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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Snails

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

2.2. Drug exposure

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

2.3. Aerial respiratory behavior

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

2.4. Standard operant conditioning procedure

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

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