What can we teach *Lymnaea* and what can *Lymnaea* teach us?

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ABSTRACT

This review describes the advantages of adopting a molluscan complementary model, the freshwater snail *Lymnaea stagnalis*, to study the neural basis of learning and memory in appetitive and avoidance classical conditioning; as well as operant conditioning of its aerial respiratory and escape behaviour. We firstly explored 'what we can teach *Lymnaea*' by discussing a variety of sensitive, solid, easily reproducible and simple behavioural tests that have been used to uncover the memory abilities of this model system. Answering this question will allow us to open new frontiers in neuroscience and behavioural research to enhance our understanding of how the nervous system mediates learning and memory. In fact, from a translational perspective, *Lymnaea* and its nervous system can help to understand the neural transformation pathways from behavioural output to sensory coding in more complex systems like the mammalian brain. Moving on to the second question: 'what can *Lymnaea* teach us?', it is now known that *Lymnaea* shares important associative learning characteristics with vertebrates, including stimulus generalization, generalization of extinction and discriminative learning, opening the possibility to use snails as animal models for neuroscience translational research.

Key words: associative learning, behaviour, brain–behaviour, complementary models, invertebrates, *L. stagnalis*, Mollusca, memory, neuroscience translational research, psychiatric disorders

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I. INTRODUCTION

Learning about the relationships between stimuli (i.e. classical conditioning) and the consequences of one's own behaviour (i.e. operant conditioning) is part of the 'survival-pack' that animals use to adapt to an ever-changing environment (Abel & Lattal, 2001). It would be tremendously ineffective if each experience encountered was considered unique, because this would require that learning must take place *de novo* each time a situation is encountered. For that reason, the ability to encode, store, and retrieve information from previous experiences is necessary for making predictions, recognizing time-based patterns and generating appropriate behaviours (Abel & Lattal, 2001).

Based on its temporal persistence, memory can be categorized as: short-term memory (STM; persisting for minutes), intermediate-term memory (ITM; lasting 2–3 h), and long-term memory (LTM; persisting >18 h) (Rosenzweig *et al.*, 1993; Abel & Lattal, 2001). The process by which the transient STM is converted to a more stable LTM is generally referred to as consolidation and is dependent on altered gene activity and new protein synthesis (Squire *et al.*, 2015). However, LTM is far from being 'fixed'. In fact, to respond in a flexible and adaptive manner to continuously changing environments, the stored memory can be weakened, disrupted, or enhanced (Alberini & Ledoux, 2013). For example, following memory retrieval, the memory reverts to a labile state and must undergo a reconsolidation process for it to be retained. However, the memory trace may be altered during the reconsolidation process in response to changes in the environment (Agren, 2014).

Over the last century, a vast repertoire of classical and operant conditioning procedures has provided a solid foundation to explain various aspects of learning and memory (Brembs, 2003; Byrne & Hawkins, 2015). The core of classical conditioning (i.e. Pavlovian conditioning) lies in the temporal-contingent association between two stimuli: an initially neutral stimulus (the conditional stimulus, CS) and a biologically relevant stimulus (the unconditional stimulus, US). By the temporal and forward pairing of the CS with the US, the CS comes to evoke a response that is similar to the response (i.e. behaviour) that the US evoked (Pavlov, 1927; Walters, Carew & Kandel, 1979).

In operant conditioning, the frequency of a behaviour is increased or decreased (depending on the reinforcer used) by the consequences of the behaviour. Reinforcement can either be negative, leading to a decrease in the frequency of the behaviour, or positive, resulting in an increase (Brembs, 2003). Thus, operant conditioning is concerned with an association between the behaviour of an organism and its environment.

II. *LYMNAEA STAGNALIS* IN NEUROSCIENCE AND BEHAVIOURAL RESEARCH

One of the most remarkable discoveries by Charles Darwin is that evolution is conservative (Griffiths *et al.*, 1999). When a mechanism is successful, natural selection tends to retain it, and it is transmitted to subsequent generations. This has taken place with cellular processes underlying learning and memory (Kandel & Schwartz, 1982). Although there are large phylogenetic differences and extensive variability in neural organization in the animal kingdom; the cellular and molecular basis of learning and memory is conserved (Byrne & Hawkins, 2015). Thus, a focus of neuroscientists is the development of translational approaches to study the conserved mechanisms underlying learning and memory (Willner, 1986; Rodgers *et al.*, 1997). In this regard, it is of great importance to identify and select the most appropriate animal models, focussing on homologous/analogous behaviours, neuronal circuits, and molecular cascades (Byrne & Hawkins, 2015).

In this context, invertebrates offer several experimental advantages over other possible model systems. Chief among these is that they possess a relatively simple nervous system compared to mammals that mediates relatively simple behaviours that are tractable (Tascedda *et al.*, 2015). Although invertebrates are often identified as 'alternative models', the term 'complementary models' might be more appropriate. That is, they can and should be used in addition to, and not as an alternative, to classic mammalian models. Invertebrate model systems are also, compared to mammalian systems, relatively inexpensive and they allow fine dissection of the neuronal circuits and underlying molecular pathways of memory and learning (Rivi *et al.*, 2020). Among a wide variety of invertebrate models, the freshwater pond snail *Lymnaea stagnalis* (henceforth *Lymnaea*), has been widely recognized as useful model for the study of the behavioural, cellular, and molecular mechanisms underlying learning and memory (Willner, 1986; Rodgers *et al.*, 1997). *Lymnaea* has a wide Holarctic distribution, mainly inhabiting ponds and lakes (Kemenes & Benjamin 2009; Fodor *et al.*, 2020a). The rich behavioural repertoire that these snails use to survive and adapt to their natural environment makes *Lymnaea* a remarkable model system with which to study associative learning and the neuronal and molecular mechanisms of memory formation (Rivi *et al.*, 2020). *Lymnaea* possess relatively simple but important homeostatic behaviours whose underlying neuronal circuitry has been well elucidated (Benjamin & Kemenes, 2010). Moreover, many of these behaviours are tractable and are relatively easy to train (Benjamin, Staras & Kemenes, 2000).

In this review, we highlight the most widely employed classical and operant conditioning procedures used in *Lymnaea*, offering the possibility to study both 'why' and 'when' associative learning occurs. In particular, by focusing on the animal's responses to internal and external stimuli at various times during behavioural training, it is possible to study the temporal dimension of memory formation and storage. Interestingly, studies from *Lymnaea* confirmed previous research conducted in *Aplysia* and other model systems demonstrating that ITM is an additional form of new-protein-dependent memory that persists for a few hours and is later transformed into LTM (Emptage & Carew, 1993; Lukowiak

et al., 2000; Braun & Lukowiak, 2011). These studies add complexity to the standard dichotomy between STM and LTM and show that what we call 'memory' is a continuum in time and space, where the past, present, and future are acting almost in parallel (Rosenzweig *et al.*, 1993).

At neuronal level, the nervous system of *Lymnaea* consists of about 20000 large (up to 150 μm in diameter) neurons, organized in a ring of interconnected ganglia, offering a relatively large amount of biological material that can be analysed molecularly, physiologically, and morphologically (Hawkins, Hon & Ren, 2010; Tascedda *et al.*, 2015). The neurons can be easily removed and placed in culture, where they reform the appropriate synaptic connections (Syed, Bulloch & Lukowiak, 1990). Thus, single neurons can be identified and analysed as part of defined circuits, allowing electrophysiological dissection of the networks involved in relatively simple rhythmic behaviours, such as feeding and aerial respiration (ter Maat, 1992; Whelan & McCrohan, 1996; Jones, Kemenes & Benjamin, 2001; Jones *et al.*, 2003; Feng *et al.*, 2009). These rhythmic movements are induced by groups of central pattern-generating neurons (CPGs) (Katz, 2016), whose characterization is critical for understanding where and how the nervous system controls these homeostatic behaviours and how the interplay between CPGs and external stimuli participates in the production of adaptive learned behaviours (Syed *et al.*, 1990; Yeoman, Brierley & Benjamin, 1996; Spencer, Syed & Lukowiak, 1999; Spencer *et al.*, 2002; Straub, 2004). These CPG circuits can be plastically reconfigured *via* environmental changes, experiences, and conditioning procedures to optimize the output to meet specific behavioural demands (Katz, 2016). Neuronal plasticity exhibited in the CPG circuits plays an important role in regulating the initiation and temporal output of behavioural rhythms in response to rewarding/aversive stimuli (as occurs in classical conditioning) and action–outcome contingencies (as occurs in operant conditioning) (Kojima *et al.*, 1997). Using *in vitro* and semi-intact preparations (which allow monitoring the behaviour and neural activity simultaneously), the CPGs controlling feeding and aerial respiration have been well studied in *Lymnaea* and learning-induced changes elucidated with cellular precision (Lukowiak, 1991; Kemenes *et al.*, 1997; Spencer *et al.*, 1999, 2002; Lukowiak & Syed, 1999; McComb, 2005). Studies such as these cannot easily be performed in most vertebrate preparations because their behaviours are more complex, and the underlying neuronal circuitries are more inaccessible to direct cellular and synaptic analyses (Kemenes, Staras & Benjamin, 1997).

An additional advantage is that molluscan neurons are unipolar and the single process (i.e. the primary neurite) emerging from the soma is the site where most synaptic interactions and normal neuronal activities and behaviours are mediated (Syed *et al.*, 1990, 1992; Scheibenstock *et al.*, 2002). Fascinating and noteworthy is the finding that the primary neurite can not only survive after the surgical ablation of the soma, but is also competent to synthesize new proteins without the soma being present (Spencer

et al., 2000). For that reason, it has been demonstrated that following removal of the soma ITM can occur but not LTM (Scheibenstock *et al.*, 2002). These studies have allowed researchers to distinguish between the sites (i.e. neurites and soma) in which memories are processed (Scheibenstock *et al.*, 2002). The characteristics of molluscan neurons differ from most vertebrate and mammal preparations, where disruption of the neuronal soma usually causes death of the entire cell (Saleuddin & Mukai, 2017).

Finally, quantitative changes in gene expression induced by conditioning can be studied at the level of single neurons, helping us to elucidate which molecules participate in the dialogue between the synapse and the nucleus and *vice versa* during memory and learning (Rivi *et al.*, 2020).

