REVIEW ARTICLE

CANADIAN ASSOCIATION OF NEUROSCIENCES REVIEW: Learning at a Snail's Pace

Kashif Parvez, David Rosenegger, Michael Orr, Kara Martens, Ken Lukowiak

ABSTRACT: While learning and memory are related, they are distinct processes each with different forms of expression and underlying molecular mechanisms. An invertebrate model system, *Lymnaea stagnalis*, is used to study memory formation of a non-declarative memory. We have done so because: 1) We have discovered the neural circuit that mediates an interesting and tractable behaviour; 2) This behaviour can be operantly conditioned and intermediate-term and long-term memory can be demonstrated; and 3) It is possible to demonstrate that a single neuron in the model system is a necessary site of memory formation. This article reviews how *Lymnaea* has been used in the study of behavioural and molecular mechanisms underlying consolidation, reconsolidation, extinction and forgetting.

RÉSUMÉ: L'apprentissage au pas d'escargot. Bien que l'apprentissage et la mémoire soient deux fonctions connexes, leurs processus sont distincts et chacun a des formes d'expression différentes et des mécanismes moléculaires sous-jacents différents. Nous utilisons un système dans un modèle invertébré, la Lymnaea stagnalis, pour étudier comment se forme une mémoire non déclarative. Nous avons utilisé ce modèle parce que : 1) Nous avons découvert un circuit neural qui assure la médiation d'un comportement intéressant et observable; 2) Ce comportement peut être conditionné en cours d'étude et la mémoire à moyen et à long terme peut être démontrée; 3) Il est possible de démontrer dans ce modèle qu'un seul neurone est nécessaire pour la formation de la mémoire. Cet article revoit comment la Lymnaea a été utilisée pour étudier les mécanismes comportementaux et moléculaires sous-jacents à la consolidation, à la reconsolidation, à l'extinction et à l'oubli.

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CLASSIFICATIONS OF LEARNING AND MEMORY

The ability to learn and form a memory enables organisms to adapt to a changing environment so as to be better able to survive. Learning and memory are behavioural manifestations of activity within individual neurons and neuronal circuits. While learning and memory are interrelated, they are separate processes each with different underlying molecular mechanisms and forms of expression. Learning can be broadly defined as the acquisition of a new behaviour, while memory is defined as the ability to both store and recall the new information.

Learning and memory (or their correlates) have been studied at behavioural, systems, neuronal, and sub-cellular levels in organisms ranging from humans to worms. In this short review we will focus on research in an invertebrate model system that has provided insight into the underlying mechanisms of memory formation. While an understanding of the causal mechanisms of learning and memory formation in snails is of heuristic interest, our main reason for employing such a molluscan model system is to gain insight as to how learning and memory occur in us. It appears that the 'substrates' for learning and memory have been fairly conserved in all organisms throughout evolution. Moreover, there are many experimental advantages (see below) to using invertebrate model systems over mammalian preparations. Thus, gaining an understanding of causal mechanisms of memory formation in a snail may help us better understand how memory systems function in higher organisms such as in humans. Since this paper will focus primarily on how memory is formed following learning we first need to describe how memory is classified.

Memory encompasses a broad spectrum of sub-types. Studies on humans have examined both declarative and non-declarative forms of memory. Declarative (or explicit) memory is the memory of facts and events, while non-declarative (implicit or procedural) memory is the memory of 'how' to do things (e.g.

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Reprint requests to: Ken Lukowiak, Hotchkiss Brain Institute, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta, T2N 4N1, Canada.

motor memory). However, there are a number of complexities associated with declarative memory that have made it more difficult to study. For example, declarative memories are initially formed in different neuronal circuits in the brain than the ones where they are ultimately stored. The distributed nature of declarative memory makes it difficult to determine if an unexpressed memory has been forgotten (i.e. no longer stored) or is not accessible at a specific time (i.e. a failure in retrieval). Moreover, whether a memory is no longer stored (i.e. forgotten) or just not able to be accessed at a specific time is problematic. These problems may be avoided by studying non-declarative memory. Non-declarative memories are stored in the circuit that mediates the behavior.[1-3] Thus, we know 'where' the memory is stored if we can determine which neurons are responsible for generating the behavior. Since the memory is stored within the circuit that mediates the behaviour, we eliminate inaccessibility as an explanation for lack of memory expression.

