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RESEARCH ARTICLE

A thermal stressor, propranolol and long-term memory formation in freshly collected *Lymnaea*

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ABSTRACT

A heat stressor (1 h at 30°C) in *Lymnaea stagnalis* before operant conditioning training of aerial respiration is sufficient to enhance long-term memory (LTM) formation in 'average' cognitive ability, laboratory-reared, inbred snails. However, in freshly collected outbred snails, the same heat stressor blocks LTM formation in 'smart' cognitive phenotype but not in average cognitive phenotype strains. Here, we hypothesize that (1) preventing the stress associated with the heat stressor before training allows LTM to form in the smart phenotype strains; and (2) alleviating the stress before a memory recall session allows a formed LTM to be recalled in the smart phenotype strains. We found that an injection of propranolol, which mitigates the stressor, before snails experience the heat stressor enabled two strains of the smart phenotype snails to form LTM, consistent with our first hypothesis. However, the injection of propranolol before a memory test session did not alleviate a memory recall block in the smart phenotype snails. Thus, our second hypothesis was not supported. Therefore, smart cognitive phenotype snails encountering a heat stressor have an inability to form LTM, but this inability can be overcome by the pre-injection of propranolol.

KEY WORDS: Thermal stress, *Lymnaea*, Cognitive alteration, Stress

INTRODUCTION

The pond snail, *Lymnaea stagnalis*, is a successful model system used to study adaptive behaviours and how ecologically relevant stressors alter their cognitive abilities (Rivi et al., 2020; Lukowiak et al., 2014). A singular characteristic of the *Lymnaea* model system is the naturally occurring variability in specific populations (i.e. strains) in their cognitive abilities. Thus, unique *Lymnaea* strains can be classified as exhibiting the following cognitive phenotypes: 'smart', 'average' and 'below-average', based on their ability to form long-term memory (LTM) following operant conditioning of aerial respiratory behaviour (Lukowiak et al., 1996; Orr et al., 2009; Sunada et al., 2017; Totani et al., 2019; Rothwell and Lukowiak, 2019). A smart cognitive phenotype strain, such as the TC1 or WSL strain, forms LTM with a single 0.5 h training session (Orr et al., 2009; Hughes et al., 2017), whereas in an average cognitive phenotype strain, such as the TC2 or W-strain, LTM requires two 0.5 h sessions (Dodd et al., 2018). Finally, a below-average cognitive phenotype strain, such as the B-strain, requires four 45 min sessions to produce LTM (Rothwell et al., 2018). Ecologically relevant stressors that are capable of altering both LTM formation and the ability of the memory to be recalled are also dependent on the snail's specific cognitive ability (Lukowiak et al., 2003; 2010; Swinton et al., 2020). For example, certain stressors enhance LTM formation in average cognitive phenotype snails, but those same stressors create an inability to recall a formed LTM in smart cognitive phenotype snails (Hughes et al., 2017; Shymansky et al., 2018). The inability to recall a memory in smart cognitive phenotype snails was first thought to indicate that those stressors blocked LTM formation (Hughes et al., 2017). One of those stressors was the heat stressor (1 h at 30°C) stimulus (Teskey et al., 2012; Sunada et al., 2016). Subsequently, it was shown that many of the stressors examined in the Hughes et al. (2017) study actually did not block LTM formation in smart cognitive phenotype snails, but rather obstructed the ability of those snails to retrieve the memory (Swinton et al., 2020). The memory block in smart cognitive phenotype snails could be (1) prevented by an injection of propranolol before the snails experienced the stressor; or (2) alleviated by an injection of propranolol just before the memory recall session. However, in Swinton et al. (2020), only combinations of stressors (e.g. food deprivation+carrot odour) that create an emotional memory in *Lymnaea* (Hughes et al., 2016) were used, as it was hypothesized that propranolol was only effective at altering emotional memories (Hughes et al., 2016; Shymansky et al., 2018). Because the heat stressor was not considered in those studies to produce an emotional memory, the effect the heat stressor had on smart cognitive phenotype snails was not further examined. Here, we examine whether propranolol can prevent and/or alleviate the suppressive effect the heat stressor has on LTM formation or its recall in freshly collected smart cognitive phenotype snails.

Propranolol was first used by the Lukowiak laboratory (Hughes et al., 2016) to determine whether it was possible to differentiate memory formed by combinations of stressors to those formed by a single stressor. They found that memories created under conditions of a combination of stressors were susceptible to disruption by propranolol whereas others were not. Subsequently, it was demonstrated that propranolol disrupted the consolidation of emotional memories in *Lymnaea* (Shymansky et al., 2018). However, as noted above, the susceptibility of the thermal shock-induced enhancement to propranolol block was not investigated. Adamo (2008) pointed out that molluscs are unique in that they use both norepinephrine [i.e. noradrenaline (NA)] and octopamine (OA) as neurotransmitters/neuromodulators. Massarsky et al. (2011) posited that the OA and NA systems are homologous. We had hypothesized that propranolol brought about its blocking effect on LTM enhancement by its effect on OA receptors (Shymansky et al., 2018; Swinton et al., 2019; 2020). This hypothesis was supported by findings showing that OA was involved in mediating stress in molluscs (Fabbri and Capuzzo, 2010). In addition, the application