The molecular mechanisms of LTM involve highly conserved signalling pathways [such as cyclic AMP-dependent protein kinase A (cAMP/PKA), nitric oxide/cyclic GMP (NO/cGMP), mitogen-activated protein kinase (MAPK), N-methyl-D-aspartate (NMDA) receptors, and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)], transcriptional regulation of gene expression by cAMP response element-binding protein (CREB) and CCAAT-enhancer-binding proteins (C/EBPs), and new protein synthesis (Kemenes, 2008; Rosenegger, Wright & Lukowiak, 2010; Korneev *et al.*, 2018; Rivi *et al.*, 2020). Moreover, studies from *Lymnaea* confirmed that the induction of ITM is protein synthesis-dependent but RNA synthesis-independent, whereas STM requires neither protein nor RNA synthesis and LTM requires both (reviewed in Rivi *et al.*, 2020).

III. CLASSICAL CONDITIONING IN *LYMNAEA STAGNALIS*

Feeding is a rhythmic behaviour that has proved remarkably useful to investigate both reward (Alexander, Audesirk & Audesirk, 1984; Kemenes & Benjamin, 1989) and aversive classical conditioning (Kojima *et al.*, 1996). Feeding behaviour in *Lymnaea* shares important aspects with vertebrates, such as a strong dependence on external and internal variables and stimulus generalization and discrimination (Kemenes & Benjamin, 2009).

The well-characterized CPG controlling feeding in *Lymnaea* has been a major focus of learning and memory studies, allowing investigators to correlate conditioning-induced changes at the behavioural level with neuronal modifications in the CPG feeding circuit. In this circuit, neuron 1 medial (N1M), neuron 2 (N2), and neuron 3 tonic (N3t) cells form part of the feeding CPG (Elliott & Benjamin, 1985; Yeoman *et al.*, 1995; Brierley, Staras & Benjamin, 1997; Straub *et al.*, 2002; Braak *et al.*, 2013) and their activity is regulated by higher order interneurons, termed cerebral giant cells (CGCs) and cerebro-buccal interneurons (CBIs) (McCrohan, 1984). When food is present, the feeding response is generated by a cascade of depolarization through the sensory neurons (SNs) to the CBIs that excite N1M

which, in turn, activates motor neurons triggering feeding behaviour. In the absence of food and in satiated snails, tonic inhibition of the feeding network is mediated by N3t which has inhibitory monosynaptic connections with N1M (Yeoman *et al.*, 1996). Because sucrose enhances feeding behaviour in *Lymnaea* (Kemenes & Benjamin, 2009), it can be used as a rewarding stimulus to evoke a feeding response in food-reward conditioning (Sadamoto *et al.*, 2010; Ito, Totani & Oike, 2017). On the other hand, potassium chloride (KCl) elicits escape behaviour, shutting down the feeding response; thus KCl can act as an aversive stimulus in conditioned taste aversion (Kojima *et al.*, 1996).

(1) Classical food-reward conditioning

Classical appetitive food-reward conditioning involves a temporal-contingent repeated presentation of a neutral CS with a US that elicits feeding. In *Lymnaea*, the US results in a sequence of rhythmic and stereotyped feeding movements consisting of opening the mouth, rasping with the radula, and closing the mouth (Kemenes & Benjamin, 1989). A sucrose solution is typically used as the US, but tactile, chemical, or visual cues can all be used as a CS (Kemenes & Benjamin, 1989, 1994; Staras, Kemenes & Benjamin, 1998). In one of the first associative learning LTM experiments using *Lymnaea* (see online Supporting Information, Appendix S1 for full details of experimental procedures used in conditioning experiments with *Lymnaea*), investigators employed a single-trial learning procedure consisting of a single pairing of amyl acetate, which typically does not elicit feeding, as the CS, with sucrose used as the US (Fig. S1). After the single-trial training session (i.e. CS–US pairing), application of the CS alone in the memory test induced feeding behaviour, whereas feeding behaviour in response to the CS was not observed in any of the control groups, demonstrating associative learning (Alexander *et al.*, 1984). This single CS–US pairing was sufficient to create a LTM trace that persisted for at least 15 days (Alexander *et al.*, 1984).

Single-trial conditioning of feeding using a visual cue (see Appendix S1 for methodological details) is also possible by adding the US (sucrose) to a black panel (the CS) (Andrew & Savage, 2000), which snails perceive using a lens capable of forming an image on the retina underwater (Seyer, 1992). After conditioning, approach to the black panel elicited more rasping movements in trained snails. It was also demonstrated that snails can learn to discriminate the black panel (CS) from a grey pattern of equal luminance, exhibiting the behavioural response only with the CS (Andrew & Savage, 2000).

Using appetitive classical conditioning, researchers were able to elucidate sites in the snails' central nervous system (CNS) involved in learning this task and the mechanisms used to store memory-related representations. In particular, some hours after training, conditioning leads to a reduction in spike activity in N3t that, in turn, makes N1M more likely to respond to the SNs (Marra *et al.*, 2010). Thus, there is a switch of the network from an inactive to a CS-evoked rhythmically active state (Staras *et al.*, 2003). At the same time, the conditioning procedure induces delayed but persistent depolarization of the CGCs, that facilitates inputs of the SN-to-CBI excitatory synapse (Kemenes *et al.*, 2006). Because delayed depolarization of the CGCs is known to be correlated with the establishment and the duration of LTM, it has been assumed that CGCs are involved in the maintenance of the late phase of LTM (Nikitin *et al.*, 2008).

Memory consolidation after single-trial chemical appetitive classical conditioning represents a dynamic process that offers the opportunity to study both at the behavioural and neurophysiological level so-called 'memory lapses'. During memory consolidation, multiple learning events often occur in rapid succession and competition between the various consolidating memories can emerge, resulting in memory lapses (Marra *et al.*, 2013). Thus, single-trial food-reward memory could result in the erasure of newly acquired information (retroactive interference) or previous learning can affect the success of the acquisition of a second memory (proactive interference) (Crossley *et al.*, 2019). Whether proactive or retroactive interference is activated depends on the timing of the second training and the underlying neuronal mechanisms. In particular, it was demonstrated that when new learning takes place during a stable period of the original memory, proactive interference only occurs if the two consolidating memories engage the same circuit mechanisms. On the other hand, when different circuits are used, both memories survive. New learning occurring during a labile period of consolidation instead promotes retroactive interference and the acquisition of the new memory.

The success of classical conditioning training depends on both internal (e.g. food deprivation/satiety) and external variables (e.g. water conditions) (Kemenes & Benjamin, 1994; Murakami *et al.*, 2013). Regarding external variables, Kemenes & Benjamin (1994) showed that snails trained in a novel environment had better appetitive learning performance compared to animals trained in the water of their home tanks (i.e. a familiar environment). Moreover, the learning-stimulating effect of the novel environment was enhanced if snails were food-deprived before testing, whereas it was strongly suppressed in snails fed *ad libitum*, suggesting a reciprocal interaction between internal and external variables (Kemenes & Benjamin, 1994).

In pivotal studies of reward conditioning, Audesirk *et al.* (1982) demonstrated that although both 'young' and 'old' starved snails acquired the association between food and the chemo-stimulus, 'old' snails had worse memory performance compared to 'young' ones and required multi-trial experiments. 'Old' snails fed *ad libitum* before training failed to learn, while their younger counterparts acquired the association, but expressed the learned response only after a period of food deprivation (Audesirk *et al.*, 1982). Further studies demonstrated that the acquisition of appetitive memory was not affected by ageing, but that memory retention and consolidation become progressively impaired with advancing age (Hermann *et al.*, 2007). Using appetitive

classical conditioning, age-associated learning and memory deficits were associated with declining electrophysiological excitability of the CGCs, most likely reflecting a selective deficit in the activity-dependent regulation of gene transcription, possibly through CREB-dependent mechanisms, as demonstrated in other models (Hermann *et al.*, 2007; Pirger *et al.*, 2010; Scutt *et al.*, 2015). Memory decline in aged *Lymnaea* is consistent with the age-dependent impairment in learning and memory functions observed in many animal species, including humans, where the molecular, cellular, and neural network functions show a decline of neuronal experience-dependent plasticity (Hermann *et al.*, 2007). Together, these results indicate that the *Lymnaea* feeding system is a powerful tool for investigators attempting to understand the cellular and molecular correlates of age-related declines in cognitive ability.

(2) Aversive classical conditioning of feeding behaviour

Learning what to eat, and what not to eat, is fundamental for well-being and survival (Curtis, de Barra & Aunger, 2011). Thus, animals evolved food-avoidance behaviours to prevent ingestion of toxic substances (Lin, Arthurs & Reilly, 2017). Because this behaviour can be conditioned by internal cues that occur after ingestion of an aversive substance [e.g. the Garcia effect (Garcia, Hankins & Rusiniak, 1974; Garcia *et al.*, 1985)], observing the aversive reaction of a peer (Chambers, 2018), or a bitter taste (Sugai *et al.*, 2006), it is often termed conditioned taste aversion (CTA).

Lymnaea can show CTA following pairing of an appetitive CS (e.g. sucrose or carrot juice) with an aversive US (e.g. KCl; Sugai *et al.*, 2007). These procedures can involve either single-trial training (i.e. single pairing of the CS–US; Fig. S2A) or multi-trial training (i.e. multiple CS–US pairings with a short inter-trial interval interposed between each pairing; Fig. S2B). CTA can be acquired rapidly, and can persist for up to 1 month after single-trial conditioning (Kojima *et al.*, 1996; Kawai *et al.*, 2004).

Changes in activity of CPG neurons mediating feeding induced by CTA conditioning have been extensively studied in *Lymnaea* (Kojima *et al.*, 1997; Ito *et al.*, 2012b). Before CTA training, SNs responsive to sucrose excite the CBIs of the feeding CPG to induce a feeding response, whereas the SNs activated by KCl depolarize motor neurons involved in the defensive withdrawal pathway, which in turn inhibits the feeding response (Kojima *et al.*, 1997). Activity of the higher-order modulatory neurons (the CGCs) is altered by the KCl-induced input and ultimately exerts a strong polysynaptic inhibitory influence on N1M both directly and indirectly *via* N3t, thereby blocking the feeding response (Kojima *et al.*, 1997).

Additionally, it has been demonstrated that after ablation of the CGC soma, the functional primary neurite continues to be a necessary participant in the mediation of CTA learning. Following soma ablation LTM cannot be formed, as it is dependent on altered gene activity that cannot take place as ablation of the soma also removes the nucleus. However, when the CGC soma is ablated after memory consolidation, snails can still retrieve the memory, indicating that while the CGC must be present for LTM formation, it is not essential for its recall (Sunada, Lukowiak & Ito, 2017).