Probably, the most basic and fundamental form of learning is non-associative learning. Habituation and sensitization are two well-known examples of non-associative learning. In the case of habituation the animal learns and remembers not to respond to a 'benign' (i.e. harmless) stimulus that has been repeatedly presented to it. The analysis of how such learning and memory are encoded in neurons was greatly aided by the development of a set of parametric characteristics of habituation that all animals exhibited[4] and the development of model systems such as *Aplysia*. Model organisms showed that habituation was similar in intact animals and in reduced semi-intact preparations, which allowed simultaneous study of behaviour and neurophysiology.[5-7] Sensitization, on the other hand, can de defined as an increase in response amplitude following the presentation of another distinct non-contingent stimulus. Again, our understanding of how sensitization is mediated at the neuronal level came from the development of model systems where behaviour and neural activity could be studied simultaneously.[8] Finally, with both habituation and sensitization it was shown that long-lasting memory was dependent on new protein synthesis and altered gene activity.[5,9]

A more complicated and important form of learning is associative learning. Associative learning can itself be divided into two types; classical conditioning and operant conditioning.[3,10,11] In classical conditioning (i.e. Pavlovian conditioning) a 'neutral' stimulus, known as the conditional stimulus (CS) is temporally paired with a stimulus, known as the unconditional stimulus (US), that inevitably elicits a response (the unconditional response; UR). With repeated CS-US pairings, but not US-CS pairings (backwards conditioning), the CS comes to elicit the UR. That is, the animal learns that the CS predicts the occurrence of the US. The most famous example of this form of learning is that of Pavlov's dogs who learned, after a number of CS-US pairings, that the presentation of a tone, signaled the presentation of a food-substance. Thus, the dogs salivated (the UR) on hearing the tone (CS) even if the US (the food-substance) was not presented.

In contrast to classical conditioning, operant conditioning involves application of a reinforcement stimulus contingent upon spontaneous performance of a specific behaviour.[12-14] With repeated contingent presentation of the reinforcing stimulus the occurrence of the behaviour changes.[10] The reinforcing stimulus can result in either increasing the occurrence of the behaviour (positive reinforcement) or decrease its occurrence (negative reinforcement). In short, operant conditioning results in an animal learning the consequence of its behaviour.

Lymnaea stagnalis as a model system

A vast variety of model systems ranging from invertebrates (e.g. *Lymnaea*,[15,16] *C. elegans*,[17] *Aplysia*,[18-20] *Apis*,[21] *Drosophila*,[22] *Hermissenda*[23]) to non-human primates are available to researchers for the study of memory formation. Each model system comes with benefits and drawbacks. For example, one benefit of using a rodent model system is that they have all the neural structures hypothesized to play key roles in human declarative memory formation and storage (e.g. the hippocampus, amygdala and cortex). The equivalent brain structures in rodents are also necessary for memory formation and recall. However, in rodents as in humans, declarative memory is stored 'somewhere' (i.e. in multiple areas) in the cortex. Attempts have been made to directly study how and where the various components of the memory (e.g. visual, olfactory, auditory, emotional, etc) are stored and accessed. There has been progress in localizing auditory fear conditioning (i.e. classical conditioning) to the lateral amygdala.[24,25] Another form of associative learning, conditioned taste aversion (CTA), has been localized to the insular and prepiriform cortices and the amygdala.[26-28] However, as of yet the necessary molecular changes in neurons that cause memory formation in the aforementioned structures have not been elucidated.

With the above caveats in mind we have taken to using an invertebrate model system to study memory formation of a non-declarative memory. We have done so because: 1) We have discovered the neural circuit that mediates an interesting, tractable behaviour; 2) This behaviour can be operantly conditioned and long-lasting memory can be demonstrated; and 3) It is possible to demonstrate that a single neuron in the model system is a necessary site of memory formation.

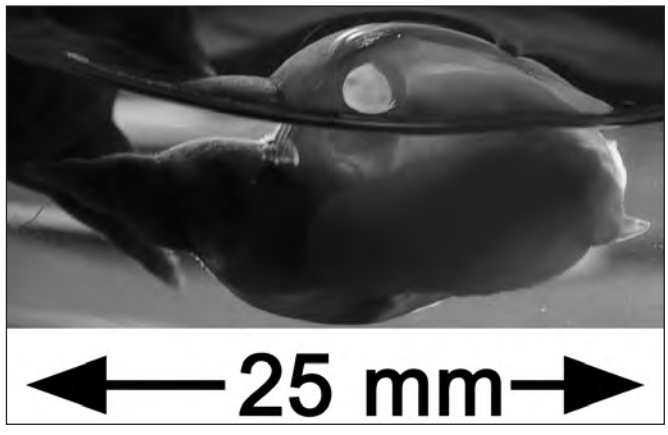

Figure 1: *Lymnaea with its pneumostome open, performing aerial respiration. Adult Lymnaea measure approximately 25 mm in shell length.*

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Lymnaea stagnalis, a freshwater pond snail, is an ideal model system for the study of learning and memory. There are many advantages to studying learning and memory in this mollusc. *Lymnaea* are bimodal breathers, meaning they can exchange O_2 and CO_2 cutaneously (through their skin) or aerially through an orifice (i.e. pneumostome; Jones, 1961; Lukowiak et al, 1996). To aerially respire, the snail approaches the water surface, opens its pneumostome and begins gas exchange. The snail must come to the air-water interface for aerial respiration and it is easy to observe this behaviour.[29]

Another advantage to *Lymnaea* is that it has a simple central nervous system consisting of several thousand neurons. Some of the unusually large neurons have been characterized and are easily identified. Most importantly, a three neuron central pattern generator (CPG) has been thoroughly characterized and proven both necessary and sufficient for controlling aerial respiratory behaviour in *Lymnaea*.[30,31]

Central Pattern Generator

The aerial respiratory CPG in *Lymnaea* consists of three identified interneurons. Right Pedal Dorsal 1 (RPeD1) is a dopaminergic interneuron that initiates the respiratory rhythm. Right Pedal Dorsal 1 receives chemosensory inputs from the pneumostome area[32] to modulate its activity. The remaining two interneurons in the CPG are Visceral Dorsal 4 (VD4) and the Input 3 Interneuron (IP3I). Visceral Dorsal 4 initiates pneumostome closure by excitation of VK closer motor neurons.

