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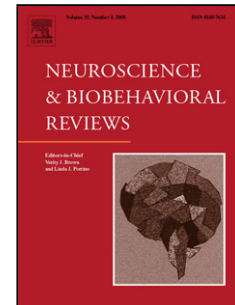
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***Lymnaea stagnalis* as model for translational neuroscience research: from pond to bench**

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Highlights

- 1) *Lymnaea stagnalis*, a reductionistic, yet sophisticated model to address fundamental questions in learning and memory
- 2) Learning and memory in snails have been highly conserved
- 3) The “molecular actors” memory are similar both across phylogenetic groups and learning paradigms
- 4) *Lymnaea stagnalis* teach us the importance of context,
- 5) *Lymnaea stagnalis* to understand in what conditions we memorize, we eat, we memorize, we age
- 6) *Lymnaea* is a valid and reliable model to move research from pond to bench to bedside

Abstract

The purpose of this review is to illustrate how a reductionistic, but sophisticated, approach based on the use of a simple model system such as the pond snail *Lymnaea stagnalis* (*L. stagnalis*), might be useful to address fundamental questions in learning and memory. *L. stagnalis*, as a model, provides an interesting platform to investigate the dialog between the synapse and the nucleus and vice versa during memory and learning. More importantly, the “molecular actors” of the memory dialogue are well-conserved both across phylogenetic groups and learning paradigms, involving single- or multi-trials, aversion or reward, operant or classical conditioning. At the same time, this model could help to study how, where and when the memory dialog is impaired in stressful conditions and during aging and neurodegeneration in humans and thus offers new insights and targets in order to develop innovative therapies and technology for the treatment of a range of neurological and neurodegenerative disorders.

1. Background

Understanding the molecular and physiological mechanisms involved in brain disorders is one of the most important challenges in neuroscience today. In the last decades, the animal models of choice used in neuroscience research have been mostly small mammals (i.e. rats and mice). However, this approach may not be always the most appropriate, and has fuelled many protests and triggered many scientific, economic, ethical and social discussions (Alberts, 2010).

During the evolutionary process that prompted the diversity among species, numerous molecular pathways have been almost entirely conserved ~~across species~~ (Ottaviani et al., 2007), which has led to the recognition of invertebrate models as a more flexible tool to study the basic and conserved mechanisms of central nervous system (CNS) physiology and pathology (Corning, Dyal and Willows, 1973; Kaang et al., 1993; Ottaviani et al., 2013; Tascedda et al., 2015), exceeding the practical and conceptual limitations of experimentation on mammals and cell cultures. Moreover, this approach, reduces the time and the costs of experimentation. On balance, using an interdisciplinary approach, that combines different methods and fields including evolution, genetics, molecular biology and behaviour, a model such as *Lymnaea stagnalis* will allow us to open new frontiers towards translational neuroscience research, starting from simple model systems, passing through more complex organisms, until arriving at *Homo sapiens sapiens*.

2. Attributes of *L. stagnalis* as model for studies in the field of Neuroscience

It is well-known that molluscan gastropods are good models to study the molecular and cellular mechanisms of neuronal function and dysfunction (Nestler et al., 2010; Burne et al., 2011; Tascedda et al., 2015; Stefano et al., 2015). Among them, attention should be paid to the pond snail *Lymnaea stagnalis* (*L. stagnalis*, Linnaeus, 1758), a pulmonate gastropod, widely used as model system in basic and applied neuroscience research (Murakami et al., 2013a; Takigami et al., 2013). The CNS of *L. stagnalis* consists of approximately 20,000 readily identifiable neurons, organized in a ring of 11 interconnected ganglia, whose functional roles within specific networks can be defined and directly attributed to observable behaviours, such as reproduction (van Minnen et al., 1989; Ter Maat et al., 1992), respiration (Syed et al., 1990; Winlow et al., 1992), feeding (Yeoman et al., 1994; Straub et al., 2002) and locomotion (Syed et al., 1991). Not only, many neurons are large in size (diameter up to ~100 μm), offering a large amount of biological material for molecular, morphological and functional analyses, which have led to the validation of the function of specific genes and the study of the molecules and metabolic pathways involved in neuronal regeneration (Hermann et al., 2000; Koert et al., 2001), synapse formation (Syed et al., 1992; Feng et al., 1997; Gardzinski et al., 2007), synaptic plasticity (Smit et al., 2001), neurodevelopment (Croll et al., 2000), aging (Wildering et al., 1991; Klaassen et al., 1998; Patel et al., 2006), adaptive responses to stress (Hermann et al., 1998; Fei et al., 2007) and, last, but not least, learning and memory formation (Benjamin et al., 2000; Lukowiak et al., 2003).