Whether CTA learning and subsequent memory formation occur is dependent on the snail's internal state. The strongest CTA occurs when snails are trained after 1 day of food deprivation (referred to as Day-1 snails), suggesting that a modest level of food deprivation acts as motivating factor for memory formation (Ito *et al.*, 2015). On the other hand, when snails were subjected to severe food deprivation for 5 days (Day-5 snails) motivation from hunger was stronger than the conditioned memory and animals continued to respond to the CS (Ito *et al.*, 2015). The memory phenotype of Day-5 snails was carefully investigated: after training CTA LTM was formed but its recall was prevented by the effects of food deprivation. Day-5 snails must eat in order to survive, even if the sucrose had become a predictor of an aversive event (Ito *et al.*, 2015). Subsequently allowing Day-5 snails *ad libitum* access to food for 7 days after CTA training was not sufficient to induce memory retrieval, ruling out the possibility that very hungry snails could not access memory. Memory retrieval in Day-5 snails was present after *ad libitum* access to food if they were food deprived for 1 day before the memory test (Day-13 snails). What enables memory recall in Day-13 snails thus is the creation of a condition similar to that of Day-1 snails (Ito *et al.*, 2015). Recently Totani *et al.* (2020) demonstrated that both Day-1 and Day-13 snails exhibit CTA memory due to the presence of an 'optimal central state' in the snail related to a critical level of insulin; the insulinergic central state of Day-5 snails did not allow LTM recall. This optimal central state can be re-established by an injection of insulin, which allowed memory to be retrieved (Totani *et al.*, 2020).

That insulin plays a role in memory formation following CTA training in *Lymnaea* was convincingly demonstrated in an important series of papers (Kojima *et al.*, 1996; Sugai *et al.*, 2006; Ito *et al.*, 2012a, 2015; Murakami *et al.*, 2013). In both *Lymnaea* and mammals, insulin, in addition to its classical role in energy metabolism, plays a role in learning and memory. Insulin is known to be beneficial in modifying the pathophysiology of Alzheimer's disease and in maintaining cognitive functions (Zhao *et al.*, 1999; Dou *et al.*, 2005; Smith *et al.*, 2010; Mita *et al.*, 2014).

CTA LTM is robust and is very resistant to a memory extinction procedure consisting of the presentation of the CS alone three times at 10 min intervals at the end of CTA training (Sugai *et al.*, 2006). In each session of this extinction procedure, and in the post-extinction session, the number of bites elicited by the CS remained significantly low (Sugai *et al.*, 2006).

Extinction is not 'unlearning' (indeed, the original memory can be spontaneously recovered) and therefore does not erase the response learned previously. On the contrary, there is evidence that extinction training results in a new memory that may compete with the original memory (Sangha *et al.*, 2003c;

Sugai *et al.*, 2006). The mechanism(s) that allow one memory to dominate over the other remain unclear, however there is a direct proportionality between the robustness of the original memory and its resistance to extinction (Sugai *et al.*, 2006), confirming that CTA memories are strong. A possible explanation for the robustness of CTA memories is that the new CS evokes a conditioned fear response. This is supported by observations of changes to heart rate in conditioned snails (Kita *et al.*, 2011). Future work should explore the neural and molecular basis of this switch from aversive to fear conditioning and the consequent robust and long-lasting memory. We will return to this theme when we discuss configural learning (Swinton *et al.*, 2019) when an appetitive food is simultaneously experienced with a stimulus that signals the presence of a predator (see Section IV.1).

Using a CTA training procedure, it was also shown that snails can distinguish between appetitive stimuli during CTA and can even acquire second-order conditioning (Fig. S2C; Sugai *et al.*, 2006). In these experiments, two appetitive CSs (sucrose and carrot juice) and an aversive US (KCl) were used in a two-phase training procedure. In the first phase snails were conditioned to avoid one of the appetitive food substances (CS1–US). In the second phase, the second CS (CS2) was paired 10 times with CS1, thus serving as the US. After this second training presentation (CS2–CS1), CS2 no longer acted as an appetitive stimulus, but instead acquired the ability to evoke CTA, even though it was never paired with the US (Fig. S2C; Sugai *et al.*, 2006). This procedure showed the presence in the *Lymnaea* model system of higher-order conditioning previously found only in vertebrates (Gewirtz, 2000).

CTA studies are extremely useful in terms of translational medicine. Food avoidance, fear conditioning, and resistance to extinction are all hallmarks of CTA in mammals. Moreover, in rodents and humans, CTA has been associated with digestive dysfunction and has been viewed as allowing rapid learning of food toxicity that is then maintained as LTM (Garcia *et al.*, 1974). However, while one-trial training is sufficient to establish CTA in mammals, in *Lymnaea* learning occurs after a single-training procedure in only about 40% of the snails (Sugai *et al.*, 2007), suggesting that some snails can acquire CTA faster than others, despite being reared under the same conditions. CTA can be established in almost all snails with multi-trial training (Sugai *et al.*, 2006, 2007).

Recently Nakai *et al.* (2020) demonstrated that in *Lymnaea* CTA is consolidated to LTM *via* protein synthesis-dependent processes, and showed that memory consolidation begins during the period of CTA training (10 CS–US pairings). Future studies of *Lymnaea* could allow us to accumulate new insights into the molecular events associated with LTM.

IV. OPERANT CONDITIONING IN *LYMNAEA STAGNALIS*

Compared with vertebrates, relatively few studies of operant conditioning have been performed on invertebrates, and in the vast majority of cases the behaviours studied are relatively simple and reflexive (Carew, 1996; Lukowiak *et al.*, 1996). Operant conditioning of aerial respiration in *Lymnaea* is a fascinating exception that has provided insights into basic and advanced cognition (Dickinson, 1987).

(1) Operant conditioning of aerial respiration behaviour

Lymnaea is a bimodal breather: under normoxic (i.e. eumoxia) conditions, gaseous exchange occurs *via* transpiration across the skin (cutaneous exchange). In hypoxic conditions [as often arise in their pond-water environment], snails come to the water surface for aerial respiration, consisting of rhythmic opening and closing of the pneumostome (Boycott, 1936; Jones, 1961). Aerial respiratory behaviour can thus be used in operant conditioning without affecting the survival of the animal, as they can obtain oxygen *via* cutaneous exchange (Lukowiak *et al.*, 1996). In a ground-breaking series of experiments the neuronal basis of the CPG that underlies aerial respiration in *Lymnaea* was elucidated (Syed *et al.*, 1990, 1992), showing that a three-neuron network was both sufficient and necessary for aerial respiratory behaviour. Few, if any, other neuronal circuits underlying an easily observable and tractable behaviour have been described, and this system has been used to investigate the neuronal mechanisms of learning and LTM.

Lukowiak *et al.* (1996) demonstrated that the aerial respiration behaviour can be operantly conditioned by applying a relatively weak tactile stimulus to the pneumostome each time snails attempt to open it. This negative reinforcement causes the snail to close its pneumostome but does not cause the animal to withdraw its foot and mantle area (i.e. the whole-animal withdrawal response). This learning undergoes consolidation into either ITM or LTM (Lukowiak *et al.*, 2000).

ITM, and LTM can be differentially produced by altering the duration, number and intervals used in training protocols for this behaviour (Lukowiak *et al.*, 2000). For example, an operant training procedure of two 45-min training sessions with a 1-h interval between training sessions, followed by a third 45-min training session 18 h later was sufficient to produce an LTM persisting for at least 5 days (McComb *et al.*, 2002); five training sessions of 30 min with an inter-session interval of at least 1 h over the course of 2.5 days was required for the establishment of a LTM that persisted for at least 1 week; eight training sessions repeated over a four-week period resulted in a LTM that persisted for at least 1 month (Lukowiak *et al.*, 1998). A LTM lasting for 48 h can be evoked with a single day of training, by exposing snails to three series of 15-min training sessions with an inter-training interval of at least 1 h (Fig. S3A), whilst a 30-min interval between sessions resulted in associative learning but memory persistence for only ~3 h, designated as ITM by Lukowiak *et al.* (2000) (Fig. S3B) (Lukowiak *et al.*, 2000; Smyth, Sangha & Lukowiak 2002). Moreover, Smyth, Sangha & Lukowiak (2002) demonstrated that if snails received the

ITM training procedure followed by 5 h in their home aquaria, subsequent training with the LTM procedure led to a LTM that persisted for at least 72 h; 24 h longer than usually produced by this LTM training procedure on its own.

It was proposed that the ITM training procedure initiates the translation of pre-existing mRNA into proteins that bring about the neuronal changes necessary for memory. Although 5 h after the ITM training session memory can no longer be demonstrated behaviourally, it appears that at a neuronal level, some changes persisted for longer constituting a 'trace' of memory that was sufficient to enhance LTM formation and maintenance. This mechanism was described as "gone but not forgotten" by Smyth *et al.* (2002). If LTM training occurred 8 h after ITM training, LTM augmentation did not occur, suggesting that this inter-training interval was too long for the memory trace to be maintained (Smyth *et al.*, 2002). These results indicate that if the ITM trace is still present during LTM conditioning, then the memory trace can be reconstructed from a pre-formed framework, enabling it to last for longer, whereas if LTM training occurred after the ITM trace had disappeared then the neurons must build a new memory framework (Smyth *et al.*, 2002).

It was also demonstrated that extinction training after ITM training did not increase memory persistence following subsequent LTM training (Smyth *et al.*, 2002). Extinction of the ITM was achieved by placing ITM-trained snails in the same hypoxic environment for 90 min without a reinforcing stimulus. The snails were then immediately subjected to LTM training, but the memory phenotype did not persist for longer than 48 h (Smyth *et al.*, 2002). Because extinction is viewed as a form of learning that co-exists with the previously learned behaviour, it was assumed that 'ITM protein' was used up or replaced with 'extinction protein', resulting in no LTM enhancement (Smyth *et al.*, 2002).

Operant conditioning of aerial respiration clearly represents a versatile behavioural procedure that can be used to elucidate what Smyth *et al.* (2002) defined as the "lingering effects" of ITM on LTM persistence in the title of their paper. Future studies using this system could investigate the neuronal and molecular changes involved in LTM enhancement and whether these are conserved in invertebrate and vertebrate models.