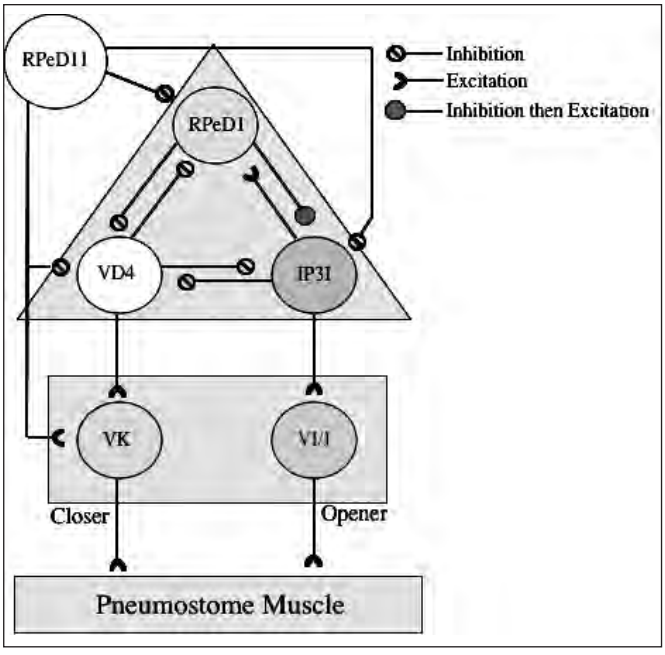

Figure 2: *A schematic diagram illustrating the connectivity of the three interneuron CPG that controls aerial respiration in Lymnaea. Included in the diagram is the neuron that regulates the whole-body withdrawal reflex (i.e. RPeD11) and the neurons responsible for the opening (i.e. VK motor neurons) and closing (i.e. VI/J motor neurons) of the pneumostome.*

Input 3 Interneuron initiates pneumostome opening by excitation of VI/J opener motor neurons. All synaptic connections between the 3 interneurons are monosynaptic. Right Pedal Dorsal 1 excites IP3I via a biphasic (inhibitory then excitatory) synaptic connection. Input 3 Interneuron and VD4 have mutually inhibitory synaptic connections as do RPeD1 and VD4. The activities of VD4 and IP3I alternate to generate the rhythmic opening and closing of the pneumostome (see Lukowiak[33] for a description of how the emergent network properties cause rhythmic activity in this circuit). The sufficiency and necessity of the 3-neuron CPG in producing rhythmogenesis was tested by reconstructing the CPG in vitro and performing cell killing and transplantation experiments.[31]

Thus *Lymnaea* was demonstrated to possess an easily observable behaviour (aerial respiration) that is driven by a 'known' neural network; it only remained to be shown that the behaviour could exhibit both associative learning and long-term memory (LTM). This was accomplished by our laboratory in 1996[15] when it was shown that aerial respiratory behaviour could be operantly conditioned and that LTM resulted following conditioning.

Operant Conditioning of Aerial Respiratory Behaviour in Lymnaea

Operant conditioning of aerial respiratory behaviour in *Lymnaea* is performed by placing snails into a beaker filled with hypoxic pond-water (PW). The PW is made hypoxic by bubbling N_2 gas through it for 20 minutes. Hypoxia is used to increase the snails' drive to perform aerial respiration. In hypoxic conditions, snails crawl to the air-water interface to open their pneumostome and perform gas exchange with the external environment. When they open their pneumostome we apply a negative reinforcement in the form of a gentle tactile stimulus. This reinforcing stimulus is of sufficient strength to cause pneumostome closure but does not elicit the whole-animal withdrawal response. With repeated application of the negative reinforcement, snails reduce the number of attempted pneumostome openings when placed in the hypoxic context.[16]

We record the number of attempted pneumostome openings during training sessions (TS1 and TS2) and a 'savings-test' (memory test; MT). We defined learning and memory operationally.[15,34,35] Learning is present if the number of attempted pneumostome openings in the last training session is significantly less than the number of attempted pneumostome openings in the first training session. In order for memory to be present when 'savings' is tested (memory test, MT), two criteria must be met: 1) the number of attempted pneumostome openings in the MT is not significantly greater than that of the last training session and 2) the number of attempted pneumostome openings in the MT is significantly less than that of the first training session.