We centred this review around two key-questions:

- 1) What changes in the brain during learning?
- 2) Once something is learned, how is that information stored ~~memorized~~ in the brain?

To answer these questions, we think it is necessary to start from the simplest examples of memory storage (Kandel, 2001) and the most experimentally manageable animal models.

We recognize that most of the molecular studies in *L. stagnalis* are the result of studies of homology and we also realise that the use of a reductionist approach in the 21st-century is an arduous undertaking, but if elementary forms of learning are common to all animals with an evolved nervous system, conserved processes must exist in the molecular mechanisms of learning that can be studied more effectively in simple invertebrate animals (Kandel, 2001). Thus, our purpose is to illustrate how this reductionistic, but not simplistic, approach based on the use of a simple model system such as *L. stagnalis*, might be useful to address fundamental questions in learning and memory. The reconstruction of the extensive dialog between the synapse and the nucleus, and the nucleus and the synapse during memory and learning, could also help to study how, where and when this dialog is impaired in stressful conditions and during aging and neurodegeneration in humans.

3. *L. stagnalis* as model for the integrative molecule-to behaviour study of memory and learning

Learning about the predictive association between events and the consequences of specific behaviours is indispensable for animals to adapt and survive in complex and ever-changing environments. Remembering these associations, animals alter their behaviour appropriately and this alteration can be defined as learning (Dalesman and Lukowiak, 2012; Lapiedra et al., 2017). While non-associative learning (i.e. habituation and sensitization) is the simplest and most primitive form of learning, associative learning is more complex and requires that stimuli occur in close temporal contiguity and in a fixed sequence (Byrne and Hawkins, 2015). Among the various categories of associative learning, classical and operant conditioning are the best known and well-studied. In particular, in classical conditioning a neutral conditioned stimulus (CS) paired with a forceful unconditioned stimulus (US) is hypothesized to evoke the unconditional response. On the other hand, operant conditioning is response-contingent and involves the presentation of a reinforcing stimulus when the animal performs a specific behaviour. If the reinforcing stimulus is negative, animals learn to avoid engaging in the behaviour, if the stimulus is positive, animals spontaneously perform the behaviour more often (Mackintosh, 1974). In this complex scenario, *L. stagnalis* has proven to be a useful model for the study of the molecular, cellular, and neuronal networks related to memory, as well as the behavioural aspects of learning and its consolidation in long-term memory (LTM) (Ito et al., 1999; Lukowiak et al., 2000; Murakami et al., 2013b).

Indeed, the knowledge of the architecture of the essential neural circuits of behaviours that can be conditioned (i.e. respiration and feeding), allowed the identification and the study of neurons exhibiting plasticity. Using intact vertebrate biological systems, these goals are more difficult to attain. Many studies in the last decades demonstrated that *L. stagnalis* can be both classically and operantly conditioned (Kojima et al., 1996; Sakakibara et al., 1998; Kobayashi et al., 1998; Spencer et al., 1999; Lukowiak et al., 2000). In particular, in food reward classical conditioning (Kemenes and Benjamin, 1989; Kemenes, et al., 2006), a neutral conditioned stimulus (CS – i.e. amyl acetate, gentle taps to the lips or visual cues) was paired with a strong unconditioned feeding stimulus (US – usually sucrose) and the temporal-contingent repeated presentation of these stimuli resulted in a sequence of rhythmic and stereotyped feeding movements when snails were exposed to the CS alone in the post-training phase.