Both ITM and LTM can be extinguished (McComb *et al.*, 2002). In this study, snails received three training sessions followed by extinction training in the same temporal sequence as the operant conditioning training but without a reinforcing stimulus. LTM was not observed when tested the following day (McComb *et al.*, 2002).

From a neuronal perspective, the CPG driving aerial respiration behaviour consists of three interneurons: right pedal dorsal 1 (RPeD1), visceral dorsal 4 (VD4) and (wide-acting) input 3 (IP3). Spiking activity in RPeD1 initiates rhythmic patterns of spiking activity of IP3 that results in pneumostome opening through monosynaptic excitatory synaptic connections to visceral J (VJ) pneumostome opener motor neurons. On the contrary, monosynaptic excitatory

connections exist from VD4 to VK pneumostome closer motor neurons (Syed *et al.*, 1990; Spencer *et al.*, 1999). After conditioning, the excitatory input from RPeD1 to IP3 is weakened, resulting in reduction of the rhythmic activity of the pneumostome (Spencer *et al.*, 1999).

Direct evidence for the role of RPeD1 in operant conditioning comes from soma-ablated snails: when the soma of RPeD1 was ablated before training, the CPG is only capable of mediating ITM, with LTM is no longer observed (Scheibenstock *et al.*, 2002; Sangha, McComb & Lukowiak, 2003a). These data indicate that ITM and LTM are not only distinct in a chronological and transcriptional manner, but are also different at the neuronal level (Braun & Lukowiak, 2011). Because ITM is dependent on new protein synthesis but not altered gene activity (Scheibenstock *et al.*, 2002), it seems that the remaining functional neurites translate the pre-existing messenger RNA (mRNA) into new proteins (Braun & Lukowiak, 2011). However, following ablation of the soma of RPeD1 after consolidation, snails could still access or retrieve a previously encoded memory, indicating that the soma was not essential for memory retention (Lukowiak *et al.*, 2003; Sangha, Scheibenstock & Lukowiak, 2003b). Thus, studies in *Lymnaea* demonstrate for the first time that a single neuron can be a site (although not necessarily the only site) for LTM formation and storage, enabling direct investigations of interactions between a single cell and a neuronal network that are necessary for memory formation, reconsolidation and extinction (Sangha *et al.*, 2003a,b,c).

Because extinction is due to new memory, ablation of the soma of RPeD1 after LTM formation could potentially prevent memory erasure (Sangha *et al.*, 2003a, 2005). Moreover, because ablating the soma of RPeD1 disrupts the reconsolidation of a LTM, the critical molecular processes occurring during both consolidation and reconsolidation not only require both new RNA and protein synthesis, but also that these take place in the same cell in *Lymnaea*. These data are consistent with studies in mammals, where both memory formation and reconsolidation use similar pathways and occur in the same brain regions as the original learning (Nader, Schafe & Le Doux, 2000; Abel & Lattal, 2001). Sangha *et al.* (2005) showed that 'forgetting' was an active process requiring altered gene activity and new protein synthesis. Ablation of the soma of RPeD1 after LTM consolidation resulted in snails that did not forget (LTM persisted for significantly longer).

Operant conditioning of *Lymnaea* aerial respiratory behaviour represents a useful tool to explore how internal and external variables affect learning and to elucidate how the context in which the snails are trained and tested interferes with associative learning (Fig. S4; Haney & Lukowiak, 2001). Haney & Lukowiak (2001) thereby demonstrated the context specificity of learning and LTM in *Lymnaea*. By training snails in a hypoxic environment with a food odorant (carrot odour), LTM was demonstrated only if carrot odour was present also at the time of the memory test (Haney & Lukowiak, 2001). Interestingly, in *Lymnaea* not only

learning, but also extinction is context dependent. In particular, if extinction training was given in a different context from that in which the learning and memory occurred (e.g. hypoxic pond water *versus* food-odorant context), then extinction did not occur (McComb *et al.*, 2002). These data confirm that extinction is itself a form of learning that occludes the original learned behaviour.

Interestingly, Lukowiak *et al.* (2003) demonstrated that snails trained in one context (context 1) but experiencing a new context (context 2) during the reconsolidation period (i.e. during the time when memory is labile) showed memory for context 2. This was defined by the authors as "memory infidelity", where "infidelity" means that snails develop a memory for something they did not undergo training for (Fig. S5). When snails were exposed to context 2 only during the reconsolidation period they incorporated that new context into LTM, even though it was not part of their training (i.e. memory infidelity). Although control snails experienced context 2 for the same interval as the experimental group, because the exposure occurred after the reconsolidation period, memory infidelity did not occur. This approach identified 'unfaithful' memory for the first time in a molluscan model system. Moreover, it was the first time that this process had been observed in a non-declarative memory in any model system (Lukowiak *et al.*, 2007). Considering that it is relatively easy to modify a memory after its reactivation, future studies could attempt to use this system to gain new insights into how memories are made, maintained and modulated.

The operant conditioning paradigm of aerial respiration has also been used to explore how memory formation in *Lymnaea* is affected by environmental stress. Environmental stressors can have a strong, and sometimes unpredictable, modulatory effect on learning and memory formation depending on the nature of the stressor and when it is encountered relative to a period of learning. In *Lymnaea*, as in vertebrates, stressors may block or enhance learning and memory formation (Lukowiak *et al.*, 2014).

We previously explored how different periods of food deprivation could alter the CTA LTM (Ito *et al.*, 2015). Studies using aerial operant conditioning also demonstrate a better ability to form memory in snails trained in the presence of kairomones of their crayfish predator (*Cambarus* spp.) (i.e. the crayfish effluent) (Forest *et al.*, 2016; see Fig. S6). Note, however, that the snails used by Forest *et al.* (2016) were first trained as juveniles, which typically are not capable of forming LTM (McComb, Varshney & Lukowiak, 2005). Interestingly, when those snails were allowed to mature into adults during 4 weeks in their home aquaria and were then trained using the ITM procedure in pond water they formed a LTM. Thus, training the juvenile snails in the presence of crayfish effluent enabled formation of LTM in response to the ITM procedure (Forest *et al.*, 2016).

Adult snails trained with the LTM procedure in the presence of crayfish effluent exhibited increased persistence of LTM, that lasted for at least 8 h, suggesting that predator-augmented memory formation allows the memory to be recalled under a broader range of challenges (Forest *et al.*, 2016). Interestingly, this memory enhancement is associated with long-lasting changes in RPeD1 (Orr & Lukowiak, 2008), suggesting that interspecific chemical communication can augment memory formation, inducing long-lasting changes in this single neuron necessary for LTM formation. This research opens the possibility to investigate how ecologically relevant stressful stimuli can alter behaviour, learning and memory at the level of a single neuron (Dalesman & Lukowiak, 2012). Enhanced LTM formation after training in the presence of crayfish effluent is not observed if snails are exposed to the serotonin blockers mianserin and methysergide, meaning that the perception of risk activates a neuronal mechanism that enhances memory formation through serotonergic modulation (Forest *et al.*, 2016). These data are consistent with previous studies in humans correlating increased serotoninergic tone with enhanced responses to anxiety-related stimuli and memory consolidation (Inoue, Koyama & Yamashita, 1993; Ji & Suga, 2007). Stress-mediated memory enhancement may not necessarily be beneficial and can be exacerbated in pathologies such as post-traumatic stress disorder (PTSD) and panic attacks (Lancaster *et al.*, 2016). Thus, *Lymnaea* represents a valid model system for elucidating how behaviourally relevant stressors can alter LTM formation and/or its persistence and may guide researchers towards possible therapeutic strategies for these psychiatric disorders.

In a recent study, Swinton *et al.* (2019) demonstrated that snails can exhibit configural learning, a higher-order form of learning and memory involving the ability to assign importance to different stimuli. In their protocol, snails simultaneously experienced the odours of crayfish effluent and carrot. Afterwards, animals were transferred for 2 h into their home aquarium where neither odour was present. Snails were then trained with a single training session lasting 30 min (ITM procedure) in hypoxic pond water containing carrot odour. Memory was tested 24 h later in hypoxic pond water and it was found that LTM was formed (Fig. S7). Enhanced LTM formation confirmed that the simultaneous exposure to the two stimuli together led carrot odour, which typically elicits feeding behaviour (Sugai *et al.*, 2006), to acquire the properties of the predator kairomone in enhancing memory-forming ability (Orr *et al.*, 2007). This result did not occur when carrot odour was paired with boiled crayfish effluent, as boiling inactivated its predator-signalling properties (Orr *et al.*, 2007), nor with simultaneous exposure to both odours for only 10 min. Finally, enhanced LTM formation was not observed if training occurred in pond water in the absence of carrot odour. These data show that snails not only possess the ability to consider the individual components of a stimulus but also can form a relationship between the two components that is treated differently from the sum of these stimuli. Interestingly, simultaneous exposure for 45 min to both odours was shown to alter the feeding responses that typically occur when snails are exposed to carrot odour (Swinton *et al.*, 2019). At the end of the conditioning procedure carrot odour no longer elicited a significant increase in

the feeding response, suggesting that after pairing of the two odours snails can assign a new meaning to carrot odour that now signals 'fear' rather than 'food'. In other words, carrot odour acquired a new motivational state (i.e. fear) as opposed to its intrinsic motivational state (i.e. enhanced rasping).

Experience with predators not only shapes learning to guide value-based behaviours, but that value can also be transferred between stimuli that perceptually or conceptually resemble one another (Orr *et al.*, 2007). Such stimulus generalization confers important adaptive advantages allowing the animal to decipher the meaning of important stimuli and to generalize the context of a predator encounter to other environmental conditions. Thus, it is not surprising that this learning process has been conserved across taxa (Swinton *et al.*, 2019).

The experiments and results described above highlight the power of behavioural training in *Lymnaea* to elucidate the neuronal–behavioural crosstalk involved in learning and to study the role of context, stress and internal variables on memory formation and retrieval. The ability of snails to undergo configural learning confirms their potential in neuroscience and behavioural research.

(2) Operant conditioning of escape behaviour

Escape behaviour is the result of a complex integration of information from sensory systems, internal states and expectations, and represents an interesting tool to investigate the molecular and behavioural mechanisms by which organisms acquire, store, and make use of their past experiences (Kobayashi *et al.*, 1998; Evans *et al.*, 2019). Benatti *et al.* (2020) demonstrated that the escape behaviour in *Lymnaea* (Fig. S8) can be operantly conditioned by repeatedly exposing snails to negative reinforcement (KCl) (see Appendix S1 for full details). From the fourth day of training snails trained with KCl attempted to escape significantly less often in both the pre- and post-test session (i.e. when KCl was not present), indicating that a memory had been formed. This effect became more pronounced over time and was accompanied by a significant increase in the latency to first escape (Benatti *et al.*, 2020).