While the 'savings-test' is a suitable method to test for memory, we also use a second memory assessment method (a 'probe-test'). In the 'probe-test' method, we compare total breathing time before and after training. Thus, memory is assessed in the absence of the presentation of reinforcing stimuli. There are both advantages and disadvantages to these two memory assessment methods. The disadvantage of the 'savings-test method' is that reinforcing stimuli are applied during the MT.

Additional learning may occur, creating the possibility that reduction in aerial respiration is due to this additional reinforcement and not the memory phenotype. The advantage of the 'savings-test' method is that, following the MT, another different context-MT (see below) can be given showing that the change in behaviour is a bona fide reflection of memory. The advantage of the 'probe-test' method is reinforcing stimuli are not applied during the MT, but there are two disadvantages. The first is that the pre-training observation session (OBS1) may trigger latent inhibition and thus affect memory formation. The second disadvantage is the post-training observation session (OBS2) is an extinction session. If another observation session in a new context is given after the post-training observation session, we cannot be sure if naïve respiratory levels are due to context specificity or occlusion by extinction. In our model system, both the 'savings-test' method and 'probe-test' method yield the same conclusions and are valid methods for the assessment of memory. Shown below is a diagram illustrating different training procedures and procedures used to assess forgetting. Figure 3A and 3B show the 'massed' and 'spaced' training procedures used to produce ITM and LTM, respectively, as measured by a savings-test method. Figure 3C and 3D show the 'massed' and 'spaced' training procedures used to produce ITM and LTM, respectively, as measured by the probe-test method. Forgetting can be assessed by giving snails memory tests at various time points after training has ceased.

In addition to using two memory assessment methods to ensure the validity of our experimental findings, we use an important control, the yoked control procedure. A yoked control snail is given a non-contingent stimulus (i.e. a stimulus when the pneumostome is not open) every time a snail from the experimental cohort receives a contingent stimulus (i.e. a stimulus when pneumostome opening is initiated). As a result, the experimental snail associatively learns and forms memory, but the yoked control snail cannot as there is no association present.[15] The yoked control procedure serves to control for phenomena that may alter behaviour or internal state of the snail such as: 1) stress associated with stimulus application, 2) handling and 3) the training environment.

In addition to the yoked control, a 'change of context' procedure is used to ensure the behaviour of snails is not altered by experimental conditions (i.e. stress, drug exposure, handling or the training environment). When trained snails are placed in a novel context, they respond as if they were naïve. That is, they show more respiratory activity in the novel context than in the trained context. Typically we train snails in hypoxic pond-water that lacks the presence of an odorant (i.e. the standard context). To create a 'novel context', N_2 is first bubbled through a flask containing, for example, a slurry of carrot and water. The resulting carrot-odorant N_2 is bubbled into the training beaker prior to training or testing. For complete details see Haney and Lukowiak[36]

