl exposure is noxious to snails, eliciting the whole-body withdrawal response. A 30 s exposure to 25 mmol l^{-1} KCl immediately before the TS has previously been shown in average snails to enhance LTM formation (Martens et al., 2006), while in smart snails by itself it does not alter LTM formation (Hughes et al., 2017). CE enhances LTM formation when average snails are trained in it (Orr and Lukowiak, 2008), but its effect on smart snails is not completely clear. Following the procedure outlined in the Hughes et al. (2017) study, we combined these two stressors. That is, the WSL snails first received the KCl bath and then were immediately trained in CE. This combination of stressors appeared to block LTM in smart snails (Hughes et al., 2017).

FD+CO

FD acts as an environmentally relevant stressor (Hughes et al., 2017). Previously it was shown that a 3 day FD combined with CO was sufficient to create an emotional memory in average *L. stagnalis* (Hughes et al., 2016; Shymansky et al., 2018). The 3 day FD snails are exposed to CO without feeding, just prior to training them in hypoxic PW (i.e. carrot odour not present). This was done through an apparatus that bubbles eumoxic air (6 ml O_2 l^{-1}) through blended carrots placed in a sealed flask, while simultaneously diverting the carrot-scented air from the sealed flask into a beaker containing PW and the snails.

Drug exposure

Lymnaea saline consists of the following dissolved in distilled water: 51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 5.0 mmol l^{-1} $MgCl_2$, 1.5 mmol l^{-1} $CaCl_2$ and 5.0 mmol l^{-1} Hepes, pH 7.9–8.l. (±)-Propranolol hydrochloride (TLC) powder was obtained from Sigma-Aldrich. The concentration of propranolol used is consistent with the published literature (Hughes et al., 2016). Immediately prior to injection (propranolol or saline), snails were placed in an ice bath for 5 min in order to anaesthetize them. Propranolol-treated snails were injected into their foot with 0.1 ml of 50 µmol l^{-1} 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 TS. If injections were done prior to the TS, snails were returned to their eumoxic (6 ml O_2 l^{-1}) home aquaria for 1 h after injection to recover before undergoing the 0.5 h TS. If injections were performed following a TS or MT1, snails were simply placed back into their eumoxic home aquaria and remained there until the memory test session. Injection of propranolol at the concentration used here does not affect homeostatic breathing behaviour in *L. stagnalis* (Hughes et al., 2016).

Statistical analyses

Statistical tests were done using Prism 8 software for the Mac OS 10.15 system. Data were first tested for normal distribution using the using the Anderson–Darling (AD) test. If the data were distributed ‘normally’ we do not report the results of the AD test and they were analysed using a mixed-effect model (REML) analysis followed by a Tukey’s *post hoc* test to determine whether there were significant differences in the number of openings between the sessions. If the data were not distributed ‘normally’, we report the outcome of the

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AD test and used the Kruskal–Wallis test, followed by a Dunn's multiple comparisons *post hoc* test. Data plotted are the mean and the s.e.m. Significance was set at $P<0.05$.

RESULTS

We first used a cohort of 46 naive, freshly collected WSL snails (Fig. 1), and performed the series of experiments described below. The snails were divided into two main groups. The first group ($n=15$) received the single 0.5 h training session in PW (TS-PW) while the second group ($n=31$) received the single 0.5 h training session in CE (TS-CE), but memory was tested in PW. In both groups, snails were then randomly chosen to be tested for memory (MT) either 24 or 72 h after the TS (MT-PW and MT-CE).

When we tested these data for normal distribution using the AD test, we found that neither the 24 h MT-PW nor the TS-CE datasets were normally distributed ($A^2=0.8520$, $P=0.0151$ for 24 h MT-PW and $A^2=0.7419$, $P=0.0477$ for TS-CE). Thus, we performed a Kruskal–Wallis (KW) test on the dataset (KW statistic=35.25, $P<0.0001$). We then compared the memory tests, using Dunn's multiple comparisons *post hoc* test. We found the following. (1) The number of attempted pneumostome openings in the 24 h MT-PW group ($n=8$) was significantly lower than the number in the TS-PW group (mean rank difference=41.66, $P=0.0053$). (2) When the remaining snails from the TS-PW cohort were tested ($n=7$) for memory 72 h after the TS (72 h MT-PW), the number of attempted openings was not statistically different from that in the TS-PW group (mean rank difference=2.176, $P>0.9999$) but was statistically greater than that in the 24 h MT-PW group (mean rank difference=−39.48, $P=0.0626$). Thus, in these WSL snails, the single 0.5 h TS does not cause the LTM phenotype to be observed when tested 72 h later.

For the TS-CE cohort, we found the following. (1) When a randomly chosen subset of these snails ($n=19$) was tested for LTM 24 h later (24 h MT-CE), the number of attempted pneumostome openings was significantly lower than the number of attempted openings in TS-CE (mean rank difference=12.63, $P=0.0476$). (2) For another subset of snails ($n=13$) tested for LTM 72 h later (72 h MT-CE), the number of attempted openings was significantly lower than that in the TS-CE group (mean rank difference=19.83, $P=0.0035$). (3) There was no significant difference in the number of attempted openings in the 72 h MT-CE versus the 24 h MT-CE session (mean rank difference=7.204, $P=0.8313$).