This behavioural-conditioned response suggested that snails learned that the CS “means” food (Kemenes and Benjamin, 2009). Interestingly, when *Lymnaea* is moderately food-deprived, it is capable to acquisition and extensive retention (for at least 19 days) of an appetitively reinforced feeding response after only a single training trial, offering the possibility to perform detailed analyses of the neural mechanisms underlying plasticity (Alexander Jr. et al. 1982, 1984; Fulton et al., 2005; Ribeiro et al., 2005).

L. stagnalis have been also trained using conditioned taste aversion (CTA) learned and subsequently formed memory to suppress the feeding response to an appetitive CS (usually sucrose) when paired with an aversive US (usually KCl or tactile stimulus) that ultimately inhibited feeding. Consequently, snails learned that the presentation of a CS signal was associated with the imminent arrival of the aversive stimulus (Kojima et al., 1997). Moreover, the aerial respiratory behaviour of snails, which is characterized by the spontaneous opening and closing of the pneumostome (the respiratory orifice) at the water surface (Boycott, 1936; Jones et al., 1961), can be operantly conditioned. When the animal attempted to open its pneumostome as a reaction to hypoxic water, it received a gentle tactile stimulus to the pneumostome area, evoking, as escape-withdrawal reflex, its closure. Prolonged tactile stimulation of the pneumostome every time the animal attempted to breathe resulted in significantly fewer attempts to open the pneumostome as training proceeded (Lukowiak et al., 1996). In a recent study, Lukowiak and co-workers demonstrated the capacity of snails to perform configural learning, that resulted in the ability to treat stimuli experienced together as different from the simple sum of their elements (Swinton et al., 2019). These results are consistent with the hypothesis that stimulus–stimulus learning is an important adaptive learning mechanism that helps animals, from invertebrates to mammals, to decipher the meaning of important stimuli in their environment. The ability of snails to undergo configurational learning is an ulterior confirmation of its potentiality in neuroscience and behavioural research (Swinton et al., 2019). Depending on the training procedure used, either intermediate term-memory (ITM; persisting up to 3 h) or LTM (persisting for at least 24 h) occurred in *L. stagnalis* (Kojima et al., 1996; Benjamin et al., 2000; Sangha et al., 2003c; Ito et al., 2013; Otsuka et al., 2013; Takahashi et al., 2013; Lukowiak et al., 2014; Sunada et al., 2014). Because the inhibition of transcription or translation blocked the formation of LTM in a variety of model systems, but did not affect short-term memory (STM), it has been concluded that this phase, lasting only minutes, does not require neither *de novo* protein nor RNA synthesis (De Zazzo and Tully, 1995). In addition to the differences in the length of time that memory persisted, a difference between LTM and ITM in requiring altered gene activity was observed. In fact, while ITM required the translation of new proteins from pre-existing RNA and only depended on new protein synthesis, LTM depended on altered gene activity and required both the transcription of new RNA, and their translation into new proteins (Lukowiak et al., 1996; McGaugh, 2000; Inda et al., 2005; Sangha et al., 2005). Thus, LTM is represented at the cellular level by activity-dependent modulation of both the function and the structure of specific synaptic connections that, in turn, depend on the activation of specific patterns of gene expression. In recent years much effort has gone into identifying the signalling cascades that ultimately lead to the production of new proteins for the process of memory formation, such as, proteins required for the maturation of particular synapses that store the remembered information. A variety of different molecular and biochemical tools have been used to measure changes in the expression or activation levels of specific molecules during LTM and ITM in *L. stagnalis* and enhanced expression or activation of these key factors has been observed in specific stages of memory formation and consolidation.

As would be expected with such vital processes, learning and memory are observable across a vast array of species. It is thus reasonable to hypothesize that such a fundamental conserved mechanism, may occur as the result of a well conserved set of underlying molecular mechanisms. On balance, in this complex and dynamic scenario, *Lymnaea* could give an enormous contribution to understand the molecular mechanism by which organisms acquire, store, and eventually use their experiences.