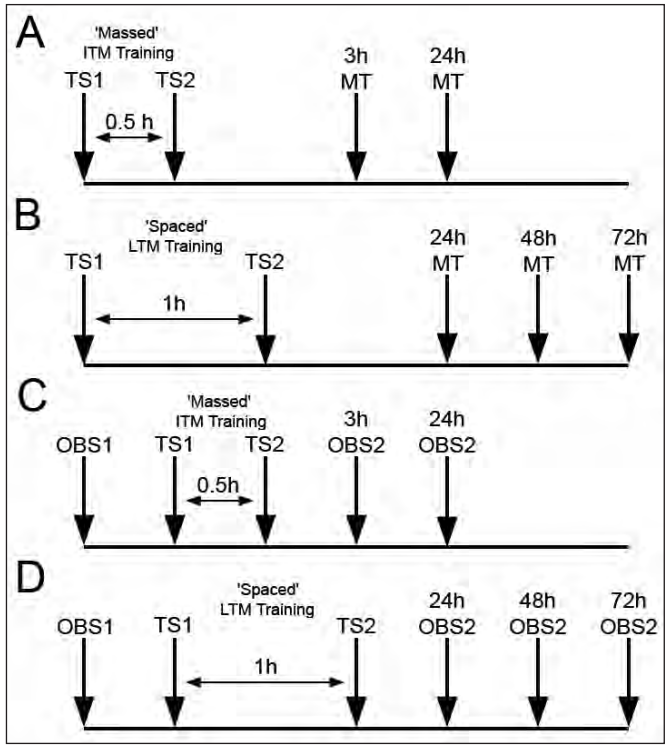

Figure 3: *A diagram illustrating training procedures used to produce memory in Lymnaea and to measure the forgetting of memory. (A) To produce ITM, snails are given a 'massed' training procedure that consists of two 0.5h training sessions separated by a 0.5h rest interval and then memory is tested for at 3h (3h MT) using the 'savings-test' method. If snails are given a MT 24h after training (24h MT), memory is no longer present and hence forgotten. (B) To produce LTM, snails are given a 'spaced' training procedure that consists of two 0.5h training sessions separated by a 1h rest interval and then memory is tested for at 24h (24h MT) using the 'savings-test' method. The procedure to assess forgetting is performed by giving snails MTs at 24h, 48h and 72h (24h MT, 48h MT and 72h MT, respectively). (C) An alternative method to the 'savings-test' method is to use the 'probe-test' method. A breathing observation (OBS1) is performed to measure mean total breathing time of a cohort of naïve snails. Twenty four hours later, the snails are given 'massed' training (i.e. ITM training procedure; two 0.5h training sessions separated by a 0.5h rest interval). Memory is tested at 3h by performing another breathing observation (3h OBS2) to measure mean total breathing times. If memory is tested using the 'probe-test' method at 24h after training (24h OBS2), memory is no longer present and hence forgotten. (D) Snails are treated as in C except they are given 'spaced' training (i.e. LTM training procedure; two 0.5h training sessions separated by a 1h rest interval) and memory is tested at 24h. The procedure to assess forgetting is performed by giving snails breathing observations at 24h, 48h and 72h (24h OBS2, 48h OBS2 and 72h OBS2, respectively).*

Memory Consolidation

As already mentioned, learning and memory, while related, are two separate and distinct processes.[1] Behaviourally learning is the acquisition of a new or altered behaviour, while memory refers to the retention of what is learned. There are also fundamental molecular differences between learning and memory and these will be discussed. The process that leads to the formation of memory following learning has come to be known as consolidation. The concept of consolidation stems from clinical observations made by Ribot in 1882. He noted that after traumatic brain injury, recent memories were more likely to be forgotten than memories of earlier events.[37] The hypothesis postulated that after learning, memory existed in a fragile state

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and was vulnerable to interference. With time the memory was stabilized and became insusceptible to interference by amnesiac treatments.[38] It was observed that the shorter the interval between the training and the amnesiac treatment, the more prominent the retrograde amnesia. Therefore, if enough time had passed after training, the amnesiac treatment was no longer able to disrupt memory. That is, the memory had become fixed and stable and, hence 'consolidated'.[39,40]

We noted above that memories could be classified as declarative or non-declarative. In addition memories may also be distinguished by their temporal characteristics as follows: 1) short-term memory (STM; lasting seconds to minutes), 2) intermediate-term memory (ITM; lasting up to 3 hours); and 3) long-term memory (LTM; lasting greater than 6 hours).[3,41,42] We found that we could in Lymnaea differentially produce ITM and LTM by specific training procedures. We can broadly classify the two procedures as 'massed' (ITM-training) or 'spaced' (LTM-training) training. For example, if we subject snails to two 30-min training sessions separated by a 0.5h interval only ITM is formed. However, if we increase the interval between the training sessions to 1h, LTM (persisting at least 1 day) forms. Thus, depending on which training procedure we choose to use we can get ITM or LTM.[16]

Short-term memory does not require the synthesis of new protein rather it results from the transient modification of already existing proteins (e.g. phosphorylation of proteins). Intermediate-term memory requires de novo protein synthesis from pre-existing mRNA but ITM can also be protein synthesis independent as shown in *Aplysia*.[43,44] Long-term memory requires both de novo protein synthesis and altered gene activity (for review see Milner et al, 1998). We first determined if memory in our *Lymnaea* model system conformed to these rules. To show that ITM and LTM require protein synthesis, snails are subjected to procedures that inhibit or reduce their rate of protein synthesis. For example, if snails are injected with the protein synthesis inhibitor anisomycin, before training neither ITM nor LTM results. As a control, snails injected with saline at the same time continue to exhibit memory. If, however, snails are injected prior to training with the transcription inhibitor, actinomycin D, ITM forms but LTM formation is blocked. A further attribute of our model system is that we are able to subject snails, without harming them, to cooling to 4ºC. Cooling has been used to interfere with memory consolidation by other several labs.[45,46] Cooling interferes with both protein synthesis and gene transcription. Thus, if snails are subjected to 1h of cooling immediately after training, neither ITM nor LTM result. If however, the same duration of cooling (1h) is delayed by 1h after training memory formation is not interfered with.[47]

One of the reasons we have used the *Lymnaea* model for studying memory formation is that we have a very through knowledge of the underlying neuronal circuitry. Thus, we should be able to show that molecular events in CPG neurons are necessary for memory formation. To do this we chose to utilize an advantage of molluscs, namely the ability to surgically remove the soma of an identified neuron while leaving behind a functional primary neurite. Since the synaptic specializations (pre and post) of invertebrate neurons all reside on the primary neurite, removal of the soma does not interfere with synaptic interactions. Moreover, the primary neurite is still capable of de novo protein synthesis and is capable of surviving for weeks after surgery.[2,48] What is missing, after this procedure is performed, are the genes. Thus, we can directly ask whether altered gene activity in the soma of a particular neuron is need for LTM formation. We made a decision to first examine the role played in memory formation by the RPeD1, the neuron that initiates CPG rhythmogenesis. Another reason for choosing RPeD1 is that we had already shown specific changes in neural activity could be correlated with changes in behaviour following memory formation.[49] We therefore removed either the soma of RPeD1 or, as a control, the soma of a similar sized neuron (LPeD1) that is not a member of the respiratory CPG from naïve snails. We found that both groups (i.e. RPeD1 and LPeD1 soma ablated snails) demonstrated learning and ITM. However, the RPeD1-soma ablated snails did not exhibit LTM. That is, removal of only the somata of a single neuron prevented LTM memory formation without altering the ability of the snail to learn or form ITM.[2] LPeD1 soma ablated animals were still capable of forming and exhibiting LTM.