We further compared the data between the two groups (i.e. those in the TS-PW and TS-CE group) and found the following. (1) The number of attempted pneumostome openings in TS-PW versus TS-CE was not significantly different (mean rank difference=18.97, $P=0.3530$). (2) There was no significant difference between the number of attempted openings in the 24 h MT session between snails trained in PW and CE (mean rank difference=−4.230, $P>0.9999$). (3) There was a significant difference in the response exhibited by snails trained in CE versus PW in the 72 h memory test session (mean rank difference=44.77, $P=0.0061$).

Together these data show that: (1) the WSL snails collected in the summer of 2019 exhibit the smart phenotype, i.e. they have the ability to form a 24 h LTM when trained with a single 0.5 h TS in PW but they do not exhibit the LTM phenotype when tested 72 h after the TS; and (2) training the snails in CE does not alter their ability to form a 24 h LTM when tested in PW and causes memory to be present when tested 72 h after the training session.

It has previously been shown (Hughes et al., 2017) in smart snails that the combination of two stressors (a 25 mmol l^{-1} KCl bath followed immediately by training in CE; KCl+CE) blocked LTM formation. Using a cohort of 54 naive smart WSL snails (Fig. 2), we determined the possible effects of pre-injection of propranolol or saline on the ability of these smart snails to form LTM following a single 0.5 h training session in PW or using the KCl+CE procedure. As a first control for subsequent experiments, we determined the effect, if any, of a propranolol pre-injection on the ability of WSL smart snails to form LTM following a single 0.5 h TS in PW. We injected WSL smart snails ($n=10$) with propranolol 1 h before training with a single 0.5 h TS in PW (prop-TS) and then tested for

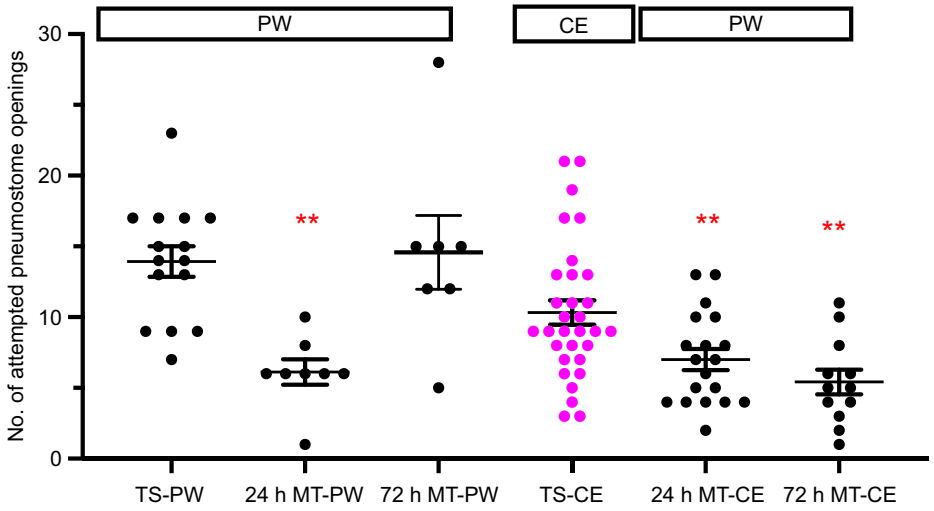

Fig. 1. Whitesand Lake (WSL) snails collected in the summer of 2019 are 'smart' and predator detection extends memory persistence. A total of 46 naive WSL snails were used. A cohort of 15 of these snails received a single 0.5 h training session in pond water (TS-PW, black dots) and, 24 h later, 8 of these snails were randomly chosen to be tested (memory test, MT) for long-term memory (LTM; 24 h MT-PW). The remaining 7 snails were tested for LTM 72 h after the TS (72 h MT-PW). A second naive cohort of 31 WSL snails received a single 0.5 h TS in crayfish effluent (TS-CE, magenta dots) and 19 of these snails were randomly chosen to be tested for LTM 24 h later (24 h MT-CE); the remaining 12 snails were tested for LTM 72 h after the TS (72 h MT-CE). All memory tests for these 31 snails were conducted in PW. The important conclusions from these data are: (1) these are smart snails; (2) WSL snails trained in PW with a single 0.5 h TS form LTM that persists for 24 h but not 72 h; (3) however, when the WSL snails are trained in CE, they form both a 24 h and a 72 h LTM. Thus, training in CE does not block LTM formation in WSL smart snails but actually extends the duration of LTM. **Significantly different from TS-PW; significantly different from TS-CE.