These results suggest that retrieval of the previously acquired memory allows snails to predict and avoid noxious stimuli. Interestingly, this behavioural protocol was used to compare the performance of adult and 'old' snails, demonstrating that ageing did not affect the acquisition of escape memory, but its consolidation in aged snails required more time (Benatti *et al.*, 2020). *Lymnaea*, unlike other well-characterized invertebrate models such as *Drosophila melanogaster* and *Caenorhabditis elegans*, has a relatively long lifespan, allowing the identification of distinct age categories that could be linked with age-related modifications to genetic, molecular, and cellular mechanisms, which often require time to manifest their effects (Rivi *et al.*, 2020). Future studies could investigate the impact of age-related changes in electrophysiological activity, motor and/or chemosensory functions, and the functionality of biochemical components of memory formation on

escape behaviour in ageing snails (Fodor *et al.*, 2020*b*). We also suggest that operant conditioning of escape behaviour, rarely employed in the last 20 years, could be used to unravel a variety of sophisticated cognitive phenomena that were previously thought to be restricted to vertebrates or humans, such as goal-directed decision-making.

V. CONCLUSIONS

(1) This review firstly explored 'what we can teach *Lymnaea*', by discussing a variety of sensitive, solid, easily reproducible, and simple behavioural tests that allow us to investigate how snails modify their behaviours to survive and adapt in an ever-changing environment. Although these conditioning paradigms are based on a restricted set of learned behaviours that snails perform to receive rewards or avoid negative reinforcers, there are other learned behaviours in *Lymnaea* and further studies will allow greater insights into the memory abilities of this model system. Learned behaviours reflect plastic alterations at both the neuronal and molecular level and are dependent on various factors including external stimuli, past experiences, and changes in internal homeostasis. From a translational perspective, *Lymnaea* and its nervous system, can help to understand the flow of neural transformation from behavioural output to sensory coding in more complex systems like the mammalian brain.

(2) So 'what can *Lymnaea* teach us'? Classical and operant conditioning of its feeding and respiratory behaviour have allowed to explore the behavioural, neuronal and molecular mechanisms of memory consolidation, reconsolidation, and extinction revealing that *Lymnaea* shares important characteristics with vertebrates related to associative learning, such as stimulus generalization, generalization of extinction and discriminative learning, and opening the possibility that *Lymnaea* can be used as animal models in translational research.

(3) *Lymnaea* can form LTMs not only after multi-trial conditioning but also after single-trial conditioning. Moreover, *Lymnaea* show 'higher forms' of learning, such as second-order conditioning, contingency learning, and configural learning. These forms of learning also have been identified in other molluscs, including *Limax* (Sahley, Rudy & Gelperin, 1981) and *Aplysia* (Hawkins, Greene & Kandel, 1998), but in *Lymnaea* the neural circuits can be analysed relatively easily. Unlike other invertebrate models (with the exception of bees), *Lymnaea* shares with vertebrates the capacity to acquire configural learning, being able to make associations regarding predation risk and making appropriate responses in similar situations (Giurfa, 2017; Swinton *et al.* 2019).

(4) *Lymnaea* is also 'teaching us' the role of context in memory formation and consolidation. Typically, snails

trained in one context only showed memory if memory testing was performed in the same context, similar to results obtained for other invertebrate models such as *Aplysia*, *C. elegans*, honeybees, *Drosophila* and ants, and in studies on mammals (Ardiel & Rankin, 2010; Bos, Guerrieri & d'Ettorre, 2010; Kahsai & Zars, 2011; Fujinaka *et al.*, 2016; Panoz-Brown *et al.*, 2016; Giurfa, 2017). However, context generalization was reported in predator-experienced *Lymnaea*.

(5) Changing the context in which memory is reconsolidated may have consequences on the accuracy of memory, resulting in 'memory infidelity'. As in 'higher' organisms, including humans, activated memories in *Lymnaea* can re-enter a labile state in which they can be modified or changed during the reconsolidation process, before reconsolidating into a stable, permanent state (Lukowiak *et al.*, 2007). These findings on memory reconsolidation have triggered great interest among mental health professionals who treat disorders based on pathological memories (Alberini & Ledoux, 2013). In recent years, classical and operant conditioning procedures have been translated into preclinical and clinical practice (Bisaz, Travaglia & Alberini, 2014). In particular, memory reconsolidation has been adopted in therapeutic settings to make learning and memory creation more efficient and adaptive, to prevent or rescue memory impairments, and to ameliorate maladaptive memories linked to psychopathologies, such as those associated with PTSD and addiction (Bisaz *et al.*, 2014).

(6) Although many such studies have focused on humans and rodents, a translational approach based on *Lymnaea* may be a rapid and cost-effective option for elucidating the causal, neuronal and molecular changes underlying memory at the level of a single cell.

VI. ACKNOWLEDGEMENTS

VII. REFERENCES

ABEL, T. & LATTAL, K. M. (2001). Molecular mechanisms of memory acquisition, consolidation and retrieval. *Current Opinion in Neurobiology* **11**, 180–187.

AGREN, T. (2014). Human reconsolidation: a reactivation and update. *Brain Research Bulletin* **105**, 70–82.

ALBERINI, C. M. & LEDOUX, J. E. (2013). Memory reconsolidation. *Current Biology: CB* **23**, R746–R750.

ALEXANDER, J., AUDESIRK, T. E. & AUDESIRK, G. J. (1984). One-trial reward learning in the snail *Lymnaea stagnalis*. *Journal of Neurobiology* **15**, 67–72.

ANDREW, R. J. & SAVAGE, H. (2000). Appetitive learning using visual conditioned stimuli in the pond snail, *Lymnaea*. *Neurobiology of Learning and Memory* **73**, 258–273.

ARDIEL, E. L. & RANKIN, C. H. (2010). An elegant mind: learning and memory in *Caenorhabditis elegans*. *Learning & Memory* **17**, 191–201.

AUDESIRK, T. E., ALEXANDER, J. E., AUDESIRK, G. J. & MOYER, C. M. (1982). Rapid, nonaversive conditioning in a freshwater gastropod: I. Effects of age and motivation. *Behavioral and Neural Biology* **36**, 379–390.

BENATTI, C., RIVI, V., COLLIVA, C., RADIGHIERI, G., TASCEDDA, F. & BLOM, J. M. C. (2020). Redefining operant conditioning of escape behaviour in *Lymnaea stagnalis*. *Invertebrate Survival Journal* **17**, 129–137.

BENJAMIN, P. & KEMENES, G. (2010). *Lymnaea* learning and memory. *Scholarpedia* **5**, 4247.

BENJAMIN, P. R., STARAS, K. & KEMENES, G. (2000). A systems approach to the cellular analysis of associative learning in the pond snail *Lymnaea*. *Learning & Memory (Cold Spring Harbor, N.Y.)* **7**, 124–131.

BISAZ, R., TRAVAGLIA, A. & ALBERINI, C. M. (2014). The neurobiological bases of memory formation: from physiological conditions to psychopathology. *Psychopathology* **47**, 347–356.

BOS, N., GUERRIERI, F. J. & D'ETTORRE, P. (2010). Significance of chemical recognition cues is context dependent in ants. *Animal Behaviour* **80**, 839–844.

BOYCOTT, A. E. (1936). The habitats of fresh-water Mollusca in Britain. *The Journal of Animal Ecology* **5**, 116.

BRAAK, H., ZETTERBERG, H., DEL TREDICI, K. & BLENNOW, K. (2013). Intraneuronal tau aggregation precedes diffuse plaque deposition, but amyloid-β changes occur before increases of tau in cerebrospinal fluid. *Acta Neuropathologica* **126**, 631–641.

BRAUN, M. H. & LUKOWIAK, K. (2011). Intermediate and long-term memory are different at the neuronal level in *Lymnaea stagnalis*. *Neurobiology of Learning and Memory* **96**, 403–416.

BREMBS, B. (2003). Operant conditioning in invertebrates. *Current Opinion in Neurobiology* **13**, 710–717.

BRIERLEY, M. J., STARAS, K. & BENJAMIN, P. R. (1997). Behavioral function of glutamatergic interneurons in the feeding system of Lymnaea: plateauing properties and synaptic connections with motor neurons. *Journal of Neurophysiology* **78**, 3386–3395.

BYRNE, J. H. & HAWKINS, R. D. (2015). Nonassociative learning in invertebrates. *Cold Spring Harbor Perspectives in Biology* **7**, a021675.

CAREW, T. J. (1996). Molecular enhancement of memory formation. *Neuron* **16**, 5–8.

CHAMBERS, K. C. (2018). Conditioned taste aversions. *World Journal of Otorhinolaryngology - Head and Neck Surgery* **4**, 92–100.

CROSSLEY, M., LORENZETTI, F. D., NASKAR, S., O'SHEA, M., KEMENES, G., BENJAMIN, P. R. & KEMENES, I. (2019). Proactive and retroactive interference with associative memory consolidation in the snail *Lymnaea* is time and circuit dependent. *Communications Biology* **2**, 1–11.

CURTIS, V., DE BARRA, M. & AUNGER, R. (2011). Disgust as an adaptive system for disease avoidance behaviour. *Philosophical Transactions of the Royal Society B: Biological Sciences* **366**, 389–401.

DALESMAN, S. & LUKOWIAK, K. (2012). How stress alters memory in 'smart' snails. *PLoS One* **7**, e32334.

DICKINSON, A. (1987). Animal conditioning and learning theory. In *Theoretical Foundations of Behavior Therapy* (eds H. J. EYSENCK and I. MARTIN), pp. 57–79. Springer US, Boston.

DOU, J.-T., CHEN, M., DUFOUR, F., ALKON, D. L. & ZHAO, W.-Q. (2005). Insulin receptor signaling in long-term memory consolidation following spatial learning. *Learning & Memory (Cold Spring Harbor, N.Y.)* **12**, 646–655.

ELLIOTT, C. J. & BENJAMIN, P. R. (1985). Interactions of pattern-generating interneurons controlling feeding in *Lymnaea stagnalis*. *Journal of Neurophysiology* **54**, 1396–1411.

