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Commentary

# Associative learning and memory in *Lymnaea stagnalis*: how well do they remember?

Ken Lukowiak*, Susan Sangha, Chloe McComb, Nishi Varshney, David Rosenegger, Hisayo Sadamoto and Andi Scheibenstock

*Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta, Canada, T2N 4N1*

*Author for correspondence (e-mail: lukowiak@ucalgary.ca)

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## Summary

**The search for 'the how and the where' of memory formation in the brain, the engram, is still one of the unattained 'Holy Grails' of neuroscience. Over the years, various paths have been trodden in attempts to attain this goal, and while tantalizing glimpses appear now and then on the scientific horizon, the Grail still has not been grasped. One of the paths that investigators have walked is the invertebrate 'model system' approach. Some invertebrates possess relatively simple nervous systems that mediate relatively simple behaviours that are both interesting and trainable. In this commentary, we would like to shed light on a relatively new player, the pond snail *Lymnaea stagnalis* L., that is being used in the quest to illuminate 'the how and the where' the nervous systems encode and store memory. We will show that it is possible to demonstrate that a single neuron is *a* site of memory formation and storage for a form of associative learning in this lowly snail. It may be that the Grail is a little closer to being grasped.**

Key words: associative learning, operant conditioning, long-term memory, *Lymnaea*, invertebrate learning, invertebrate memory.

## Molluscan model systems for the study of learning and memory

This is not by any means an exhaustive review of invertebrate model systems that have been used to study the neuronal and molecular mechanisms of learning and memory; the interested reader is directed to two recent excellent reviews on this subject (Sahley and Crow, 1998; Chase, 2002). We have also restricted our discussion to gastropod molluscs, but this should in no way be taken to mean that the truly ground-breaking work on the mechanisms of learning and memory using 'worms' (e.g. *Caenorhabditis elegans*) or insects such as *Drosophila* is not worth mentioning or pursuing but rather that space limitations preclude their inclusion. For much the same reason, the fascinating studies using cephalopod molluscs (e.g. octopus), which exhibit very sophisticated learning and memory capabilities, will also not be reviewed here.

Some of the first studies in the 'modern' search for the engram took the comparative physiological and psychological approach. For example, in the early 1900s, Piéron (1911), Dawson (1911) and Thompson (1917) used snails in attempts to discover how learning occurred. However, these studies were, by and large, forgotten, and it really was not until tests that were more natural and meaningful to the organisms were used that a full appreciation of the learning capabilities of gastropods became apparent.

The 1960s saw a burst of activity that is still evident today to study how the nervous system is 'wired-up' to mediate specific behaviours and how changes in the behaviour brought about by training procedures are reflected or caused (the real goal) by changes in the activity of specific neurons. Thus, preparations such as *Aplysia*, *Hermissenda*, *Pleurobranchaea* and *Tritonia* gained popularity. Eric Kandel, with his share of the Nobel Prize for Medicine and Physiology in 2000, attained one of the pinnacles of science in part by using *Aplysia*.

All the species mentioned above are marine creatures, and, with few exceptions (e.g. the land slug *Limax* and the land snail *Helix*), conventional wisdom from the 1970s through to the early 1990s was that the freshwater gastropods just did not have the 'right stuff' to be used in the quest for the Holy Grail. This review will focus on why that conventional wisdom was incorrect and why using *Lymnaea* might just be a very useful path to take to grasp the Grail in hand.

## *Lymnaea* as a model system for neurobiology

Until the Dutch, under the inspired leadership of Professors Lever and Joose in The Department of Biology at Vrije Universeit in Amsterdam, adopted *Lymnaea* as their animal of choice for study in the early 1970s, *Lymnaea* was not often used in neurobiological research. The natural history of the

snail and several of its important homeostatic behaviours were described early on (Jones, 1961). Notably, the *Lymnaea* central nervous system (CNS) was given almost as much space in Bullock and Horridge's masterpiece *Structure and Function in the Invertebrate Nervous System* as was the *Aplysia* CNS (interestingly, the most space was given to the nervous system of *Helix*; Bullock and Horridge, 1965). Despite the demonstration of a neural correlate of behavioural habituation in the early 1970s (Cook, 1975), pond snails were primarily used in studies examining their feeding behaviours, championed by Benjamin's group at the University of Sussex. Readers are directed to an excellent recent review of this literature (Elliott and Susswein, 2002).