4. Toward a molecular pathway for memory and learning in *L. stagnalis*

4.1 Lym-CREB in the synaptic enhancement of memory consolidation

One transcription factor that plays a major role in LTM formation in *Lymnaea* is the homologous of cAMP response element-binding protein, LymCREB (Silva et al., 1998). The cDNA sequences for the activator type of CREB, LymCREB1, and the repressor type, LymCREB2, in *L. stagnalis* have successfully been cloned and analysed (Sadamoto et al., 2004). In particular, LymCREB homodimers were demonstrated at both the mRNA and protein level in cerebral giant cells (CGCs) (Ribeiro et al., 2003; Sadamoto et al., 2004) and in the right pedal dorsal 1 interneuron (RPeD1), which are necessary for CTA (Kojima et al. 1997; Nakamura et al. 1999; Scheibenstock et al., 2002) and operant conditioning of respiration (Taylor and Lukowiak, 1992; Scheibenstock et al., 2002; Sangha et al. 2003b), respectively. In particular, memory training in these cells enhanced both the levels of phosphorylated LymCREB1 (Ribeiro et al., 2003) as well as LymCREB1 gene expression (Sadamoto et al. 2010), indicating that memory training increased both the gene expression of LymCREB transcriptional activator and the level of its activation by phosphorylation. After phosphorylation, LymCREB1, in turn, initiated a cascade of altered gene activity and new protein synthesis, necessary for synaptic enhancement in memory consolidation (Nakamura et al., 1999; Ribeiro et al., 2003; Sadamoto et al., 2004). In contrast, LymCREB2 inhibited the function of LymCREB1 (Nakamura et al., 1999) and the ratio of activator/repressor LymCREBs has been proposed to act as a “molecular switch” in determining whether LTM is formed (Sadamoto et al., 2004). Similar findings have been obtained in invertebrates, including *D. melanogaster* (Perazzona et al., 2004) and *A. californica* (Bartsch et al., 1995), and in mammals (Karpinski et al., 1992; Yin et al., 1994; Josselyn et al., 2001). Most of the upstream signalling cascade leading to the activation of CREB appears to be conserved through evolution, and many aspects of the role of CREB in synaptic plasticity described in invertebrates have also been observed in the mammalian CNS (Barco et al., 2003).

Evidence from numerous model systems indicate that CREB-driven transcription results downstream of the activation of Cyclic Adenosine Monophosphate (cAMP), which mediates almost all of its actions through protein kinase A (PKA). Furthermore, in various model systems ranging from invertebrates to mammals, CREB1 works as a transcriptional activator only after its phosphorylation by either PKA, mitogen-associated protein kinase (MAPK) or calcium calmodulin-dependent protein kinase II (CaMKII) (Montminy, 1997). Similar to mammals and *Aplysia*, LymCREB1 contains a kinase inducible domain which presents consensus sequences of several kinases (Pinna and Ruzzene, 1996), such as LymPKA, protein kinase C (LymPKC), LymCaMKII and protein kinase G (PKG) (Sadamoto et al., 2004). On the other hand, LymCREB2 has two consensus sequences for the LymMAPK phosphorylation site and one PKC recognition site (Sadamoto et al., 2004), suggesting that these kinases act as memory promoter genes that up-regulate the expression of LymCREB or down-regulate the suppressor activity of LymCREB2.

The contribution to synaptic plasticity and memory of these kinases, together with highly conserved molecular targets, such as N-methyl-D-aspartate (NMDA) glutamate receptors (GRINs) and nitric oxide (NO), have been investigated in *Lymnaea*.