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of propranolol reversibly blocked the synaptic response elicited by tactile stimulation of the foot in *Lymnaea* (Anokhin et al., 1973). This synaptic response was thought to use either NA or OA as the transmitter. More recently, Samarova et al. (2005) showed in *Lymnaea* that propranolol reversibly blocked synaptic potentials elicited by both photic and tactile stimuli. Propranolol was also shown in a related snail (*Helisoma*) to block serotonin receptors, but it only did so at significantly higher concentrations than used here (Goldberg et al., 1994). Finally, we previously measured the OA content of the *Lymnaea stagnalis* central nervous system (Aonuma et al., 2017) and found it to be ∼21 pmol per brain. Thus, OA can play a role in modulating/mediating stress-related behaviours.

Temperature influences a large number of behavioural and physiological traits in many different ways in aquatic organisms (Cloyed et al., 2019). *Lymnaea* reside in northern Eurasia and North America, and are particularly sensitive to higher temperatures (Vaughn, 1953; Leicht et al., 2013; Salo et al., 2019). *Lymnaea* typically inhabit shallow, slow-moving streams, small ponds, or bays of larger lakes (Dillon, 2000), and experience large temperature shifts both seasonally and daily in summer. For example, in the Alberta and Saskatchewan ponds in which we collect *Lymnaea* (the TC1, TC2 and WSL strains), the snails experience temperatures at or lower than 4°C for at least 6 months of the year (i.e. winter). In the summer months, they may experience temperature fluctuations from 8°C to 27°C on a daily basis (K.L., unpublished data-log recordings). Moreover, the upper thermal limit of these *Lymnaea* is a few hours at 30°C (Vaughn, 1953; Salo et al., 2019).

Consequently, sudden changes in temperature are an acute stressor (Martens et al., 2006). We define stress as a condition that alters the physiological or psychological homeostasis of an organism (Kim and Diamond, 2002). Temperature effects on behaviour and physiology in *Lymnaea* should be viewed in relation to the Yerkes–Dodson/Hebb law (Yerkes and Dodson, 1908; Hebb, 1955; Ito et al., 2015). This law attempted to explain the effect of different levels of stress on learning and memory-forming performance. It posits that the ability to form or recall a memory differs with the perception of stress (Hebb, 1955). Thus, it could be that snails living in ponds experiencing large, naturally occurring temperature changes may respond differently from snails raised in the laboratory. Moreover, whether the stress occurs before learning (i.e. acquisition), before or immediately after the memory consolidation process, or before the retrieval process significantly impacts memory formation and/or its recall (Roozendaal, 2002; Sandi and Pinelo-Nava, 2007; Swinton et al., 2020).

Here, we test two hypotheses: (1) a pre-injection of propranolol (i.e. prevention strategy) before snails experience the heat shock stressor (HS) will allow the smart snails to form LTM; (2) an injection of propranolol just prior to a memory retrieval session (i.e. alleviation strategy) in smart snails that experienced the HS before training will enable the formed memory to be retrieved.

MATERIALS AND METHODS

Snails

Three strains of *Lymnaea stagnalis* (Linnaeus 1758), were used in these experiments. We used freshly collected smart cognitive phenotype snails from Alberta and Saskatchewan (the TC1 and WSL strains, respectively; Dodd et al., 2018). A third strain, TC2, is also from Alberta and possesses average cognitive ability (Braun et al., 2012). All three strains used (2.5–3.0 cm shell length) were maintained at room temperature (∼20°C) in the laboratory for at least 2 weeks after being collected in the spring and summer and had continuous access to romaine lettuce in their home eumoxic aquaria. The aquaria contained oxygenated artificial pond water [i.e. eumoxic pond water 6 ml O_2 l^{-1}, containing 0.25 g l^{-1} Instant Ocean (Spectrum Brands, Madison, WI, USA) and 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. It is important to note that a snail was only used once in an experiment.

Operant conditioning training

Operant conditioning of aerial respiration for the data presented here was conducted under hypoxic conditions as previously described (Lukowiak et al., 1996, 2000). Before the training session (TS) and memory test session (MT), 100% N_2 gas was vigorously bubbled into 500 ml pond water in order to create the hypoxic environment (<0.1 ml O_2 l^{-1}). This bubbling was maintained at a lower rate during all TSs and MTs. A 10 min acclimation period was given before each TS and MT to allow the animals to acclimate to this new hypoxic environment. Immediately prior to each TS and MT, all animals were gently pushed underwater in the test beaker. During the 0.5 h TS and MT, 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 causing the snail to retract into its shell. The number of pokes was recorded for each snail.