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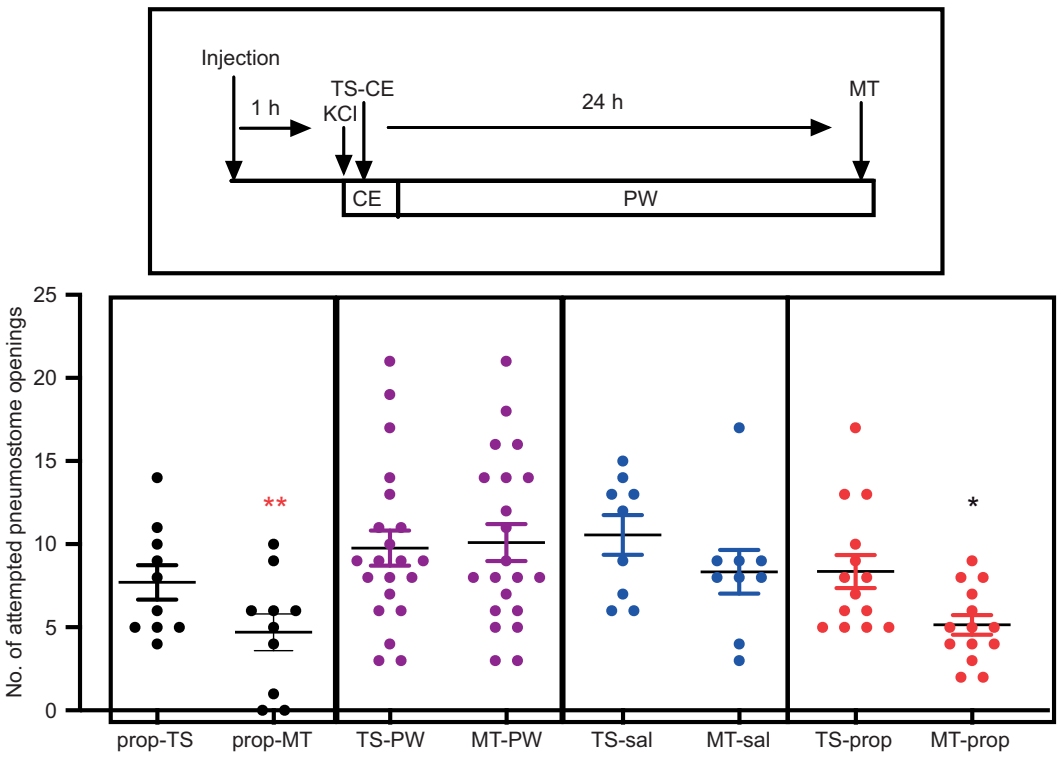

Fig. 2. Pre-injection of propranolol mitigates the memory-blocking effect of the KCl+CE training procedure. A time line of the experiments is shown above the data points. A total of 54 naive WSL snails were used. Each snail was randomly assigned to one of four groups and received a single training session (TS) and a single memory test session (MT) 24 h after the TS. In this graph, the TS and the MT for each of the four cohorts are grouped together (e.g. prop-TS and prop-MT) for easier viewing. One group was trained in just PW and received a propranolol injection 1 h beforehand (prop-TS, n=10, black dots); the second group (TS-PW, n=21, magenta dots) did not receive any injection but was trained using the KCl+CE procedure; the third group (TS-sal, n=9, blue dots) received a pre-injection of saline 1 h before training with the KCl+CE procedure; the fourth group (TS-prop, n=14, red dots) received a pre-injection of propranolol 1 h before training with the KCl+CE procedure. In all four groups, memory (MT) was tested 24 h later in PW. The important conclusions from these data are: (1) propranolol injection before training in PW in the WSL smart snails does not prevent LTM from forming; and (2) WSL snails subjected to the KCl+CE training procedure do not form LTM unless they receive a propranolol injection before training. Thus, propranolol mitigates the blocking effects of the KCl+CE stressors on memory formation in WSL smart snails. **Significantly different from prop-TS; *significantly different from TS-prop.

memory 24 h later (prop-MT). We then repeated the Hughes et al. (2017) experiment (TS-PW and MT-PW; n=21); in this group, no injection took place. In two other groups, snails were injected with saline (TS-sal and MT-sal; n=9) or propranolol (TS-prop and MT-prop; n=14) 1 h before exposure to the KCl+CE stressors. In all snails, the MT occurred in PW 24 h after the TS.

A mixed-effects model (REML) ($F_{3.823,43.69}$=3.729; P=0.0117) followed by a Tukey's *post hoc* test on each of the memory tests was performed on these data. To summarize the important points: (1) propranolol pre-injection does not alter the ability of smart snails to form LTM when trained in PW, i.e. the number of attempted openings in prop-MT was significantly lower than that in prop-TS (P=0.0014); (2) LTM does not form in smart snails subjected to the KCl+CE combination of stressors in the absence of injection, i.e. the number of attempted pneumostome openings in MT-PW was not significantly different from that in TS-PW (P>0.9999); (3) LTM does not form in snails subjected to the KCl+CE combination and a saline pre-injection, i.e. the number of attempted pneumostome openings in MT-sal was not significantly different from that in TS-sal (P=0.8486); and (4) LTM forms in snails subjected to the KCl+CE combination and a propranolol pre-injection, i.e. the number of attempted pneumostome openings in MT-prop was statistically lower than that in TS-prop (P=0.0424). Other comparisons made from this analysis show that: (1) MT-prop was not different from prop-MT (P=0.9998) but was different from MT-PW (P=0.0361) and MT-sal (P=0.0412); and (2) MT-sal was not different from MT-PW (P=0.9814). Together, these data allow us to conclude that propranolol has on its own no blocking effect of LTM formation in smart snails trained in pond water but that propranolol pre-injection alleviates the negative effects of the KCl+CE training procedure on LTM formation.