The Audersirks in the early 1980s (Alexander et al., 1982) were probably the first to really appreciate that *Lymnaea* had the capacity for associative learning, but, for reasons not understood by us, this path was not taken up by others until very recently. Now, at least three different laboratories in the UK and Japan (e.g. Ito et al., 1999; Ono et al., 2002; Staras et al., 1999) have embarked on studies examining associative learning and its long-term memory in *Lymnaea*, following the pathway opened up by the Audersirk's pioneering studies. Again, readers are directed to a recent review on these studies (Benjamin et al., 2000). If we might editorialize here, we think that the feeding circuitry is just too complicated for investigators to show that changes in neuronal activity are causal for memory, and that is why we did not go down that particular pathway in search of the engram. However, we could be wrong.

Our main reason for moving from *Aplysia* to *Lymnaea* to study learning and memory was the finding that a three-neuron network drove an important homeostatic function in *Lymnaea*, aerial respiratory behaviour. By using a combination of cell culture and *in vivo* transplantation techniques, Syed et al. (1990, 1992) were able to directly demonstrate both the sufficiency and necessity of the three-neuron central pattern generator (CPG) to drive aerial respiratory behaviour. Few, if any, other neural circuits have been described that meet both the sufficiency and necessity tests. If this behaviour could undergo associative learning and its consolidation into long-lasting memory [intermediate term memory (ITM), lasting 3–4 h, and long-term memory (LTM), lasting longer than 5 h] then we would have a preparation where we might be able to study the causal neuronal mechanisms of learning and memory directly.

*Lymnaea* are bi-modal breathers, obtaining oxygen either through cutaneous or aerial respiration. Typically, in eumoxic conditions, cutaneous respiration dominates and aerial respiration seldom occurs. To 'motivate' *Lymnaea*, we make the pond water hypoxic by bubbling $N_2$ through the training beaker for 20 min (Fig. 1) and, in these hypoxic conditions, aerial respiration predominates. Briefly, to operantly condition (a form of associative learning) the snails, we apply a relatively weak tactile stimulus (a sharpened wooden applicator is used) to their pneumostome, the respiratory orifice, each time they 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). Pneumostome stimulation also does not cause the snails to sink to the bottom of the beaker.