A question that has arisen is whether LTM formation first requires ITM formation (i.e. LTM formation occurs serially) or can it occur without ITM formation (i.e. a parallel process). If ITM and LTM are the result of parallel molecular processes one could block the ITM producing process and not interfere with the LTM molecular process. This strategy has been attempted by a number of different laboratories.[50-56] For example in a one-trial step down inhibitory avoidance task studied in rats, 11 different treatments blocked what we have termed ITM but not LTM and 18 treatments either blocked or enhanced LTM alone and left ITM unaffected.[50]

However, if ITM and LTM are the result of parallel processes, then an ITM training procedure no matter how many times presented should only produce ITM and never result in the formation of LTM. Studies in our lab and others have shed doubt on the notion that ITM and LTM formation occur in parallel by demonstrating that training procedures that would not normally result in LTM (i.e. ITM-training) can in fact be made to produce LTM.[57-62] If an ITM training protocol is given to snails, the memory produced persists for 3h but not 24h (i.e. ITM). If the parallel hypothesis was correct, then a subsequent ITM training procedure the following day could only result only in ITM since the molecular pathways underlying ITM and LTM formation occur in parallel. However, we found that a subsequent bout of ITM training a day after the first bout of ITM training produces LTM. This suggests that the first ITM training protocol produced a residual molecular memory trace that persists longer than the behaviourally observable memory. Activation of the residual molecular memory trace with a second bout of ITM training was able to boost this trace into LTM consistent with the hypothesis that ITM and LTM are serial processes.[58,63]

Another demonstration of how the processes that lead to LTM occur in series was demonstrated in *Drosophila*. In *Drosophila*, behavioural experiments have shown that olfactory conditioning (i.e. a form of classical conditioning) can produce an associative LTM. Training is performed by repeated pairings of an odor and electrical shock and a different odor and no electrical shock. To test for conditioned odor avoidance responses (i.e. memory) fruit flies are placed in a T-maze and allowed to distribute themselves into the arms of the maze containing the two odors. Memory is

observed if fruit flies avoid the odor that was paired to electrical shock. LTM is produced by multiple 'spaced' training sessions separated by rest intervals, a training paradigm that we also use. In contrast, 'massed' training is unable to produce LTM. To show that they could boost memory, they created transgenic flies that carried a heat shock inducible activator CREB2 isoform. Three hours after heat shock, massed training produced LTM in the transgenic flies. This demonstrated that activating CREB2 in transgenic flies permitted LTM formation by a procedure that would not normally produce LTM.[64] These findings suggest that activation of the activator isoform of CREB2 is part of a serial pathway that allows LTM formation to occur.

While the role of the activator isoform of CREB2 was explored in LTM formation, the repressor isoform of CREB2 was also studied in another invertebrate model system. In *Aplysia*, a repressor isoform of CREB was identified and characterized and termed ApCREB2. The repressor isoform of ApCREB2 is targeted for phosphorylation by protein kinases such as PKA, PKC, MAPK and CAMK. Normally, a single pulse of 5-HT produces short-term facilitation that lasts minutes. But when anti-ApCREB2 was injected into the nucleus of sensory neurons to 'inactivate' the repressor isoform of ApCREB2, a single 5-HT pulse produced long-term facilitation.[65] This long-term facilitation required protein synthesis and altered gene activity. These experiments showed that induction of CREB2 de-repression could permit the formation of long-term facilitation by a procedure that would normally produce short-term facilitation. These findings suggest that the de-repression of the repressor isoform of CREB2 is part of a serial pathway that allows LTM formation to occur.

While the previous two studies manipulated CREB activity, another study activated an upstream kinase, PKA, to produce LTM. In the honeybee, the associative olfactory conditioning (i.e. classical conditioning) of the proboscis extension response results in the formation of LTM that was associated with persistent PKA activation. A single pairing of the odor stimulus (carnation; CS) followed by presentation of sucrose solution (US) does not result in LTM formation. However, multiple pairings produce a LTM that was associated with persistent PKA activation. If photo-release of caged cAMP in the antennal lobes is coupled with a single conditioning trial, LTM is formed.[66] These findings suggest that activation of the PKA pathway is also a part of the serial pathway that allows LTM formation to occur.

All the above mentioned results in *Lymnaea, Drosophila, Aplysia* and the honeybee are consistent with the hypothesis that LTM formation involves processes that occur in series.