Having shown that a propranolol pre-injection before the single 0.5 h TS mitigated the effect of the combined stressors (KCl+CE) in smart snails, we next determined whether propranolol altered the memory retrieval process following this combination of stressors. That is, was LTM formed but occluded by the effect of the combined stressors on the retrieval process?

A naive cohort of smart snails (Fig. 3, n=29) was subjected to the combination of the two stressors (KCl+CE) and then trained with a single 0.5 h TS. We then tested for memory 24 h later (MT1) in PW. Following an additional 24 h period, these snails were injected with either saline (n=13) or propranolol (n=16) and then memory was tested again 1 h later (MT-sal or MT-prop) in PW. Thus, all 29 snails in this experiment received the TS, a memory test 24 h after the TS (MT1) and a second memory test session (MT) 25 h after MT1. A mixed-effects model (REML) analysis ($F_{2.680,49.14}$=4.899; P=0.0062) followed by Tukey's *post hoc* tests on each of the memory tests indicated the following: (1) LTM was not present in MT1, i.e. the number of attempted openings in MT1 was not statistically different from that in TS (P=0.9621); (2) LTM was not present in the saline-injected cohort as the number of attempted pneumostome openings in MT-sal was not significantly different

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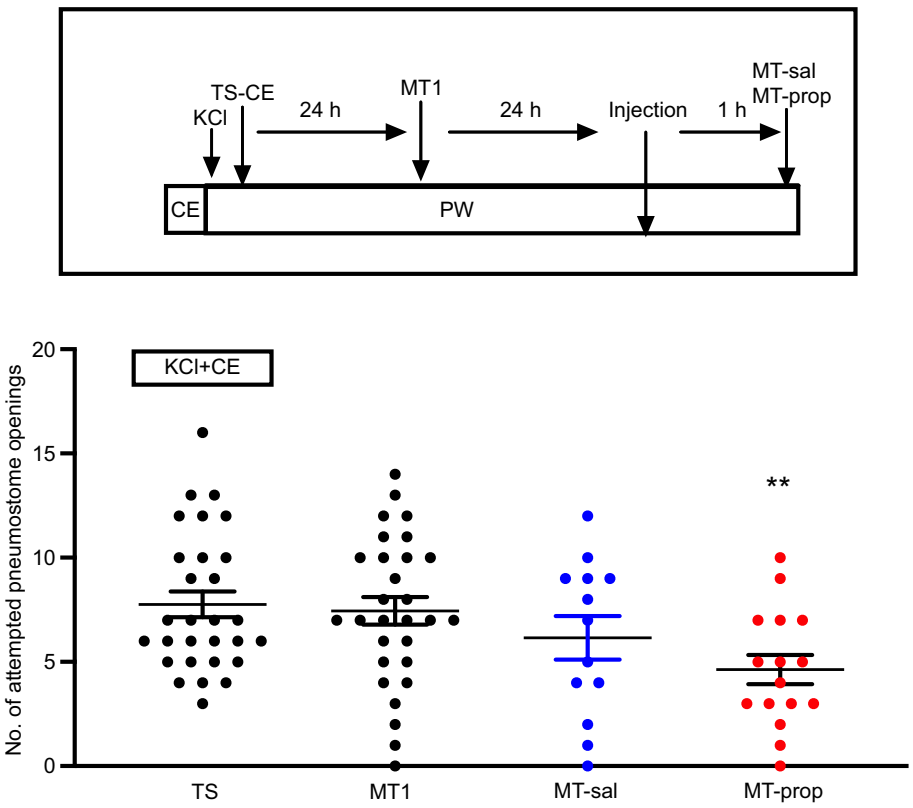

Fig. 3. Propranolol injection but not saline injection before a memory retrieval session allows the memory to be retrievable. A time line of the experiment is shown above the data points. A total of 29 naive WSL smart snails were used. Each snail was trained using the KCl+CE procedure (TS) and then tested for memory 24 h later in PW (MT1). The snails were then randomly divided into two groups. One group (MT2-sal, *n*=13, blue dots) received a saline injection 1 h before a second memory test while the remaining snails (MT2-prop, *n*=16, red dots) received a propranolol injection 1 h before a second memory test. Thus, each snail received the TS, a first memory test (MT1) and then a second memory test (MT-sal or MT-prop). The important conclusion from these data is that propranolol injection 1 h before a second memory test allows memory retrieval, while saline injection does not. **Significantly different from TS, MT1 and MT2-sal.

from that in TS (P=0.2054) or MT1 (P=0.6396); (3) LTM was present in the propranolol-injected cohort as the number of attempted pneumostome openings in MT-prop was statistically lower than that in TS (P=0.0141) and MT1 (P=0.0337); and (4) saline injection did not allow memory retrieval as the number of attempted pneumostome openings in MT-prop was significantly lower than that in MT-sal (P=0.0453). We conclude that in these smart snails subjected to the KCl+CE training procedure, LTM forms but its retrieval is blocked. The retrieval block can be relieved by injecting propranolol 1 h before the memory test. Thus, following the propranolol injection, the LTM phenotype was revealed.