EMPTAGE, N. J. & CAREW, T. J. (1993). Long-term synaptic facilitation in the absence of short-term facilitation in *Aplysia* neurons. *Science (New York, N.Y.)* **262**, 253–256.

EVANS, D. A., STEMPEL, A. V., VALE, R. & BRANCO, T. (2019). Cognitive control of escape behaviour. *Trends in Cognitive Sciences* **23**, 334–348.

FENG, Z.-P., ZHANG, Z., VAN KESTEREN, R. E., STRAUB, V. A., VAN NIEROP, P., JIN, K., NEJATBAKHSH, N., GOLDBERG, J. I., SPENCER, G. E., YEOMAN, M. S., WILDERING, W., COORSSEN, J. R., CROLL, R. P., BUCK, L. T., SYED, N. I., et al. (2009). Transcriptome analysis of the central nervous system of the mollusc *Lymnaea stagnalis*. *BMC Genomics* **10**, 451.

FODOR, I., HUSSEIN, A. A., BENJAMIN, P. R., KOENE, J. M. & PIRGER, Z. (2020a). The unlimited potential of the great pond snail, *Lymnaea stagnalis*. *eLife* **9**, e56962.

FODOR, I., URBÁN, P., KEMENES, G., KOENE, J. M. & PIRGER, Z. (2020b). Aging and disease-relevant gene products in the neuronal transcriptome of the great pond snail (*Lymnaea stagnalis*): a potential model of aging, age-related memory loss, and neurodegenerative diseases. *Invertebrate Neuroscience* **20**, 9.

FOREST, J., SUNADA, H., DODD, S. & LUKOWIAK, K. (2016). Training *Lymnaea* in the presence of a predator scent results in a long-lasting ability to form enhanced long-term memory. *Journal of Comparative Physiology A* **202**, 399–409.

FUJINAKA, A., LI, R., HAYASHI, M., KUMAR, D., CHANGARATHIL, G., NAITO, K., MIKI, K., NISHIYAMA, T., LAZARUS, M., SAKURAI, T., KEE, N., NAKAJIMA, S.,

WANG, S.-H. & SAKAGUCHI, M. (2016). Effect of context exposure after fear learning on memory generalization in mice. *Molecular Brain* **9**, 2.

GARCIA, J., HANKINS, W. G. & RUSINIAK, K. W. (1974). Behavioral regulation of the milieu interne in man and rat. *Science* **185**, 824–831.

GARCIA, J., LASITER, P. S., BERMUDEZ-RATTONI, F. & DEEMS, D. A. (1985). A general theory of aversion learning. *Annals of the New York Academy of Sciences* **443**, 8–21.

GEWIRTZ, J. C. (2000). Using Pavlovian higher-order conditioning paradigms to investigate the neural substrates of emotional learning and memory. *Learning & Memory* **7**, 257–266.

GIURFA, M. (2017). Behavioral analysis of learning and memory in honeybees. In *Learning and Memory: A Comprehensive Reference* (ed. J. H. BYRNE), pp. 407–426. Academic Press, Cambridge.

GRIFFITHS, A. J., GELBART, W. M., MILLER, J. H. & LEWONTIN, R. C. (1999). *Darwin's Revolution. Modern Genetic Analysis.* W. H. Freeman, New York.

HANEY, J. & LUKOWIAK, K. (2001). Context learning and the effect of context on memory retrieval in *Lymnaea*. *Learning & Memory (Cold Spring Harbor, N.Y.)* **8**, 35–43.

HAWKINS, R. D., GREENE, W. & KANDEL, E. R. (1998). Classical conditioning, differential cond*itioning, and second-order conditioning of the *Aplysia* gill-withdrawal reflex in a simplified mantle organ preparation. *Behavioral Neuroscience* **112**, 636–645.

HAWKINS, R. D., HON, G. C. & REN, B. (2010). Next-generation genomics: an integrative approach. *Nature Reviews. Genetics* **11**, 476–486.

HERMANN, P. M., LEE, A., HULLIGER, S., MINVIELLE, M., MA, B. & WILDERING, W. C. (2007). Impairment of long-term associative memory in aging snails (*Lymnaea stagnalis*). *Behavioral Neuroscience* **121**, 1400–1414.

INOUE, T., KOYAMA, T. & YAMASHITA, I. (1993). Effect of conditioned fear stress on serotonin metabolism in the rat brain. *Pharmacology, Biochemistry, and Behavior* **44**, 371–374.

ITO, E., OKADA, R., SAKAMOTO, Y., OTSHUKA, E., MITA, K., OKUTA, A., SUNADA, H. & SAKAKIBARA, M. (2012a). Insulin and memory in *Lymnaea*. *Acta Biologica Hungarica* **63**(Suppl 2), 194–201.

ITO, E., OTSUKA, E., HAMA, N., AONUMA, H., OKADA, R., HATAKEYAMA, D., FUJITO, Y. & KOBAYASHI, S. (2012b). Memory trace in feeding neural circuitry underlying conditioned taste aversion in *Lymnaea*. *PLoS One* **7**, e43151.

ITO, E., TOTANI, Y. & OIKE, A. (2017). Necessity knows no law in a snail. *The European Zoological Journal* **84**, 457–464.

ITO, E., YAMAGISHI, M., HATAKEYAMA, D., WATANABE, T., FUJITO, Y., DYAKONOVA, V. & LUKOWIAK, K. (2015). Memory block: a consequence of conflict resolution. *Journal of Experimental Biology* **218**, 1699–1704.

JI, W. & SUGA, N. (2007). Serotonergic modulation of plasticity of the auditory cortex elicited by fear conditioning. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **27**, 4910–4918.

JONES, J. D. (1961). Aspects of respiration in *Planorbis corneus* and *Lymnaea stagnalis* (Gastropoda: Pulmonata). *Comparative Biochemistry and Physiology* **4**, 1–29.

JONES, N., KEMENES, G. & BENJAMIN, P. R. (2001). Selective expression of electrical correlates of differential appetitive classical conditioning in a feeding network. *Journal of Neurophysiology* **85**, 89–97.

JONES, N. G., KEMENES, I., KEMENES, G. & BENJAMIN, P. R. (2003). A persistent cellular change in a single modulatory neuron contributes to associative long-term memory. *Current Biology: CB* **13**, 1064–1069.

KAHSAI, L. & ZARS, T. (2011). Learning and memory in *Drosophila*: behavior, genetics, and neural systems. In *International Review of Neurobiology* (ed. N. ATKINSON), pp. 139–167. Academic Press, Cambridge.

KANDEL, E. & SCHWARTZ, J. (1982). Molecular biology of learning: modulation of transmitter release. *Science* **218**, 433–443.

KATZ, P. S. (2016). Evolution of central pattern generators and rhythmic behaviours. *Philosophical Transactions of the Royal Society B: Biological Sciences* **371**, 20150057.

KAWAI, R., SUNADA, H., HORIKOSHI, T. & SAKAKIBARA, M. (2004). Conditioned taste aversion with sucrose and tactile stimuli in the pond snail *Lymnaea stagnalis*. *Neurobiology of Learning and Memory* **82**, 164–168.

KEMENES, G. (2008). Molecular mechanism of associative learning in *Lymnaea*. In *Learning and Memory: A Comprehensive Reference* (Volume 4, ed. J. H. BYRNE), pp. 133–148. Elsevier, Oxford.

KEMENES, G. & BENJAMIN, P. R. (1989). Appetitive learning in snails shows characteristics of conditioning in vertebrates. *Brain Research* **489**, 163–166.

KEMENES, G. & BENJAMIN, P. R. (1994). Training in a novel environment improves the appetitive learning performance of the snail, *Lymnaea stagnalis*. *Behavioral and Neural Biology* **61**, 139–149.

KEMENES, G. & BENJAMIN, P. R. (2009). Lymnaea. *Current Biology* **19**, R9–R11.

KEMENES, G., STARAS, K. & BENJAMIN, P. R. (1997). In vitro appetitive classical conditioning of the feeding response in the pond snail *Lymnaea stagnalis*. *Journal of Neurophysiology* **78**, 2351–2362.

KEMENES, I., KEMENES, G., ANDREW, R. J., BENJAMIN, P. R. & O'SHEA, M. (2002). Critical time-window for NO-cGMP-dependent long-term memory formation after one-trial appetitive conditioning. *The Journal of Neuroscience* **22**, 1414–1425.

KEMENES, I., STRAUB, V. A., NIKITIN, E. S., STARAS, K., O'SHEA, M., KEMENES, G. & BENJAMIN, P. R. (2006). Role of delayed nonsynaptic neuronal plasticity in long-term associative memory. *Current Biology* **16**, 1269–1279.

KITA, S., HASHIBA, R., UEKI, S., KIMOTO, Y., ABE, Y., GOTODA, Y., SUZUKI, R., URAKI, E., NARA, N., KANAZAWA, A., HATAKEYAMA, D., KAWAI, R., FUJITO, Y., LUKOWIAK, K. & ITO, E. (2011). Does conditioned taste aversion learning in the pond snail *Lymnaea stagnalis* produce conditioned fear? *The Biological Bulletin* **220**, 71–81.

KOBAYASHI, S., KOJIMA, S., YAMANAKA, M., SADAMOTO, H., NAKAMURA, H., FUJITO, Y., KAWAI, R., SAKAKIBARA, M. & ITO, E. (1998). Operant conditioning of escape behavior in the pond snail, *Lymnaea stagnalis*. *Zoological Science* **15**, 683–690.

KOJIMA, S., NANAKAMURA, H., NAGAYAMA, S., FUJITO, Y. & ITO, E. (1997). Enhancement of an inhibitory input to the feeding central pattern generator in *Lymnaea stagnalis* during conditioned taste-aversion learning. *Neuroscience Letters* **230**, 179–182.

KOJIMA, S., YAMANAKA, M., FUJITO, Y. & ITO, E. (1996). Differential neuroethological effects of aversive and appetitive reinforcing stimuli on associative learning in *Lymnaea stagnalis*. *Zoological Science* **13**, 803–812.

KORNEEV, S. A., VAVOULIS, D. V., NASKAR, S., DYAKONOVA, V. E., KEMENES, I. & KEMENES, G. (2018). A CREB2-targeting microRNA is required for long-term memory after single-trial learning. *Scientific Reports* **8**, 1–12.

LANCASTER, C., TEETERS, J., GROS, D. & BACK, S. (2016). Posttraumatic stress disorder: overview of evidence-based assessment and treatment. *Journal of Clinical Medicine* **5**, 105.