In this study, two different training protocols were employed to operantly condition aerial respiratory behaviour. The first was a single 0.5 h TS followed by a MT 24 h later. This procedure produces LTM in smart cognitive phenotype snails (e.g. TC1 strain) but not average cognitive phenotype strains (Orr and Lukowiak, 2008; Orr et al., 2009; Dalesman et al., 2011). The second procedure utilized the 0.5 h TS followed 24 h later by a memory test (MT1). This was followed 24 h later by an injection of propranolol and, 1 h later, a second memory test (MT2). This was the successful procedure used in the Swinton et al. (2020) study on propranolol's effects on memory retrieval.

We used an operational definition of LTM formation: the number of attempted pneumostome openings in the MT had to be significantly less than the number in the TS.

HS

A 1000 ml beaker filled with 500 ml pond water heated to and maintained at 30°C served as the apparatus used to deliver the HS. Up to 12 snails were kept in the beaker for the entirety of the 1 h heating period; this is referred to as the HS (Teskey et al., 2012).

Propranolol

We obtained ±-propranolol hydrochloride (TLC) powder from Sigma-Aldrich. The concentration of propranolol used here was as previously used (∼0.015 mg ml^{-1}; Hughes et al., 2016). Immediately prior to injection, snails were placed in an ice bath for 5 min to anesthetize them. Propranolol-treated snails were injected into their foot with 0.1 ml of 50 µmol l^{-1} propranolol dissolved in *Lymnaea* saline (*Lymnaea* saline composition: 24 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 2 mmol l^{-1} $MgCl_2$, 4 mmol l^{-1} $CaCl_2$, 0.3 mmol l^{-1} D-glucose, 0.1 mmol l^{-1} NaH_2PO_4 and 35.4 mmol l^{-1} Hepes–NaOH; pH 7.9). Injections were either performed prior to snails experiencing the HS (prevention experiments) or 1 h prior to MT2 (alleviation experiments). Snails were returned to their eumoxic home aquaria for 1 h after injection to recover before undergoing either the 0.5 h TS or MT2. Injection of propranolol or *Lymnaea* saline has previously been demonstrated to not affect homeostatic breathing behaviour in *Lymnaea* (Hughes et al., 2016).

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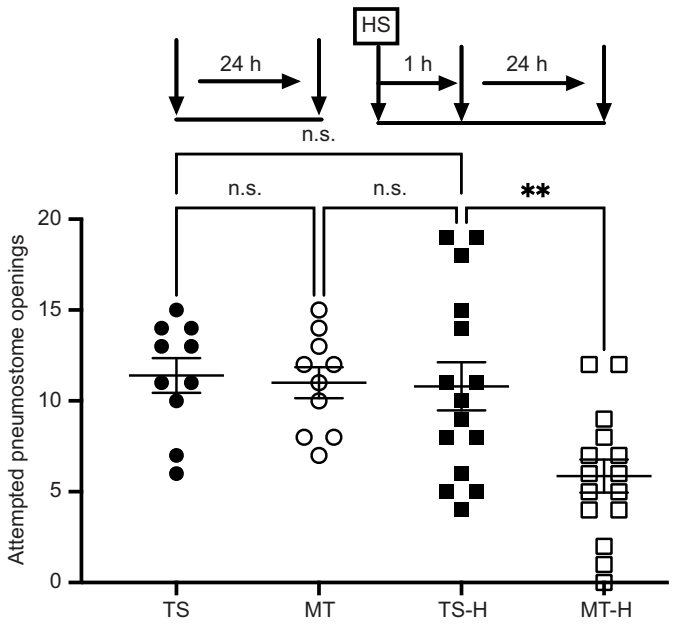

Fig. 1. The TC2 average cognitive phenotype strain of *Lymnaea stagnalis* and the heat shock stressor. The timelines for each experimental cohort are presented above the data points. Freshly collected snails (N=10) from the TC2 pond (filled and open circles) received a single 0.5 h training session (TS), and long-term memory (LTM) was then tested 24 h later (MT). The TC2 snails did not exhibit LTM. However, if a second cohort of TC2 snails (N=15; filled and open squares) first experienced the heat shock stressor (HS) 1 h before a single 0.5 h training session (TSA-H) they formed LTM when tested 24 h later (MT-H). n.s., not significant; **P=0.0059 (Tukey's *post hoc* test).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 9 software for the Mac OS 11.2.3 system. Data were first tested for normal distribution using the using the Anderson–Darling (A-D) test. If the data were distributed 'normally' we do not report the results of the A-D test. We also present the effect size number (e.g. R^2). In Figs 1, 2 and 4, the data were analysed using an ordinary one-way ANOVA followed by Tukey's *post hoc* tests on each of the training and memory tests. The data in Fig. 3 were analysed using a repeated-measures one-way ANOVA followed by Tukey's *post hoc* tests on each of the training and memory tests. Data plotted are the means±s.e.m. Actual P-values are given.