A second combination of two stressors (FD+CO) has previously been shown to cause an emotional memory in average snails but obstruct LTM formation in smart snails (Hughes et al., 2016, 2017; Shymansky et al., 2018). Here, we both confirmed these earlier findings and then determined whether a saline or propranolol pre-injection before the TS altered the memory phenotype (Fig. 4). We used 28 naive smart WSL snails and divided them into three cohorts: control (TS-control), pre-injection of saline (TS-sal) and pre-injection of propranolol (TS-prop). All snails were food deprived for 3 days and then challenged with the carrot odour (i.e. they are hungry, smell carrot but there is no food to eat) just before the single 0.5 h TS. A mixed-effects model (REML) analysis ($F_{2.432,18.49}$=4.354; P=0.0226) followed by Tukey's *post hoc* test on each of the memory tests indicated the following. (1) In TS-control (n=13), the snails did not receive any injection. When memory was tested 24 h later (MT-control), there was no significant difference in the number of attempted pneumostome openings in these two sessions (P=0.4961). (2) In the group pre-injected with saline (n=8) 1 h before training, there was a significant difference between the memory test and training session; but it was a significant increase in the number of attempted openings in MT-sal compared with TS-sal (P=0.0394). This means that LTM did not form. (3) Finally, in the cohort that received the propranolol pre-injection 1 h before the training session (n=7) and was tested for LTM 24 h later, there was a significant decrease in the number of attempted openings in MT-prop versus TS-prop (P=0.0102). It is important to further note that the number of attempted openings in MT-prop was significantly lower than that in both MT-sal (P=0.0465) and MT-control (P=0.0372). Finally, the number of attempted openings in TS in the three groups was not different. Together, these data allow us to conclude that the pre-injection of propranolol 1 h before training with the FD+CO procedure mitigates the stressors' effect on the snails, such that LTM is now observable.

Our next task was to determine whether these two stressors (FD+CO) also blocked the ability of the snails to retrieve a formed LTM. In this dataset, we used WSL snail data from both the 2018 and 2019 summers. We had performed our initial studies ultimately using 17 snails in the summer of 2018. We combined those data with the data obtained in 2019 (n=22). Thus, we subjected a naive cohort of 39 smart WSL snails (Fig. 5) to those two stressors. These snails were then trained (TS) and tested for LTM 24 h later (MT1). Then, 24 h later, the snails were injected with either saline (n=19, MT-sal) or propranolol (n=20, MT-prop) and again tested for LTM 1 h later, to see whether this injection unmasked LTM. Thus, all 39 snails were tested for LTM twice (i.e. MT1 and MT-sal or MT-prop). When we tested these data for normal distribution using the AD test, we found that neither the TS nor the MT1 dataset was normally distributed (A^2=1.622, P=0.0003 for TS and A^2=0.8842, P=0.0215 for MT1). Thus, we performed a KW test on the dataset (KW statistic=18.91, P=0.0003). We then compared the memory tests, using Dunn's multiple comparisons *post-hoc* test. We found the following. (1) The LTM phenotype was not present in MT1; that is, there was no significant difference in the number of attempted pneumostome openings in TS compared with MT1 (mean rank

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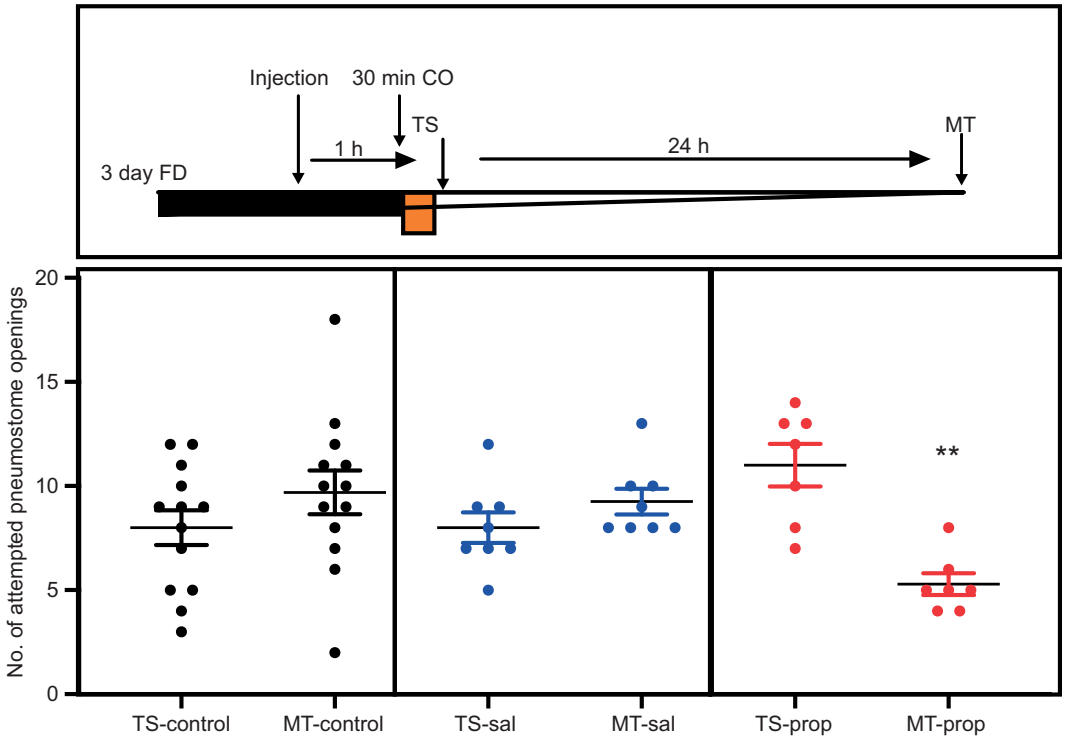