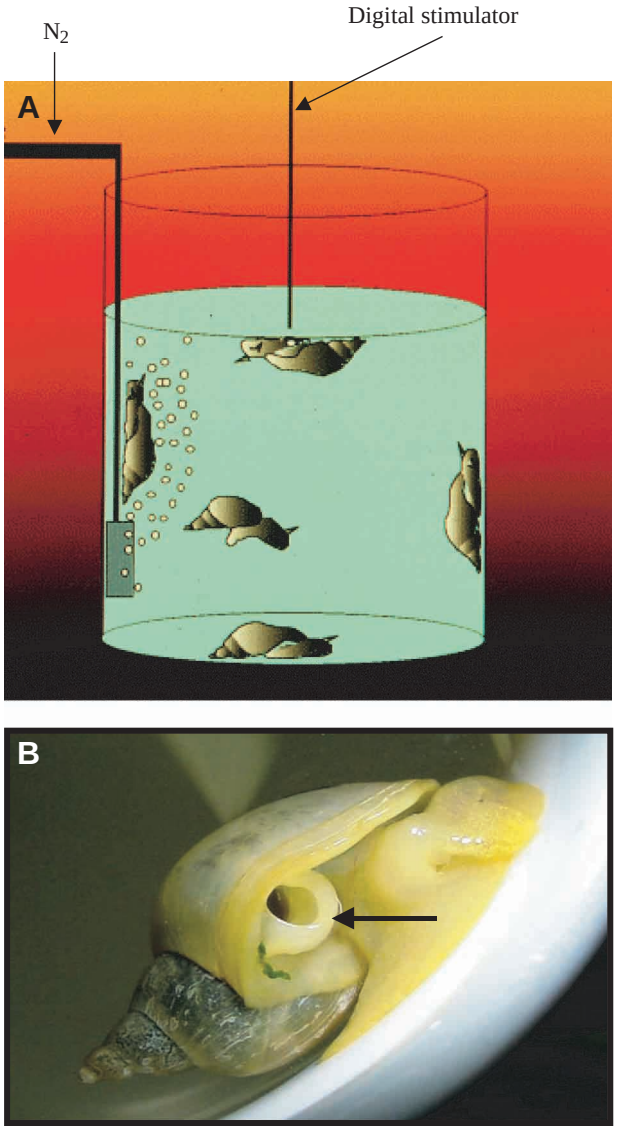


Fig. 1. *Lymnaea*, the pneumostome and training. (A) Cartoon of the training apparatus used to operantly condition aerial respiratory behaviour in *Lymnaea*. A beaker is filled with pond-water and then $N_2$ is bubbled through it for 20 min in order to make the pond water hypoxic. Snails are then added, given a 10-min acclimatization period and then training begins. $N_2$ is continuously bubbled throughout the training period. Each time the snail attempts to open its pneumostome, the respiratory orifice, it receives a tactile stimulus to the pneumostome area, which causes the pneumostome to close. The tactile stimulus is delivered by means of a sharpened wooden stick, the 'digital stimulator'. (B) Photograph of a 2.5 cm (i.e. adult) snail. The pneumostome (arrow) is open. The pneumostome only opens when the snail is at the air–water interface.

We found that *Lymnaea* have the ability to be operantly conditioned (Lukowiak et al., 1996) and that this learning