RESULTS

To confirm that the HS is sufficient to enhance LTM formation in freshly collected TC2 average cognitive phenotype snails, we used two naïve cohorts of these snails (Fig. 1). One cohort (N=10; Fig. 1, filled and open circles) received a single 0.5 h TS and memory was tested 24 h later (MT). The other cohort (N=15; Fig. 1, filled and open squares) received the HS 1 h before the single 0.5 h training session (TS-H) and memory was tested 24 h later (MT-H). An ordinary one-way ANOVA was performed on these data ($F_{3,46}$=6.243; P=0.0012; R^2=0.2893) followed by Tukey's *post hoc* tests on each of the training and memory test sessions. The important points from the analyses are that (1) LTM did not form in the TC2 snails following a single 0.5 h training session; and (2) LTM formed in TC2 snails when they were trained 1 h after experiencing the HS, as MT-H was significantly less than TS-H (P=0.0059). The number of attempted openings in the 0.5 h TSs was not statistically different between the two cohorts (i.e. TS is not different from TS-H; P=0.9813). Thus, the HS enhances LTM formation in average cognitive phenotype, freshly collected snails.

Next, we addressed the effect of the HS on LTM formation and the possible role of propranolol in preventing or alleviating stress in freshly collected smart cognitive phenotype snails. We first determined whether a pre-injection of propranolol (i.e. stress prevention) or saline would prevent the HS from blocking LTM formation in the TC1 snails (Fig. 2). Two naïve cohorts of TC1 snails were pre-injected with either saline (N=9; Fig. 2, filled and open circles) or propranolol (N=11; Fig. 2, filled and open squares) 1 h before they were subjected to the HS. These snails then received the single 0.5 h training session 1 h later (sal-TS and prop-TS, respectively) and memory was tested 24 h later (sal-MT and prop-

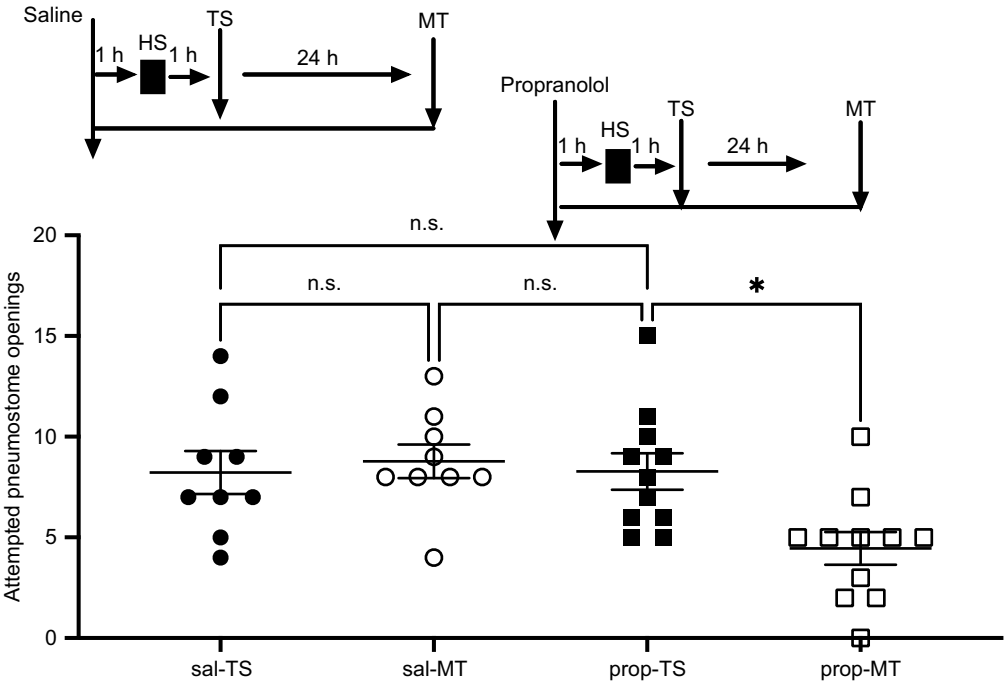

Fig. 2. Propranolol, saline, the HS and LTM formation in the TC1 smart cognitive phenotype *L. stagnalis* strain. The timeline for each experiment is presented above the data points. Freshly collected snails from the TC1 pond were used. A naïve cohort of TC1 snails (N=9; filled and open circles) received an injection of saline 1 h before the HS and, 1 h later, was operantly conditioned with a single 0.5 h training session (filled circles, sal-TS). These snails were then tested for memory 24 h later (open circles, sal-MT), and LTM formation was not apparent. A second cohort of naïve TC1 snails (N=10; filled and open squares) was injected with propranolol 1 h before the HS and, 1 h later, was operantly conditioned with a single 0.5 h training session (prop-TS). They were then tested for LTM 24 h later (open squares, prop-MT). LTM formed in these snails. n.s., not significant; *P=0.0172 (Tukey's *post hoc* test).