Fig. 4. Propranolol pre-injection mitigates the memory blocking effect of the FD+CO training procedure in smart snails. A time line of the experiment is shown above the data points. A cohort of 28 WSL naive smart snails was used in the experiment. The snails were randomly divided up into three groups: control (TS-control, *n*=13, black dots), a saline injection group (TS-sal, *n*=8, blue dots) and a propranolol injection group (TS-prop, *n*=7, red dots). All snails were subjected to the food deprivation+carrot odour (FD+CO) procedure before training (TS) and were then tested for memory once (MT). The important conclusion from these data is that propranolol injection before the FD+CO training procedure allows the memory phenotype to be expressed. **Significantly different from TS-prop.

difference=8.795, $P>0.9999$). (2) Likewise, the memory phenotype was not present in the saline-injected snails (MT-sal), i.e. the number of openings in MT-sal was not significantly different from that in either TS (mean rank difference=10.02, $P>0.9999$) or MT1 (mean rank difference=1.221, $P>0.9999$). (3) The memory phenotype was present in the MT-prop group, i.e. there was a significant difference between the number of openings in MT-prop compared with TS (mean rank difference=39.93, $P=0.0001$) and with MT1 (mean rank difference=31.14, $P=0.0049$). (4) Finally, the number of openings in MT-prop is significantly lower than the number in MT-sal (mean rank difference=29.92; $P=0.0347$). Thus, the propranolol injection enables LTM to be retrievable.

DISCUSSION

In the WSL strain (i.e. a smart, predator-experienced snail strain; Dalesman et al., 2011; Shymansky et al., 2018), it was previously concluded that encountering two different combinations of stressors (KCl+CE and FD+CO) blocked LTM formation (Hughes et al., 2017). Those authors suggested this was a cost of being smart. However, our new data using the same strain of freshly collected snails show that the conclusion reached in the Hughes et al. (2017) paper regarding a cost of being smart was incorrect. As shown here, an actual cost of being smart is not the inability to form LTM but rather an inability to retrieve the memory. Using the KCl+CE or the FD+CO training procedures in smart snails impeded the memory retrieval process. Propranolol, whether injected into snails before training or just before a memory test session, allowed the memory to become retrievable. Those two training procedures cause an emotional memory in average snails (Hughes et al., 2016). Thus, using procedures in smart snails that result in emotional memory formation leads to blockage of the memory retrieval process. However, the neuronal mechanism(s) underlying retrieval block is overcome by propranolol. We believe this is the first demonstration that a memory retrieval block of a non-declarative memory occurs in an invertebrate model system.

Our conclusion that smart snails make LTM following that specific combination of stressors but cannot access it bears some basic similarity to the exam scenario constructed in the Introduction. There, a student, presumably under stress during the learning process (i.e. before the exam, maybe even the night before!), taking a final exam was not able to correctly recall a memory during the exam but was able to do so after handing in the paper (i.e. removal of the stress of taking the test).

In the above scenario, the information needed to be recalled was presumably a hippocampal-dependent, declarative, episodic memory, whereas, in the data presented, here a non-declarative memory was in a non-retrievable state because of a specific combination of stressors. The majority of the literature on stress-induced retrieval blockade has focused on hippocampal declarative memory (Lupien and Lepage, 2001; Lupien et al., 2007). However, memory in rodents and humans is not a single entity (Milner et al., 1998). Memory is composed of multiple hippocampal and non-hippocampal memory systems that are all potentially altered by stressful stimuli (Squire, 2006; Squire and Zola, 1996). Each of the different phases of memory (i.e. acquisition, consolidation, recall and reconsolidation) can be differentially altered by stress (Sandi and Pinelo-Nava, 2007; Roozendaal et al., 2010). One of the non-hippocampal memory systems that has received increased attention in recent years is the striatum. For a long time, the striatum was