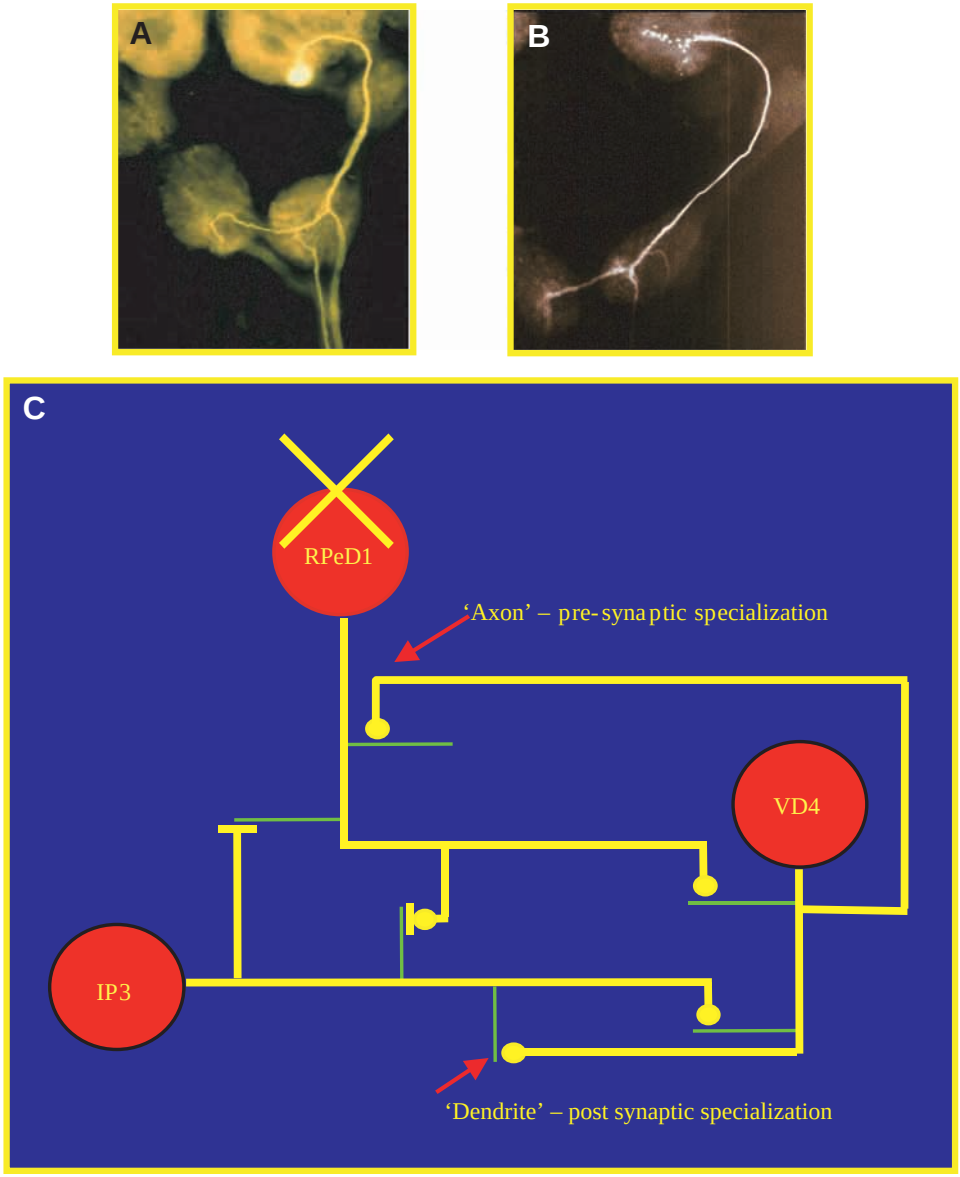


Fig. 2. Soma ablation in *Lymnaea*. (A) Photomicrograph of a Lucifer yellow-filled RPeD1. RPeD1 sends neuritic branches to other ganglia, where it synapses with other members of the aerial respiratory network, and also sends processes to the pneumostome area, where it receives both tactile and chemosensory input. The soma diameter of RPeD1 is approximately 75 μmol $l^{-1}$. (B) A similar RPeD1 Lucifer yellow fill, except that gentle poking of it with a glass microelectrode has ablated its soma. The isolated neurite remains viable, as detected electrophysiologically for at least 2 weeks and behaviourally for at least 1 month. (C) A cartoon of the aerial respiratory central pattern generator (CPG; RPeD1, VD4 and IP3; see Syed et al., 1990) showing how synaptic connections are made in these unipolar neurons. Removal of RPeD1's soma does not disrupt either the pre- or postsynaptic specialization areas of the neuron. The closed circles represent an inhibitory chemical synaptic input, the bars represent excitatory synaptic input, and a combined bar and filled circle represents a conjoint inhibitory/excitatory input. All synapses are chemical and involve, at a minimum, the classical transmitters acetylcholine and dopamine and peptide transmitters of the RFamide family.

undergoes consolidation into either ITM or LTM (Lukowiak et al., 2000). Depending on the training procedure used, LTM persistence could be as long as one month (Lukowiak et al., 1998). All the necessary controls to show that this change of behaviour is a *bona fide* example of associative learning have been performed. We are now at the point where we can begin to determine the causal neuronal basis of learning and its consolidation into memory.

## Definitions of learning and memory

We define 'learning' as the acquisition of a skill, while 'memory' is the ability to retain that skill. Memory (both ITM and LTM) is operationally defined as meeting the following two criteria: (1) the number of attempted pneumostome openings in the memory-test session is not significantly greater than the number of attempted openings in the last training session and (2) the number of attempted openings in the memory-test session is significantly less than the number of attempted openings in the first training session.

## Where is the non-declarative memory of operant conditioning formed and stored?

We are studying a form of memory known as non-declarative memory, and this form of memory is stored within the same neural circuit that mediates the behaviour (Milner et al., 1998; Scheibenstock et al., 2002). We thus avoid the problem of whether memory is forgotten or rather just inaccessible (McGeoch, 1932; Capaldi and Neath, 1995; Schacter, 2001) because if the snail can perform the behaviour (i.e. access the neural circuit) the memory *cannot* be inaccessible. Since we know the neural circuit that drives aerial respiration (i.e. the respiratory CPG), we had a good idea where we would see neural correlates of the learning and its memory. Neural correlates of learning and

LTM were shown in RPeD1 (the cell that initiates CPG activity) in both isolated ganglionic and semi-intact preparations obtained from previously trained snails (Spencer et al., 1999, 2002). But, these studies do not tell us if RPeD1 is a necessary site of memory formation and/or storage (i.e. correlation is not causality). To attempt to show that changes in RPeD1 were causal for memory formation, we made use of an amazing attribute of our model system, the ability of the axons and dendrites of *Lymnaea* neurons to survive for long periods of time without their cell body (i.e. the soma).

Molluscan neurons are unipolar. That is, they have a single process emerging from the soma (properly known as the primary neurite, but more often than not called the axon) and this is where the majority of synaptic interactions occur (Bullock and Horridge, 1965; Kandel, 1979; Fig. 2). Moreover, molluscan neurons possess an ability to function 'normally' for long periods of time without their soma. It is therefore possible to surgically remove the soma of RPeD1, leaving behind a functional primary neurite sufficient to mediate normal neuronal activity and aerial respiratory behaviour (Haque, 1999; Scheibenstock et al., 2002). The isolated primary neurites (i.e. without the soma) of *Lymnaea* are also capable of *de novo* protein synthesis of injected novel mRNA, and the newly synthesized protein can be functionally integrated into the membrane (van Minnen et al., 1997; Martin et al., 1997; Spencer et al., 2000). Because LTM is dependent on both altered gene activity and new protein synthesis (Kandel, 2001), our working hypothesis is that, if RPeD1 is a site for either the formation or storage of LTM, removal of its soma, and thus its nucleus, *before* operant conditioning training should prevent the formation of LTM. We therefore determined whether selective ablation of the RPeD1 soma (i.e. leaving intact its primary neurite) would result in an inability to encode or access LTM of the operantly conditioned aerial respiratory behaviour.