LIN, J.-Y., ARTHURS, J. & REILLY, S. (2017). Conditioned taste aversions: from poisons to pain to drugs of abuse. *Psychonomic Bulletin & Review* **24**, 335–351.

LUKOWIAK, K. (1991). Experimental reconstruction of neuronal pattern generators. *Current Opinion in Neurobiology* **1**, 577–582.

LUKOWIAK, K., ADATIA, N., KRYGIER, D. & SYED, N. (2000). Operant conditioning in *Lymnaea*: evidence for intermediate- and long-term memory. *Learning & Memory (Cold Spring Harbor, N.Y.)* **7**, 140–150.

LUKOWIAK, K., COTTER, R., ROSE, J., RINGSEIS, E. & SPENCER, G. (1998). Long-term memory of an operantly conditioned respiratory behaviour pattern in *Lymnaea stagnalis*. *The Journal of Experimental Biology* **201**, 877–882.

LUKOWIAK, K., FRAS, M., SMYTH, K., WONG, C. & HITTEL, K. (2007). Reconsolidation and memory infidelity in *Lymnaea*. *Neurobiology of Learning and Memory* **87**, 547–560.

LUKOWIAK, K., RINGSEIS, E., SPENCER, G., WILDERING, W. & SYED, N. (1996). Operant conditioning of aerial respiratory behaviour in *Lymnaea stagnalis*. *The Journal of Experimental Biology* **199**, 683–691.

LUKOWIAK, K., SANGHA, S., SCHEIBENSTOCK, A., PARVEZ, K., McCOMB, C., ROSENEGGER, D., VARSHNEY, N. & SADAMOTO, H. (2003). A molluscan model system in the search for the engram. *Journal of Physiology, Paris* **97**, 69–76.

LUKOWIAK, K., SUNADA, H., TESKEY, M., LUKOWIAK, K. & DALESMAN, S. (2014). Environmentally relevant stressors alter memory formation in the pond snail *Lymnaea*. *Journal of Experimental Biology* **217**, 76–83.

LUKOWIAK, K. & SYED, N. (1999). Learning, memory and a respiratory central pattern generator. *Comparative Biochemistry and Physiology - Part A, Molecular & Integrative Physiology* **124**, 265–274.

MARRA, V., KEMENES, I., VAVOULIS, D., FENG, J., O'SHEA, M. & BENJAMIN, P. R. (2010). Role of tonic inhibition in associative reward conditioning in *Lymnaea*. *Frontiers in Behavioral Neuroscience* **4**, 161.

MARRA, V., O'SHEA, M., BENJAMIN, P. R. & KEMENES, I. (2013). Susceptibility of memory consolidation during lapses in recall. *Nature Communications* **4**, 1578.

McCOMB, C. (2005). Juvenile *Lymnaea* ventilate, learn and remember differently than do adult *Lymnaea*. *Journal of Experimental Biology* **208**, 1459–1467.

McCOMB, C., SANGHA, S., QADRY, S., YUE, J., SCHEIBENSTOCK, A. & LUKOWIAK, K. (2002). Context extinction and associative learning in *Lymnaea*. *Neurobiology of Learning and Memory* **78**, 23–34.

McCOMB, C., VARSHNEY, N. & LUKOWIAK, K. (2005). Juvenile *Lymnaea* ventilate, learn and remember differently than do adult *Lymnaea*. *The Journal of Experimental Biology* **208**, 1459–1467.

McCROHAN, C. R. (1984). Properties of ventral cerebral neurones involved in the feeding system of the snail, *Lymnaea stagnalis*. *Journal of Experimental Biology* **108**, 257–272.

MITA, K., YAMAGISHI, M., FUJITO, Y., LUKOWIAK, K. & ITO, E. (2014). An increase in insulin is important for the acquisition conditioned taste aversion in *Lymnaea*. *Neurobiology of Learning and Memory* **116**, 132–138.

MOROZ, L., HASAN, S., BULLOCH, A., LUKOWIAK, K. & SYED, N. (1993). Behavioral analysis of respiration in *Lymnaea stagnalis*. *Society for Neuroscience – Abstracts* **23**, 183.

MURAKAMI, J., OKADA, R., SADAMOTO, H., KOBAYASHI, S., MITA, K., SAKAMOTO, Y., YAMAGISHI, M., HATAKEYAMA, D., OTSUKA, E., OKUTA, A., SUNADA, H., TAKIGAMI, S., SAKAKIBARA, M., FUJITO, Y., AWAJI, M., MORIYAMA, S., LUKOWIAK, K. & ITO, E. (2013). Involvement of insulin-like peptide in long-term synaptic plasticity and long-term memory of the pond snail *Lymnaea stagnalis*. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **33**, 371–383.

NADER, K., SCHAFE, G. E. & LE DOUX, J. E. (2000). Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature* **406**, 722–726.

Nakai, J., Totani, Y., Kojima, S., Sakakibara, M. & Ito, E. (2020). Features of behavioral changes underlying conditioned taste aversion in the pond snail *Lymnaea stagnalis*. *Invertebrate Neuroscience* **20**, 8.

Nikitin, E. S., Vavoulis, D. V., Kemenes, I., Marra, V., Pirger, Z., Michel, M., Feng, J., O'Shea, M., Benjamin, P. R. & Kemenes, G. (2008). Persistent sodium current is a nonsynaptic substrate for long-term associative memory. *Current Biology* **18**, 1221–1226.

Orr, M. V., El-Bekai, M., Lui, M., Watson, K. & Lukowiak, K. (2007). Predator detection in *Lymnaea stagnalis*. *Journal of Experimental Biology* **210**, 4150–4158.

Orr, M. V. & Lukowiak, K. (2008). Electrophysiological and behavioral evidence demonstrating that predator detection alters adaptive behaviors in the snail *Lymnaea*. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **28**, 2726–2734.

Panoz-Brown, D., Corbin, H. E., Dalecki, S. J., Gentry, M., Brotheridge, S., Sluka, C. M., Wu, J.-E. & Crystal, J. D. (2016). Rats remember items in context using episodic memory. *Current Biology: CB* **26**, 2821–2826.

Pavlov, I. P. (1927). *Conditioned Reflexes: An Investigation of the Physiological Activity of the Cerebral Cortex*. Oxford University Press, Oxford.

Pirger, Z., László, Z., Kemenes, I., Tóth, G., Reglődi, D. & Kemenes, G. (2010). A homolog of the vertebrate pituitary adenylate cyclase-activating polypeptide is both necessary and instructive for the rapid formation of associative memory in an invertebrate. *Journal of Neuroscience* **30**, 13766–13773.

Rivi, V., Benatti, C., Colliva, C., Radighieri, G., Brunello, N., Tascedda, F. & Blom, J. M. C. (2020). *Lymnaea stagnalis* as model for translational neuroscience research: from pond to bench. *Neuroscience & Biobehavioral Reviews* **108**, 602–616.

Rodgers, R. J., Cao, B. J., Dalvi, A. & Holmes, A. (1997). Animal models of anxiety: an ethological perspective. *Brazilian Journal of Medical and Biological Research = Revista Brasileira De Pesquisas Medicas E Biologicas* **30**, 289–304.

Rosenegger, D., Wright, C. & Lukowiak, K. (2010). A quantitative proteomic analysis of long-term memory. *Molecular Brain* **3**, 9.

Rosenzweig, M. R., Bennett, E. L., Colombo, P. J., Lee, D. W. & Serrano, P. A. (1993). Short-term, intermediate-term, and long-term memories. *Behavioural Brain Research* **57**, 193–198.

Sadamoto, H., Kitahashi, T., Fujito, Y. & Ito, E. (2010). Learning-dependent gene expression of CREB1 isoforms in the Molluscan brain. *Frontiers in Behavioral Neuroscience* **4**, 25.

Sahley, C., Rudy, J. W. & Gelperin, A. (1981). An analysis of associative learning in a terrestrial mollusc. *Journal of Comparative Physiology* **144**, 1–8.

Saleuddin, S. & Mukai, S. (2017). *Physiology of Molluscs: A Collection of Selected Reviews, Two-Volume Set*. CRC Press, Boca Raton.

Sangha, S., McComb, C. & Lukowiak, K. (2003a). Forgetting and the extension of memory in *Lymnaea*. *The Journal of Experimental Biology* **206**, 71–77.

Sangha, S., Scheibenstock, A. & Lukowiak, K. (2003b). Reconsolidation of a long-term memory in *Lymnaea* requires new protein and RNA synthesis and the soma of right pedal dorsal 1. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **23**, 8034–8040.

Sangha, S., Scheibenstock, A., Martens, K., Varshney, N., Cooke, R. & Lukowiak, K. (2005). Impairing forgetting by preventing new learning and memory. *Behavioral Neuroscience* **119**, 787–796.

Sangha, S., Scheibenstock, A., Morrow, R. & Lukowiak, K. (2003c). Extinction requires new RNA and protein synthesis and the soma of the cell right pedal dorsal 1 in *Lymnaea stagnalis*. *Journal of Neuroscience* **23**, 9842–9851.

Scheibenstock, A., Krygier, D., Haque, Z., Syed, N. & Lukowiak, K. (2002). The Soma of RPeD1 must be present for long-term memory formation of associative learning in *Lymnaea*. *Journal of Neurophysiology* **88**, 1584–1591.

Scutt, G., Allen, M., Kemenes, G. & Yeoman, M. (2015). A switch in the mode of the sodium/calcium exchanger underlies an age-related increase in the slow afterhyperpolarization. *Neurobiology of Aging* **36**, 2838–2849.

Seyer, J.-O. (1992). Resolution and sensitivity in the eye of the winkle *Littorina littorea*. *Journal of Experimental Biology* **170**, 57–69.

Smith, M., Riby, L., Eekelen, J. & Foster, J. (2010). Glucose enhancement of human memory: a comprehensive research review of the glucose memory facilitation effect. *Neuroscience and Biobehavioral Reviews* **35**, 770–783.

Smyth, K., Sangha, S. & Lukowiak, K. (2002). Gone but not forgotten: the lingering effects of intermediate-term memory on the persistence of long-term memory. *Journal of Experimental Biology* **205**, 131–140.

Spencer, G. E., Kazmi, M. H., Syed, N. I. & Lukowiak, K. (2002). Changes in the activity of a CpG neuron after the reinforcement of an operantly conditioned behavior in *Lymnaea*. *Journal of Neurophysiology* **88**, 1915–1923.