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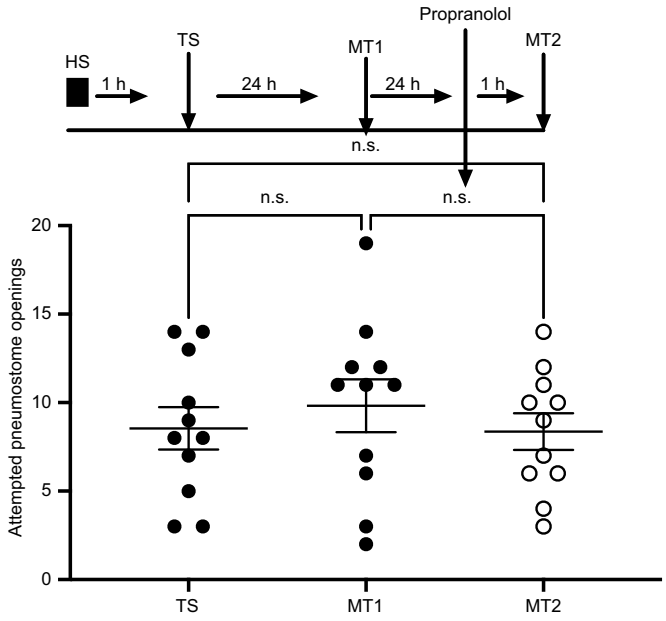

Fig. 3. Propranolol does not relieve a memory retrieval block in the TC1 smart cognitive phenotype *L. stagnalis* strain following the HS and operant conditioning training. The timeline for each experiment is presented above the data points. A naïve cohort of TC1 snails (N=11) experienced the HS and, 1 h later, was operantly conditioned with a single 0.5 h training session (filled circles, TS). The snails were then tested for LTM 24 h later (filled circles, MT1), and LTM was not apparent. They were injected with propranolol 24 h later and, 1 h later, received a second memory test (open circles, MT2). LTM was also not apparent in the MT2. n.s., not significant (Tukey's *post hoc* test).

MT, respectively). An ordinary ANOVA was performed on these data ($F_{3,36}$=5.173; P=0.0045; R^2=0.3012) followed by Tukey's *post hoc* tests on each of the training and memory test sessions. To summarize the important points from the analyses: (1) in snails pre-injected with saline, LTM did not form, as sal-MT was not significantly less than sal-TS (P=0.9760); (2) in snails pre-injected with propranolol, LTM formed, as prop-MT was significantly less than prop-TS (P=0.0172); (3) the number of attempted pneumostome openings in sal-TS was not significantly different from that in prop-TS (P<0.9999); and (4) the number of attempted openings in prop-MT was significantly less than that in sal-MT (P=0.0095). We therefore concluded that the pre-injection of saline did not alter the blocking effect the HS has on LTM formation, but the pre-injection of propranolol allows LTM to form in these smart cognitive phenotype snails. These data are consistent with the prevention hypothesis. That is, an injection of propranolol before smart cognitive phenotype snails experience the HS enables LTM to form.

We next wished to determine whether LTM formed following the HS, but the ability to retrieve the memory was blocked (i.e. the alleviation hypothesis) (Fig. 3). A naïve cohort (N=11; Fig. 3, filled circles) of TC1 smart cognitive phenotype snails received a single 0.5 h operant conditioning training session (TS) 1 h after HS. LTM was then tested 24 h later (MT1). After another 24 h, the snails received an injection of propranolol and, 1 h later, a second memory test was given (MT2; Fig. 3, open circles). A repeated-measures one-way ANOVA was performed on these data ($F_{1.947,19.47}$=0.4762; P=0.6232; R^2=0.04545) followed by Tukey's *post hoc* tests on each of the training and memory test sessions. To summarize the important points from the analyses: (1) LTM was not formed, as the number of attempted openings in MT1 was not statistically different from that in TS (P=0.6953); and (2) following the injection of propranolol, LTM was also not observed, as MT2 was statistically not different from TS (P=0.9941) and the number of attempted openings was not different from that in MT1 (P=0.6384). Thus, the injection of propranolol did not alleviate the obstruction of memory retrieval in smart cognitive phenotype snails.

Similar experimental procedures were performed on a different strain of smart cognitive phenotype snails, the WSL strain (Fig. 4). Two independent cohorts of WSL smart cognitive phenotype snails either received the propranolol pre-injection before the HS (N=14; Fig. 4, filled and open squares) or the HS first and the propranolol injection before the MT2 (N=12; Fig. 4, filled and open circles). An ordinary ANOVA was performed on the combined data ($F_{4,59}$=10.74; P<0.001; R^2=0.4213) followed by Tukey's *post hoc* tests on each of the training and memory test sessions. To summarize the important points: (1) in the cohort that received the pre-injection of propranolol (Fig. 4, filled and open squares) 1 h before the HS, LTM formed [MT was significantly less than in snails that received the pre-injection of propranolol before the HS and a single 0.5 h training session 1 h later (TSa); P=0.0311]; thus, propranolol prevented the HS block of memory formation; (2) following the HS, LTM was not formed, as the number of attempted openings in MT1 was not significantly less than that in snails that did not receive the pre-injection of propanol before the single 0.5 h training session (TSb; P=0.9928); (3) following the injection of propranolol before the MT2, LTM was also not observed, as MT2 was statistically not different from TSa (P=0.9626) and the number of attempted openings in MT2 was not different from that in MT1 (P=0.9993); thus, the propranolol injection did not alleviate an obstruction of a memory retrieval process; (4) the number of attempted openings in TSa was not significantly different from that in TSb before the thermal stressor (P=0.3359); and (5) the number of attempted openings in the memory test session in snails receiving the pre-injection of propranolol (i.e. MT) was statistically lower than that in MT1 (P<0.0001) and MT2 (P<0.0001). Thus, in the WSL smart cognitive phenotype snails, as in the TC1 smart cognitive phenotype snails, the HS procedure blocks LTM formation, but the effects of this stressor on LTM formation can be prevented with the pre-injection of propranolol before the snails experience the stressor. In addition, the propranolol injection did not relieve a possible memory block caused by the HS, as LTM was not observed in the MT2 following the propranolol injection.