We found that RPeD1 soma-ablated snails (Fig. 3) had the ability to associatively learn and could form ITM (which is dependent on new protein synthesis but not altered gene activity – remember, an isolated neurite is still capable of *de novo* protein synthesis) but they could not consolidate the learning into LTM (Scheibenstock et al., 2002). Based on these data, it would appear that RPeD1 is a necessary site for LTM formation.

However, it was possible that in RPeD1 soma-ablated snails, LTM was encoded but either could not be accessed or retrieved without the soma. We therefore ablated the soma of RPeD1 *after* learning and memory consolidation had occurred (*N*=10) and found that LTM was present (Fig. 4). As an extra control, we challenged these snails with a 'different-context' (Haney and Lukowiak, 2001) test. The snails responded as they did in the initial training session (when the soma of RPeD1 was present). Thus, RPeD1 soma-ablated snails still had the ability to access or retrieve a previously encoded memory. Thus, we concluded that RPeD1 is *a* site for LTM formation and storage and we think this is the first instance where a single neuron has been shown to be a necessary site for LTM formation. Whether it is the only necessary site for this memory remains to be determined.

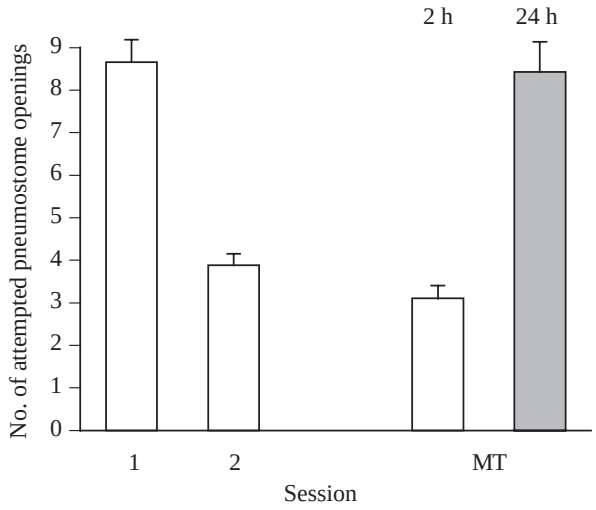


Fig. 3. RPeD1 soma ablation and memory formation. Ablating RPeD1 somata 2 days before training does not affect the snails' (*N*=20) ability to learn. That is, there is a significant difference between Session 1 and Session 2. In addition, these snails (*N*=20) can form intermediate term memory (ITM). That is, in a memory test (MT) 2 h after the last training session, the criteria for memory are met. However, if the memory is tested 24 h later in these same snails (*N*=20), the criteria for memory are not met. We conclude that RPeD1 soma-ablated snails do not form long-term memory (LTM).

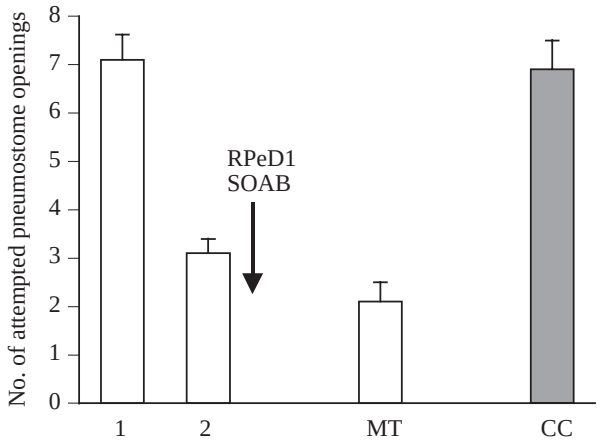


Fig. 4. RPeD1 soma-ablated snails can still access long-term memory (LTM). A cohort (*N*=10) of snails received two 45-min training sessions, with a 1 h interval between the sessions. One hour following Session 2, all snails had their RPeD1 soma ablated (RPeD1 SOAB). When we tested for memory 3 days later (MT), it was present. That is, the criteria for memory were met. However, to be certain that the apparent memory was truly memory and not unresponsiveness of the snails, they were challenged 1 h later with a 45-min change-of-context test (CC). These snails are still capable of responding, and thus RPeD1 soma-ablated snails can still access previously encoded LTM.