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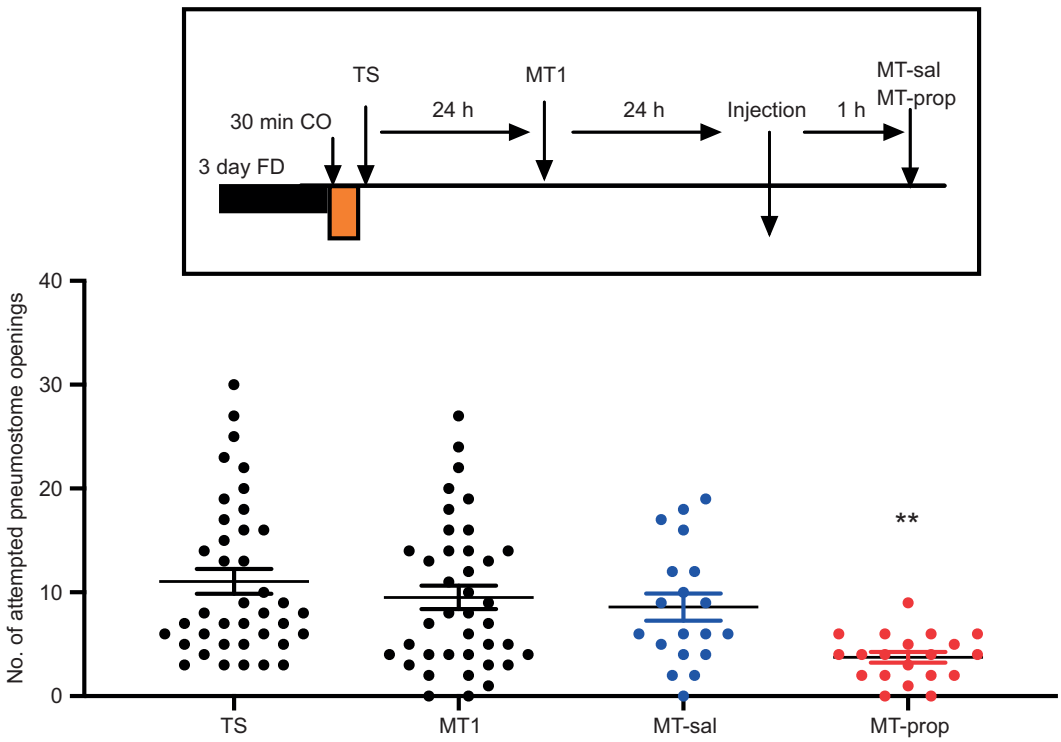

Fig. 5. Propranolol injection but not saline injection before a memory retrieval session removes the retrieval block. A time line of the experiment is shown above the data points. A cohort of 39 naive WSL smart snails was used. Each snail was trained using the FD+CO procedure (TS) and then tested 24 h later in PW (MT1). The snails were then randomly divided into two groups. One group (MT-sal, *n*=19, blue dots) received a saline injection 1 h before a second memory test while the remaining snails (MT-prop, *n*=20, red dots) received a propranolol injection 1 h before a second memory test. Thus, each snail received a training session, a 24 h memory test and then a second memory test. The important conclusion from these data is that propranolol injection 1 h before a second memory test allows memory retrieval, while saline injection does not. **Significantly different from TS, MT1 and MT-sal.

considered primarily a motor area but there is by now a broad consensus that it also has memory functions (Graybiel, 2008; Packard and Knowlton, 2002; White, 1997). As was recently shown (Atsak et al., 2016), a stimulus (an injection of either corticosterone or just saline) that raised the corticosterone level impaired the retrieval of a stimulus–response (S–R) memory. Thus, in that study, the occluding effects of stress on memory retrieval of a non-hippocampal memory were shown. Likewise, here we also show that retrieval of a non-declarative memory can be occluded by specific stressors in smart snails.

Declarative memories, such as hippocampal memory, are stored in neural circuits that can be different from the circuit where learning and the initial memory consolidation process occurred (Milner et al., 1998). This was famously illustrated in the case of the patient HM, following the bi-lateral removal of temporal lobe structures to treat intractable seizure activity (Scoville and Milner, 1957). Following this surgery, he could not form a new declarative memory but could remember declarative memories made before the surgery (Milner et al., 1998). Consequently, those accessible declarative memories must have been stored in different brain areas other than the temporal lobes. In contrast, it was generally assumed in the literature that non-declarative memories were stored in the same neural circuit that mediated the behaviour being studied (Milner et al., 1998; Lukowiak et al., 2003). It was thought that the ability to activate the neural circuit mediating the behaviour meant that one had access to the circuit where the memory was stored. Hence, unlike the situation regarding a hippocampus-dependent declarative memory, if a non-declarative memory in a snail was not present, it was assumed it had not been formed or had been forgotten.