DISCUSSION

We tested two hypotheses: (1) a pre-injection of propranolol in the two smart cognitive phenotype strains (i.e. prevention strategy) before snails experience the HS enables smart cognitive phenotype snails to form LTM; and (2) an injection of propranolol just prior to a memory retrieval session (alleviation strategy) in smart cognitive phenotype snails that experienced the HS before training enables successful memory retrieval. Our data are consistent with the first hypothesis but disprove the second hypothesis. That is, a propranolol injection did not alleviate a blockage of the memory that had formed but could not be retrieved.

Our thoughts on how stress alters LTM formation in smart cognitive phenotype snails compared with average cognitive phenotype snails have evolved since 2017, and are an interesting journey through sometimes apparently contradictory conclusions. Initially, it was concluded that a cost of being cognitively smart compared with being cognitively average was less resilience to certain stressors (Hughes et al., 2017). Thus, the same stressors (e.g. thermal, shell clip and certain combinations of stressors) that

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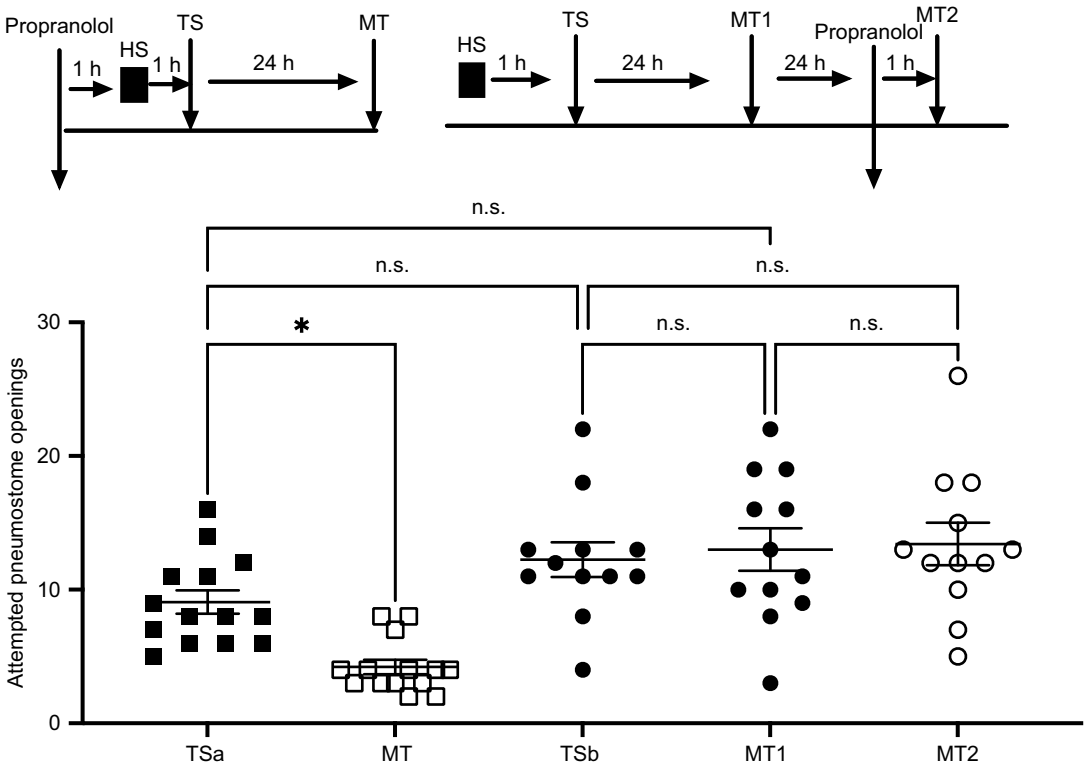

Fig. 4. The HS, propranolol and memory formation in the WSL smart cognitive phenotype *L. stagnalis* strain. The timeline for each experiment is presented above the data points. A naïve cohort of WSL snails (*N*=14; filled and open squares) was injected with propranolol 1 h before the HS and, 1 h later, was operantly conditioned with a single 0.5 h training session (filled squares, TSa). They were then tested for LTM 24 h later (MT). LTM formed in these snails. A second cohort of naïve WSL snails (*N*=12; filled and open and circles) received the HS and, 1 h later, was operantly conditioned with a single 0.5 h training session (TSb). When tested for LTM 24 h later (MT1), memory was not apparent. These snails were injected with propranolol 24 h later and, 1 h later, received a second memory test (open circles, MT2). LTM was not apparent in the MT2. n.s., not significant; **P*=0.0311 (Tukey's *post hoc* test).