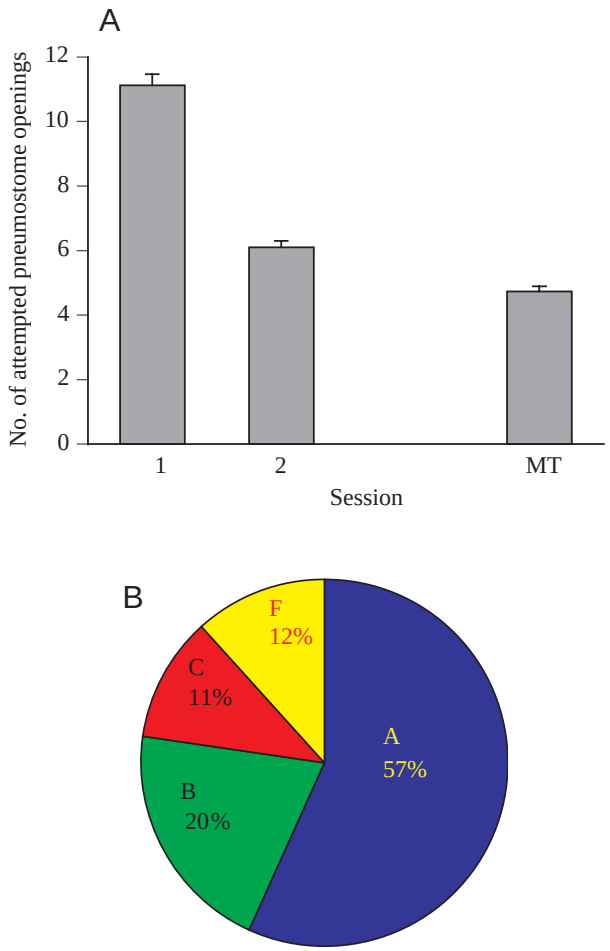


Fig. 5. Learning, memory and the assignment of marks. (A) The learning and memory curves for snails ($N$=1490) that receive two 45-min training sessions, with an interval of 1 h between the two training sessions. Memory (MT) was tested 24 h later in 490 of these snails. The other 1000 snails were used in extinction, forgetting, change-of-context and memory-extension experiments and thus could not contribute to the 24 h memory test. An analysis of variance (ANOVA; $F_{1490,1}$=1460, $P$<0.0001) shows that learning occurred. When we tested for memory 24 h later we found that the number of attempted pneumostome openings in the memory test session was not significantly greater ($P$>0.05) than the number in Session 2 and was significantly less than the number in Session 1 ($P$<0.001). Thus, the criteria for memory were met. (B) The assignment of marks to individual snails. The vast majority of snails (77%) received either an A or a B mark, while 12% 'flunked' (i.e. received an F).

## Behavioural learning and memory: the assignment of marks

As is readily apparent, we employ behavioural training to bring about changes in neuronal and molecular activity that *cause* memory formation. While this seems the obvious way to go, it is in fact not often done! In many cases, neural analogues (i.e. electrical stimulation of inputs to neurons or exogenous application of presumed transmitters/neuromodulators to cultured neurons) of training are used. Often, too, the neurons used have not been shown to be either necessary or sufficient for the behaviour, learning or memory.

We have shown above that a single neuron is a site of memory formation and storage. But the inquisitive reader might ask: 'how robust is the learning, and how robust is the memory in our model system?'

The approach we have taken to answer this question is to combine all of our data obtained using one specific training procedure and then examine this population as to how well or how poorly each individual learned and remembered. Since we teach and give examinations to students in a university, it seemed appropriate to give 'marks' or to 'grade' each snail and then view the performance level of 'the class as a whole'. The data set below ($N$=~1500 snails) uses the following marking system: a mark of 'A' is assigned if the last training session is greater than a 50% reduction compared with the initial training session; a mark of 'B' is assigned if the last training session is a 35–50% reduction of the initial session; a mark of 'C' is assigned is there is a 20–35% decrease; and an 'F' is assigned if the decrease is less than 20%.