Memory Reconsolidation

While the idea of memory consolidation has existed for a long time, what happens to the memory afterwards has only been under recent investigation. Upon activation, a stable and consolidated memory becomes active and labile. Reconsolidation, first observed by Misanin et al,[67] is the process by which an activated memory undergoes another process of stabilization to return it to an inactive state. While undergoing reconsolidation, the memory can be updated and/or changed to incorporate new information.[68,69] More recent studies have examined the mechanisms underlying reconsolidation.[46,70-76]

There exist some similarities between consolidation and reconsolidation. CREB activation has been shown to be a requirement in both consolidation[77-82] and reconsolidation [83]. As well, both processes have been shown to require new protein synthesis.[69,71,72,74,83,84] Finally, NMDA receptors have been shown to be involved in consolidation,[77,85,86] and in reconsolidation.[72,73,87] While it appears that some molecular cascades are involved in both consolidation and reconsolidation, some differences have been identified. BDNF has been shown to be required for consolidation of contextual fear memory but not its reconsolidation. In the same study, Zif268 was shown to be involved in the reconsolidation of contextual fear memory but not its consolidation.[88] C/EBP (CAAT/enhancer-binding protein) is required for consolidation.[76,89] However C/EBP does not seem to be needed for reconsolidation.[76] This suggests that consolidation and reconsolidation may trigger similar initial molecular cascades, resulting in the activation of CREB. Downstream from CREB however, the various molecular cascades and proteins involved may be different.

To demonstrate reconsolidation in Lymnaea, we activate a previously formed memory by placing the snails in the same context-training beaker, and then attempt to block newly labile memory using a variety of experimental methods. Application of protein synthesis inhibitors, RNA transcription blockers or ablation of the soma of RPeD1 after activation of the memory perturbs the reconsolidation process. In all of these scenarios, long-term memory was no longer present or abolished. However, if the memory is not activated before the application of these interventions, LTM is still present.[74] Thus, perturbations of protein synthesis and RNA transcription inhibit both consolidation and reconsolidation suggesting that similar molecular mechanisms underlie both processes. Interestingly, when snails were over-trained to produce a well-rehearsed memory, the memory eventually becomes independent of protein synthesis and transcription. This suggests that, somehow, repeated reconsolidation transforms the memory into a very stable state to the point that it can no longer be modified.[90]

Extinction

Extinction typically occurs when reinforcing stimuli are no longer applied.[91,92] Extinction research is of great interest as it potentially can be used as a therapeutic tool to treat substance addiction and fear disorders. A question that has arisen is whether extinction is unlearning or is it new learning and memory formation that occludes the original memory.[93-98] We have attempted to directly determine whether extinction is unlearning. To cause extinction, snails that possess LTM are placed in the training context and are allowed to perform pneumostome opening without any reinforcement. That is, they learn that it is 'acceptable' to open their pneumostomes. If extinction occurs snails will perform as if they are naïve snails. This is what we have found.[96,99] However, this still does not address the question as to whether extinction is unlearning. The original studies on extinction performed by Pavlov[92] demonstrated the phenomenon, which he termed 'spontaneous recovery'. That is, following extinction training, the original memory was not seen. However, with time the original memory could be evoked again by presentation of the CS, suggesting that extinction training is not unlearning of the original learning. We have also seen spontaneous recovery in our studies.[99]

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One theory of extinction postulates that extinction involves the weakening of pre-existing connections. Support for this theory of extinction assumed that the original conditioning was erased.[91] However, a defining characteristic of extinction is that spontaneous recovery can be observed.[92] If extinction training-sessions are given after LTM has formed, the extinction memory occludes the original memory. That is, extinction is not unlearning but a new memory that acts in opposition of the original LTM. However, the extinction memory itself can be forgotten causing the original memory to return (i.e. spontaneous recovery).

Mechanisms of Extinction

There is strong support to demonstrate that extinction is an active process that involves new learning and, therefore, new protein synthesis, to produce a memory that occludes the original conditioning.[93-95] Extinction is an active process that involves new learning to produce a memory that occludes the original conditioning. Extinction has been observed in many model systems.[17,96-100] Using a number of the experimental attributes of our model system outlined above we set out to rigorously test the hypothesis that extinction is new learning and memory that occludes the older memory. We were able to show that the process of extinction requires the somata of RPeD1 to be present (just as LTM formation requires RPeD1's somata).[99] While we have identified a neuron necessary for extinction (RPeD1), no one particular brain structure has been identified in mammalian systems to be responsible for extinction.[97] Thus highlighting another advantage to the *Lymnaea* model system.

Forgetting

Of all the topics that we deal with in our laboratory the one that we are most often questioned about by non-neuroscientists is forgetting. The most frequently asked question is, "How can I stop forgetting things and improve my memory?" We often answer, much to the chagrin of the questioner, that the biggest problem with forgetting is 'not forgetting' but rather the inability to forget! We first need to define what we mean by forgetting. The Oxford English Dictionary defines the verb forget as "To lose remembrance of; to cease to retain in one's memory" or "To fail to recall to mind; not to recollect." Because we are dealing with non-declarative memory that is stored within the neural circuit that mediates the behaviour (i.e. the CPG network)[2,49,101,102] we define forgetting as the obliteration of the memory and therefore the associated learned behaviour.