enhanced LTM formation in an average cognitive phenotype snail blocked LTM formation in smart cognitive phenotype snails. However, that conclusion was incorrect, as it was more recently shown (Swinton et al., 2020) that the stressors that created an emotional memory (combination of stressors such as food deprivation coupled with the smell of unattainable food) did not block LTM formation but rather obstructed the ability of the smart cognitive phenotype to recall the formed memory (i.e. a memory retrieval obstruction). That was the first instance in our model system of a non-declarative associative memory being formed, but being in an inaccessible state. The obstruction of the memory retrieval process could be alleviated by the injection of propranolol 1 h before the memory test. Thus, a cost of possessing the smart cognitive phenotype was not an inability to form LTM, but the inability to retrieve the formed LTM. This inability of smart cognitive phenotype snails to retrieve an emotional memory could be mitigated in two ways: (1) by a pre-injection of propranolol 1 h before smart cognitive phenotype snails experienced the combination of stressors (i.e. prevention); or (2) by an injection of propranolol 1 h before the memory test session (i.e. alleviation). As shown here, however, there is still more to this story. It had been hypothesized that propranolol only had significant effects on emotional memories in *Lymnaea* (Hughes et al., 2016; Shymansky et al., 2018). Thus, the Swinton et al. (2020) study did not test the effect of propranolol on the thermal stressor in smart cognitive phenotype snails, as it was thought that the thermal shock stressor did not cause an emotional memory to be formed. In our initial study (Hughes et al., 2016), we found that propranolol only had effects on memory formation when snails experienced selective stressors. That is, although the memories enhanced by all of the stressors looked similar (e.g. when graded with an objective marking scheme), a propranolol injection only blocked the enhancing effect of certain stressors. For example, a propranolol injection blocked the enhancing effect of the combination of food deprivation+the scent of a carrot, but did not block the enhancing effect brought about by the exposure of the snails to the scent of a crayfish predator. Subsequently, we found that the pre-injection of propranolol only disrupted memories enhanced by a combination of stressors leading to an emotional memory (Shymansky et al., 2018). The idea of an emotional memory in an invertebrate such as a snail or an insect has been questioned by some. However, Darwin (1872), Damasio (2010) and LeDoux (2012) certainly thought they could express emotion. This is not to suggest 'consciousness'. Emotion as written by the aforementioned authors is a reflection of the autonomic system activity or its equivalent in invertebrate systems. We believe that the findings in a number of invertebrates (see Anderson and Adolphs, 2014; Perry et al., 2016; Baciadonna and Perry, 2017; Shymansky et al., 2018) show that an emotional memory is present in a number of invertebrate species. In *Lymnaea*, the emotional memories are propranolol sensitive. Our working hypothesis is that propranolol exerts its action on an OA transmitter/neuromodulatory system.

Here, we examined how, in both smart and average cognitive phenotype snails, the HS affects LTM. We first showed, consistent with Hughes et al. (2017), that the HS procedure blocks LTM formation in smart cognitive phenotype strain snails, while

enhancing LTM formation in average cognitive phenotype snails. Three important points were found: (1) a pre-injection of propranolol 1 h before the application of the HS mitigates the effect of the HS in both the TC1 and WSL smart cognitive phenotype strains, as LTM is now formed (i.e. stress prevention); (2) a propranolol injection 1 h prior to a memory retrieval session does not alleviate the obstruction of the retrieval process; thus, in the absence of a pre-injection of propranolol, the HSD blocks LTM formation; and (3) although propranolol was previously hypothesized to only affect the formation, reconsolidation or retrieval of emotional memories in *Lymnaea* (Hughes et al., 2016; Shymansky et al., 2018), it appears that the HS has properties that induce both propranolol-sensitive and propranolol-insensitive memory states in smart cognitive phenotype *Lymnaea*.

Hughes et al. (2016) showed that although the enhanced memory forming phenotype exhibited by average cognitive phenotype snails (the W-strain of laboratory-reared, inbred snails) following their exposure to various stressors may appear phenotypically similar – for example, by the application of 'pass-fail' analysis of memory performance – only certain memories were susceptible to disruption by propranolol. They concluded that propranolol only had significant effects on emotional memories. That conclusion was further supported in studies looking at a range of other stressors in W-strain, average cognitive phenotype *Lymnaea* (Shymansky et al., 2018). Those authors reported that the injection of propranolol before snails were exposed to only crayfish effluent (CE) or only the KCl-bath procedure had no effect on the enhancing properties of those two stressors. That is, in average cognitive phenotype snails, pre-injecting propranolol before the snails experienced either of those stressors did not counteract their enhancing effect on LTM formation. They concluded that neither training in CE nor experiencing the KCl bath before training produced an emotional memory. However, the injection of propranolol immediately after snails were trained in only CE or only the KCl-bath procedure blocked LTM formation. That is, propranolol interfered with the memory consolidation processes underlying the enhancing ability of those two stressors. At this time, it is unclear how propranolol accomplishes that.