Consequently, it was thought that there were a number of advantages of studying a non-declarative memory, such as occurs following operant conditioning of aerial respiration in *L. stagnalis*. First, as just discussed, if memory formed, it would be stored within the neural circuit that mediated the behaviour (i.e. aerial respiration). Data consistent with this notion were obtained by showing that a neuron, RPeD1, in the three-cell neural circuit that drives this behaviour (Syed et al., 1990, 1992), was a necessary site for LTM formation (Scheibenstock et al., 2002). Thus, LTM was shown to be stored in this circuit. Further, it was shown that differences in cognitive ability between different strains (average versus smart) of *L. stagnalis* (i.e. ease of forming LTM and its longer duration following operant conditioning training; Dalesman et al., 2011) have been found and the behavioural differences are reflected in RPeD1's activity in the naive state (Braun et al., 2012). Second, if memory was not present when tested, it was taken to mean that the training procedure used was insufficient to cause memory to be formed. For example, in average snails, there is a requirement for at least two 0.5 h training sessions separated by a 1 h interval to enable LTM formation (Smyth et al., 2002). Consequently, a single 0.5 h training session only resulted in a memory persisting less than 3 h and did not require altered gene activity (Sangha et al., 2003). In contrast, if memory was not apparent following training it could also indicate a context-specific memory (i.e. a different context was employed for testing the memory; Haney and Lukowiak, 2001), or possibly the memory was forgotten (McComb et al., 2002). Here, however, we showed that there is another reason why memory may not be observed: the ability to retrieve it was occluded. Naively, we had previously thought that retrieval block could not occur for a

non-declarative memory because if it was possible to activate the behaviour we would have access to the storage of that memory. We are presently attempting to discover how this retrieval occlusion occurs at the neural circuit level. Presumably, neurons that are not integral members of the circuit that drives aerial respiratory behaviour are somehow involved in altering the 'state' of the circuit such that memory is not retrievable.

Here, we showed that in smart snails two combinations of stressors, KCl+CE and FD+CO, lead to an inability to retrieve a memory. We define stress following the definition put forward by Kim and Diamond (2002) as a condition that alters the physiological or psychological homeostasis of an organism. The so-called Yerkes–Dodson/Hebb law, which attempted to explain the effect of stress on learning and memory, posits that the ability to form or recall a memory differs with the perception of stress (Hebb, 1955). Stress, occurring at any point from the acquisition phase to the consolidation phase to the retrieval phase, alters memory formation, storage and retrieval properties (Roozendaal, 2002; Sandi and Pinelo-Nava, 2007). Thus, whether the stress occurs before learning (i.e. acquisition), before or immediately after the memory consolidation process, or before the retrieval process will impact memory formation and memory recall (Sandi and Pinelo-Nava, 2007).

The combination of stressors used here that obstruct memory retrieval has previously been shown in average *L. stagnalis* to both enhance LTM formation and cause the formation of an emotional memory (Hughes et al., 2016; Shymansky et al., 2018). It may be surprising to some to discuss the concept of an emotional memory in a snail. However, as Ledoux (2012) suggested, neuronal 'survival circuits' that mediate the responses of animals, such as snails, to predator detection may underlie emotional memory. Other authors such as Damasio (2010) and Darwin (1872) posited that the response exhibited by invertebrates such as insects to a predator is homologous to terror states (i.e. emotion) in humans. Interestingly, the Oxford English Dictionary's definition of emotion is 'an agitation of mind or instinctive feeling (e.g. fear) deriving from one's circumstances (i.e. experienced environment)'. Thus, there is good reason to accept the premise that a snail can have an emotional memory. The emotional memories in *L. stagnalis* have been shown here and in previous publications (Hughes et al., 2016; Shymansky et al., 2018) to be modified by propranolol.

Propranolol has been shown to protect the impairing effects of stress on memory retrieval (De Quervain et al., 2007a,b). It is also clear in the mammalian literature that emotional memories and their retrieval are more susceptible to stress, as is the process of reconsolidation (Delorenzi et al., 2014; Larrosa et al., 2017). In *L. stagnalis*, a similar situation exists as the reconsolidation of emotional memories is blocked by propranolol, whereas reconsolidation of a non-emotional memory is not (Hughes et al., 2016). In addition, propranolol only alters the consolidation process of emotional memories in *L. stagnalis* while it has little or no effect on non-emotional memories (Shymansky et al., 2018). Our data on smart snails show that only stressors that cause emotional memory formation lead to retrieval block, which is mitigated by the pre-injection of propranolol or the injection of propranolol just before a memory test session. Thus, our new findings add to our previous findings to demonstrate that in *L. stagnalis*, propranolol has the capability to alter four different phases (i.e. acquisition, consolidation, retrieval and reconsolidation) of emotional memory (Hughes et al., 2016; Shymansky et al., 2018; Swinton et al., 2019). Finally, we presently do not understand the neuronal basis for these differences in propranolol sensitivity between smart versus average snails in the consolidation, reconsolidation and retrieval processes between emotional versus non-emotional memory but that is the thrust of on-going research.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: T.S., C.S., K.L.; Methodology: T.S., K.L.; Validation: K.L.; Formal analysis: E.S., K.L.; Investigation: E.S., T.S., C.S., K.L.; Resources: K.L.; Data curation: E.S.; Writing - original draft: K.L.; Writing - review & editing: K.L.; Supervision: K.L.; Project administration: K.L.; Funding acquisition: K.L.

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