Fig. 5A shows the overall learning and memory curves for snails trained using the procedure of two 45-min training sessions (each session separated by a 1-h interval) and testing for memory 1 day later. As a group, there was a 46% decrease in the number of attempted pneumostome openings in Session 2 compared with Session 1. An analysis of variance (ANOVA) showed that there was a significant effect ($P$=0.0001, $N$=1490) of training on the number of attempted pneumostome openings (i.e. learning occurred). Overall, the class mark assigned would be a B+. Not too bad! As is typical (sad to say) in the overwhelming majority of schools (including universities), following training (i.e. lectures) a test is given (i.e. memory test) and marks assigned based on performance. We tested 490 snails for memory 24 h later (the remaining 1000 snails were used for other experiments and were not tested for 24 h memory, thus they are not included here) and found that the criteria for memory were met. As a class, we would have assigned a mark of A, since, as a group, there was greater than a 50% decrease in the number of attempted pneumostome openings compared with Session 1. Most teachers and school boards we know would be extremely satisfied with this overall class result. When we assigned individual grades based on the memory test, we found the following grade distribution (Fig. 5B): 57% of snails received an A, 20% a B; 11% a C and 12% an F. Thus, the vast majority of our snails (77% received an A or a B) show very good learning and memory. We conclude that learning and memory in these snails is both reproducible and robust.

What is also interesting to us is why one in 10 snails do not form memory. We do not know what is different about these snails. These 'memory-challenged' snails can serve as a positive control when we begin to examine the molecular changes that cause memory to be formed and stored. We may also be able to give 'remedial education' to these snails in the form of more training or training using a different procedure

in an attempt to get them to a pass level. This may also allow us to determine what is different about their nervous systems compared with those of the other 90% of the snails.

## Where to next?

We do not know what physically constitutes LTM in our *Lymnaea* model system. However, we now know that, once established, the changes that constitute LTM do not require the soma of RPeD1, as its functional surviving primary neurite has the necessary molecular machinery to maintain LTM. We do not know how this happens. Possibly, surrounding glia 'donate' the necessary mRNA to maintain the 'memory-proteins'. If this is the case, then it suggests that memory is encoded by 'everyday' proteins that could be maintained by 'housekeeping' mRNAs from glia, etc. This is only speculation and needs to be directly tested.

We may now be able to directly test in an identified single neuron which genes are turned on or off, what proteins constitute the physical basis of memory storage, and where within the cell these proteins are located. Transcription factors analogous to those found in *Aplysia* (CREB1a, CREB2 and C/EBP; Alberini et al., 1994: Bartsch et al., 1995; Carew and Sutton, 2001; Kandel, 2001; Silva et al., 1998) have now been cloned in *Lymnaea*, and preliminary data have shown them to be present in RPeD1 (Hatakeyama et al., 2002; Sadamoto et al., 2002). Thus, by taking genomic and proteomic approaches, we may be able to specify the nature of the changes that encode memory in RPeD1.

Finally, although we have shown that RPeD1's soma is *necessary* for LTM we have not shown that changes in it are *sufficient* for LTM. It may be possible to directly determine if RPeD1's soma is both necessary and sufficient for LTM by transplantation experiments (see Syed et al., 1992). If RPeD1's soma is both necessary and sufficient for LTM then transplantation of an 'educated' RPeD1 to a naïve snail would result in the recipient snail exhibiting the behavioural phenotype of a trained snail with LTM.

## Concluding remarks

*Lymnaea* has proven itself to be a remarkable model system in which to study associative learning and its long-term memory. In a few short years, at least four different laboratories have shown at least three different forms of associative learning and its memory and have initiated studies of the molecular causal mechanisms of memory formation. If the *Lymnaea* learning and memory groups can couple their findings with those produced by the powerhouse Syed laboratory (who use sophisticated culture and microscopic techniques in determining synapse formation and functioning), the future looks bright for *Lymnaea* to lead the way in search of the engram.

Will the engram be found? Possibly, but as in any quest there will be interesting and important diversions along the way that will grab our attention and make us rethink many of our accepted beliefs. The next few years will certainly bring lots of fun, but also frustration, as we again rediscover the axiom that 'the more we really know the *less we know* about our subject'. It may be that all we will ever get are just more tantalizing glimpses of the Grail, which always appears to be just a little ahead and around the corner.

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