Spencer, G. E., Syed, N. I., van Kesteren, E., Lukowiak, K., Geraerts, W. P. & van Minnen, J. (2000). Synthesis and functional integration of a neurotransmitter receptor in isolated invertebrate axons. *Journal of Neurobiology* **44**, 72–81.

Spencer, G. E., Syed, N. I. & Lukowiak, K. (1999). Neural changes after operant conditioning of the aerial respiratory behavior in *Lymnaea stagnalis*. *The Journal of Neuroscience* **19**, 1836–1843.

Squire, L. R., Genzel, L., Wixted, J. T. & Morris, R. G. (2015). Memory consolidation. *Cold Spring Harbor Perspectives in Biology* **7**(8), a021766.

Staras, K., Kemenes, G. & Benjamin, P. R. (1998). Pattern-generating role for motoneurons in a rhythmically active neuronal network. *Journal of Neuroscience* **18**, 3669–3688.

Staras, K., Kemenes, G. & Benjamin, P. R. (1999). Cellular traces of behavioral classical conditioning can be recorded at several specific sites in a simple nervous system. *Journal of Neuroscience* **19**, 347–357.

Staras, K., Kemenes, I., Benjamin, P. R. & Kemenes, G. (2003). Loss of self-inhibition is a cellular mechanism for episodic rhythmic behavior. *Current Biology* **13**, 116–124.

Straub, V. A. (2004). Central localization of plasticity involved in appetitive conditioning in *Lymnaea*. *Learning & Memory* **11**, 787–793.

Straub, V. A., Staras, K., Kemenes, G. & Benjamin, P. R. (2002). Endogenous and network properties of Lymnaea feeding central pattern generator interneurons. *Journal of Neurophysiology* **88**, 1569–1583.

Sugai, R., Azami, S., Shiga, H., Watanabe, T., Sadamoto, H., Kobayashi, S., Hatakeyama, D., Fujito, Y., Lukowiak, K. & Ito, E. (2007). One-trial conditioned taste aversion in *Lymnaea*: good and poor performers in long-term memory acquisition. *The Journal of Experimental Biology* **210**, 1225–1237.

Sugai, R., Shiga, H., Azami, S., Watanabe, T., Sadamoto, H., Fujito, Y., Lukowiak, K. & Ito, E. (2006). Taste discrimination in conditioned taste aversion of the pond snail *Lymnaea stagnalis*. *The Journal of Experimental Biology* **209**, 826–833.

Sunada, H., Lukowiak, K. & Ito, E. (2017). Cerebral giant cells are necessary for the formation and recall of memory of conditioned taste aversion in *Lymnaea*. *Zoological Science* **34**, 72–80.

Swinton, C., Swinton, E., Shymansky, T., Hughes, E., Zhang, J., Rothwell, C., Kakadiya, M. & Lukowiak, K. (2019). Configural learning: a higher form of learning in *Lymnaea*. *The Journal of Experimental Biology* **222**, jeb190405.

Syed, N. I., Bulloch, A. G. & Lukowiak, K. (1990). In vitro reconstruction of the respiratory central pattern generator of the mollusk *Lymnaea*. *Science (New York, N.Y.)* **250**, 282–285.

Syed, N. I., Ridgway, R. L., Lukowiak, K. & Bulloch, A. G. M. (1992). Transplantation and functional integration of an identified respiratory interneuron in *Lymnaea stagnalis*. *Neuron* **8**, 767–774.

Tascedda, F., Malagoli, D., Accorsi, A., Rigillo, G., Blom, J. M. C. & Ottaviani, E. (2015). Molluscs as models for translational medicine. *Medical Science Monitor Basic Research* **21**, 96–99.

ter Maat, A. (1992). Egg laying in the hermaphrodite pond snail *Lymnaea stagnalis*. *Progress in Brain Research* **92**, 345–360.

Totani, Y., Nakai, J., Dyakonova, V. E., Lukowiak, K., Sakakibara, M. & Ito, E. (2020). Induction of LTM following an insulin injection. *eNeuro* **7**, ENEURO.0088-20.2020. https://doi/org.10.1523/ENEURO.0088-20.2020.

Walters, E. T., Carew, T. J. & Kandel, E. R. (1979). Classical conditioning in *Aplysia californica*. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 6675–6679.

Whelan, H. A. & Mccrohan, C. R. (1996). Food-related conditioning and neuronal correlates in the freshwater snail *Lymnaea stagnalis*. *Journal of Molluscan Studies* **62**, 483–494.

Willner, P. (1986). Validation criteria for animal models of human mental disorders: learned helplessness as a paradigm case. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **10**, 677–690.

Yeoman, M. S., Brierley, M. J. & Benjamin, P. R. (1996). Central pattern generator interneurons are targets for the modulatory serotonergic cerebral giant cells in the feeding system of *Lymnaea*. *Journal of Neurophysiology* **75**, 11–25.

Yeoman, M. S., Vehovszky, A., Kemenes, G., Elliott, C. J. & Benjamin, P. R. (1995). Novel interneuron having hybrid modulatory-central pattern generator properties in the feeding system of the snail, *Lymnaea stagnalis*. *Journal of Neurophysiology* **73**, 112–124.

Zhao, W., Chen, H., Xu, H., Moore, E., Meiri, N., Quon, M. J. & Alkon, D. L. (1999). Brain insulin receptors and spatial memory. Correlated changes in gene expression, tyrosine phosphorylation, and signalling molecules in the hippocampus of water maze trained rats. *The Journal of Biological Chemistry* **274**, 34893–34902.

VIII. Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article. **Appendix S1**. Methods used to investigate the neural basis of learning and memory in *Lymnaea stagnalis*.

Fig. S1. Training procedures for chemical food-reward classical conditioning (adapted from Kemenes *et al.*, 2002). Snails were pre-tested for feeding responses in the presence of water (W) followed by the conditional stimulus (CS). Conditioned snails were exposed to a single pairing of the CS with the unconditional stimulus (US), whereas the three control groups were subjected to an unpaired CS and US trial or to CS- or US-alone trials. The memory test for the CS was performed 24 h after training.

Fig. S2. Conditioning procedures for conditioned taste aversion (CTA) (adapted from Sugai *et al.*, 2006, 2007). (A) Single-trial conditioning procedure; (B) multi-trial conditioning procedure. The CS and US were added to the dish for 15 s with an inter-stimulus interval of 15 s. In the multi-trial conditioning procedure (10 trials) the inter-trial interval lasted 10 min. The feeding response was measured for 1 min in both the pre-test and post-training test. A backward-conditioned (US+CS pairing) and a naive (DW only) control group was also employed. (C) Second-order CTA. In the first phase, the first-order CTA was established by pairing the first CS (CS1) with the US. In the second phase, a second CS (CS2) was paired in a forward manner with CS1, which now served as the US. CS, conditional stimulus; DW, distilled water; US, unconditional stimulus.

Fig. S3. Training procedures for aerial respiration behaviour (adapted from Smyth *et al.*, 2002). (A) LTM conditioning procedure; (B) ITM conditioning procedure. Animals were trained on a single day with three sessions lasting 15 min and an inter-session interval of 1 h (A) or 30 min (B). During the training period, poking of the pneumostome area was applied each time the animal attempted to open its pneumostome. Snails of the yoked control group were exposed to the same procedures as the conditioned group but pneumostome stimulation was not contingent upon its opening movements. ITM, intermediate-term memory; LTM, long-term memory; PW, pond water. ITM and LTM can be differentially produced by altering the duration, number and intervals used in training protocols for this behaviour.

Fig. S4. Training procedures for studying context learning (adapted from Haney & Lukowiak, 2001). Snails were trained with three 15-min training sessions in hypoxic pond water (PW) and carrot odour (CO) with a 1-h interval between training sessions in eumoxic PW. During the training period snails received a tactile stimulus as described in the legend to Fig. S3. A yoked control procedure (see legend to Fig. S3) was also employed. In both the conditioned and the yoked control groups memory testing was performed in hypoxic PW + CO. Snails of the CO + PW control group

were trained in hypoxic PW + CO but memory testing was performed in hypoxic PW.

Fig. S5. Training procedures for studying memory infidelity (adapted from Lukowiak *et al.*, 2007). Snails were operantly conditioned not to perform aerial respiratory behaviour (by poking, see legend to Fig. S3) in a specific context (i.e. context 1). During the 1-h reconsolidation period snails were exposed to a new context (i.e. context 2). Snails of the control group were exposed to context 1 during the reconsolidation period, whereas in the post-reconsolidation period they were placed for 1 h in context 2. A yoked control procedure (see legend to Fig. S3) was also employed. All snails were tested for memory 24 h later in context 2. PW, pond water.

Fig. S6. Training procedures for operant conditioning of aerial respiration behaviour in the presence of the crayfish effluent (adapted from Forest *et al.*, 2016). The behavioural protocol (training by poking, see legend to Fig. S3) consisted of 10 min of acclimation in hypoxic pond water (PW) containing crayfish effluent (CE), followed by a single 30-min training session. A yoked control procedure (see legend to Fig. S3) was also employed. Snails of the CE control group were exposed to hypoxic PW + CE for 40 min without receiving any training. Snails of the PW control group were trained in hypoxic PW only. All snails were tested for memory 24 h later in hypoxic PW.

Fig. S7. Conditioning procedures for studying configural learning (adapted from Swinton *et al.*, 2019). During the pre-test, snails of the conditioned group were simultaneously exposed to CO + CE for 45 min, 2 h before being trained in CO alone. Training sessions involved poking, as described in the legend to Fig. S3. Snails of the boiled CO + CE control group were exposed to CO + boiled CE for 45 min, whereas snails of the CO + CE 10 min control group were exposed to CO + CE for only 10 min. Snails of the PW control group were trained in hypoxic PW alone. All snails were tested for memory 24 h after training in hypoxic PW. CE, crayfish effluent; CO, carrot odour; PW, pond water.

Fig. S8. Training procedures for operant conditioning of escape behaviour (adapted from Benatti *et al.*, 2020). For each session, the number of escapes performed in a 20-min interval and the time necessary for the first escape to occur were recorded. Snails of the conditioned group were exposed to a KCl solution soaked into a sheet of filter paper (i.e. negative reinforcement) during the training session. For pre-training and during testing, and in the control group, distilled water (DW)-soaked paper was used instead. The behavioural procedure was repeated over four days.