Exposing *Lymnaea* to the HS, either before, during or immediately after operant conditioning, was initially shown to enhance LTM formation in an inbred, laboratory-reared strain of average cognitive phenotype *Lymnaea* (Teskey et al., 2012; Sunada et al., 2016). It was later shown in those *Lymnaea* that this stressor leads to the rapid upregulation of heat shock proteins HSP40 and HSP70 (Foster et al., 2015). This finding was consistent with the fact that operant conditioning training could be delayed for hours to days after the HS and enhanced LTM formation would still be observed. It was then shown that the enhancing effect of the HS was dependent on the elaboration of the HSPs and involved a DNA methylation process in neurons necessary for LTM formation (Sunada et al., 2016). The results obtained here led us to hypothesize that there may be important differences in the ability to upregulate HSP production between average cognitive phenotype and smart cognitive phenotype snails, and that the pre-injection of propranolol may alter HSP activity in the smart cognitive phenotype snails. These hypotheses will be examined in future experiments.

High temperatures alter many important homeostatic functions in *Lymnaea*, including immune system functioning, mortality and reproduction (Vaughn, 1953; McDonald, 1969; 1973; Seppala and Jokela, 2011). In addition, higher temperatures increase parasite virulence in snails (Seppala and Leicht, 2013; Leicht et al., 2013). *Lymnaea* also reduce their innate response to predation when reared at higher environmental temperatures (Dalesman and Rundle, 2010). Inbred, laboratory reared W-strain snails at 30°C also perform homeostatic aerial respiration at a significantly higher rate than at room temperature (~20°C), but locomotor activity is not significantly increased (Teskey et al., 2012). We have not attempted to acclimatize *Lymnaea* in the laboratory to higher temperatures close to their thermal tolerance upper limit of 30°C (Vaughn, 1953; Salo et al., 2019) and then determine whether the HS has the same effect on LTM formation. Rearing temperature was shown by Hoefnagel and Verberk (2017) not to alter the upper limit of heat tolerance in *Lymnaea*, and the authors concluded that long-term acclimation had limited capacity to improve heat tolerance. In the freshly collected snails used here, however, although they may experience temperatures greater than 25°C for hours during daytime in summer, that experience does not prevent heat from acting as a stressor.

It is worth noting that in a related species of snail (*Radix balthica*) that co-exists in an Icelandic lake in stable different temperature environments – a cold environment (6°C) and a stable warm environment (23°C) – the snails survive differently when housed at different temperatures (Johansson et al., 2016). The 'warm' snails survive poorly at 6°C and have a higher survival rate in warm temperatures. Likewise, the 'cold' snails survive better in cold than in warm temperatures. But to us, the most interesting group of snails, the so-called 'seasonal' snails (i.e. they experience a range of temperatures over the course of the year in the lake) had high survival rates across all temperatures. Similar differences were also found for the growth rates of these snails. The warm snails grew faster than cold and seasonal snails, especially at higher temperature treatments, in both field and laboratory experiments. Thus, there are important differences in thermal sensitivity (i.e. survival, growth, etc.) owing to life-history experiences. Whether such a 'natural history' experiment is possible in *Lymnaea* remains to be determined, but it was noted (Mozely, 1928) that *Lymnaea* living in a large glacier-fed lake in the Canadian Rockies (Jasper National Park) were 27–60% smaller than those living in a nearby pond with a higher maximum temperature than that in the lake.

We defined stress here following the definition of Kim and Diamond (2002) as a condition that alters the physiological or psychological homeostasis of an organism. We view the blocking of LTM formation in smart cognitive phenotype snails by the HS as being consistent with the Yerkes–Dodson/Hebb law (Yerkes and Dodson, 1908; Hebb, 1955; Ito et al., 2015). In essence the 'law' states that the ability to form memory differs with the perception of stress made by the organism at that specific time. This is the most important parameter in determining the stressor's effect on memory formation. As seen in the inverted U-shaped 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. We hypothesize that, in smart cognitive phenotype snails, the inverted U-shape curve is both steep and narrow, compared with the curve exhibited by average cognitive phenotype snails. Thus, the same stressor will alter memory formation differently between the two groups. In addition, the response of the snail (i.e. memory formation or recall) will depend on whether it is subjected to either a single stressor or a combination of stressors (Dalesman et al., 2013). We speculate that the steep and narrow inverted U-shape curve we believe to exist in the smart cognitive phenotype snails may (1) serve as a model to better study how central 'states' such as anxiety alter cognitive functioning, and (2) be the result of differences in ongoing spontaneous neuronal activity in a neuron, RPeD1, shown

to be necessary for LTM formation in *Lymnaea* and to be in a different state from that neuron in average cognitive phenotype snails (Scheibenstock et al., 2002; Braun et al., 2012).

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.L.; Methodology: E.S., K.L.; Validation: K.L.; Formal analysis: K.L.; Investigation: E.S., C.S., I.P.; Resources: I.P., K.L.; Data curation: E.S., C.S., I.P.; 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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