While forgetting is correlated with time, it is not caused by the passage of time.[103] There are several theories on forgetting with the two most prominent being: 1) Failure to retrieve; and 2) Retroactive interference. The retrieval failure hypothesis states that the memory cannot be accessed and hence it is dubbed 'forgotten'. Certainly, we have all experienced this form of forgetting. While a failure to retrieve a memory might explain how forgetting of a declarative memory may occur, retrieval failure cannot explain forgetting of a non-declarative memory. We study a non-declarative memory that is formed and forgotten both in the same circuit.[2] Since we know the circuit, we can test for retrieval failure. To test this, we can produce two different context specific memories in the snail (i.e. standard and carrot). If we use a training procedure (two 45 min training sessions on two successive days) in the standard context to produce a LTM that persists for seven days and a training procedure in the carrot context to produce a LTM that persists for only one day (two 30 min sessions on one day) we can then directly show that memory is not due to an inability to retrieve the memory. If the memory in the carrot context was forgotten due to retrieval failure, then placing the animals in the standard context should not manifest the original long-lasting memory. If the animal still has the LTM for the standard context, then we know that the memory is accessible and forgetting is not due to retrieval failure. We observe the latter finding that demonstrates forgetting is not due to retrieval failure.

The interference theory states that related events that occur after LTM formation cause forgetting. When they are trained, snails make the association that pneumostome opening results in the delivery of a tactile stimulus. When snails are not being trained, they are free to perform aerially respiration *ad libitum*. Animals learn anew that pneumostome opening will not result in the delivery of a tactile stimulus. Interfering events can be prevented by submerging animals after training to prevent spontaneous opening of their pneumostome. Thus, animals will not learn the new association and therefore retain the original association. We found that preventing interfering events extended the persistence of LTM (i.e. delayed forgetting).[35] The molecular mechanisms of forgetting are still unknown. We have hypothesized that forgetting is an active process. That is, forgetting requires altered gene activity and new protein synthesis in order to obliterate the memory. Thus, cooling snails after the completion of consolidation can extend the persistence of memory (i.e. delay forgetting). Cooling reduces the snails' metabolic rate and subsequently reduce the amount of protein synthesis that occurs.[47,58] Most importantly, we can directly demonstrate that forgetting resembles learning something new and remembering it since if we remove the soma of RPeD1 after the LTM consolidation process, the snails are unable to forget (they are also unable to form new LTM if operantly conditioned in a new context) indicating that forgetting requires access to the genome.[104]

Future Directions

It has been demonstrated that stress and emotions can be strong modulators of memory formation; however, experimental results have been varied and often contradictory.[105] For example, rats that were exposed to uncontrollable restraint and tail-shock stress demonstrated normal learning during water maze training, but animals showed impaired memory compared to unstressed controls when tested the next day.[106] However, memories for very traumatic or highly emotional events are often extremely long-lasting.[107,108] Given the complexity of animal behaviour and the many diverse ways different stressors could act on learning and memory formation, the disagreement in the literature is not surprising. Preliminary evidence has shown that a single, highly aversive stimulus (submersion in KCl), contingent with pneumostome opening, is sufficient to alter the breathing behaviour of *Lymnaea*.[109] Yoked controls, change of context controls, and handling controls indicate that this change is a bona fide example of single-trial operant conditioning.

Stressors such as predators, inter- and intra-specific competition for food, habitat and procreational resources can

impart a high strain on the energy allocation of an organism. Learning about predators is expected to have adaptive payoffs in any species that can alter their behaviour during times of predation risk.[110] *Lymnaea* respond to crayfish predators by using predator-avoidance behaviours such as the full body withdrawal response when under attack.[111] It is possible that snails which can detect the presence of a predator, in a presumably stressful situation in which they respond with defensive behaviors, will show a change in their ability to learn and remember when given an operant training paradigm in the presence of that predator. Preliminary experiments in our laboratory suggest when *Lymnaea* are operantly conditioned in the presence of a crayfish predator memory formation is enhanced.[112] How this crayfish exposure affects the neural substrates responsible for this behavior and to what extent this occurs is currently under investigation. In future work, we hope to investigate the alteration of memory formation by stress.

Another avenue of research we would like to pursue is to explore anatomical studies associated with memory formation. We have as yet to determine what changes in synapse profile and cytoarchitecture occur throughout neurons that are involved in memory formation (i.e. the 3 interneurons of the CPG mediating aerial respiratory behaviour). However, we have undertaken a functional proteomics approach to identify changes in protein expression associated with LTM formation using the entire CNS of the snail. Preliminary analyses have indicated that LTM formation results in a reduction in some specific membrane proteins and an increase in the expression of many specific cytosolic proteins. Once these proteins have been identified, we can begin to construct a possible picture of what changes occur in the Central nervous system of *Lymnae*a to form LTM.

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