4.2 Involvement of LymGRIN in associative plasticity processes

NMDA receptors are required for memory formation across several types of memory and numerous species (Szapiro et al., 2003; Xia et al., 2005; Zhang et al., 2005; Glanzman et al., 2008; Kano et al., 2008). The main characteristics of NMDA receptors, such as the permeability to calcium, the voltage dependent magnesium block, the slow kinetics, together with the numerous binding sites for cofactors, make them well suited for associative plasticity processes, that are specifically mediated by the entry of calcium, which, in turn, activates a variety of cell signalling cascades, involving PKC and CaMKII pathways and NO synthase (NOs), which all contribute to memory formation (Ha et al., 2006; Wan et al., 2010; Rosenegeer et al., 2010). Data obtained from *L. stagnalis* using agents that block the receptors suggest that the activation of the homologous of NMDA receptors, LymGRINs, are required in order to allow LTM formation following conditioning (Rosenegeer and Lukowiak, 2010; Wan et al., 2010). Evidence of this, is the effect of ketamine, a NMDA blocker, which compromised the consolidation phase of memory, by acting on transcriptional events that are exclusive for early LTM but not for ITM (Browning and Lukowiak, 2008), or late LTM (Wan et al., 2010). These findings are consistent with the vertebrate and invertebrate literature on learning (Shimizu et al., 2000; Silva et al., 2003; Irvine et al., 2005; Bevilacqua et al., 2005), where the entry of calcium through NMDARs during robust synaptic stimulation triggered synapse-to-nucleus signalling cascades that resulted in the activation of CREB through PKA and MAPK-mediated phosphorylation (Montminy, 1997).

4.3 Lym-PKC in memory acquisition and maintenance

The entrance of Ca^{2+} through NMDARs activates directly or indirectly numerous protein kinases, including PKC (Malinow et al., 1988). The role of the PKC family has been investigated in many learning paradigms and animal models, including numerous invertebrate models (Choi et al., 1991; Muzzio et al., 1997). For more than two decades, PKC activation has been implicated in the formation of associative memory in a variety of species, including the mollusc, *Hermissenda crassicornis*, rodents and rabbits (Bank et al., 1988, Olds et al., 1989; Nelson et al., 1990), providing a valid support for a mechanism that has been conserved across the evolution of species ranging from invertebrate molluscs to higher mammals (Takigami et al., 2014a). Interestingly, data from *Lymnaea* indicated that the administration of bryostatin, a PKC activator, before the conditioning training procedure enhanced both ITM and the length of LTM (Rosenegeer et al., 2008; Takigami et al., 2014), whereas injection of a PKC inhibitor (GF109203X) blocked both ITM and LTM formation (Rosenegeer et al., 2008). In this regard, Rosenegeer, Parvez, and Lukowiak (2008) demonstrated that pre-treatment of *Lymnaea* with bryostatin before operant conditioning of aerial respiration not only makes it easier to produce LTM, but also makes the memory persist much longer (Rosenegeer et al., 2008).

Moreover, Tagikami and colleagues (2014) demonstrated that the mechanism by which STM becomes consolidated in LTM involved the activation of PKC-mediated phosphorylation, following CTA (Tagikami et al., 2014a). In fact, when bryostatin was injected within the early time period following CTA trials, memory consolidation was progressively enhanced, suggesting that PKC initiated the synthesis of new proteins necessary for LTM formation and enhanced mRNA translation following DNA transcription (Tagikami et al., 2014a). In turn, protein synthesis, seems to be critical for providing essential biochemical and structural components to the synaptic apparatus required for the implementation of the memory storage process. This hypothesis is fully supported by similar findings indicating that bryostatin-induced PKC phosphorylation extended memory duration in both *Hermissenda* and mammals following Pavlovian-conditioning (Alkon et al., 2005; Kuzirian et al., 2006; Hongpaisan and Alkon, 2007; Sun and Alkon, 2008).

Because PKC dysfunctions are involved in several types of memory impairments in both humans and rodents (Pascale et al., 1998) and considering the highly-conserved involvement of this pathway in memory, *L. stagnalis* represents an attractive tool to elucidate the potentiality of PKC as pharmacological target for the treatment of memory decline and dementias.

4.4 LymCaMKII in late consolidation of associative memory

The involvement of CaMKII in memory acquisition has been well documented in several organisms (Cammarota et al., 2002; Silva, 2003; Elgersma et al., 2004). CaMKII, like other CaM-kinases, is activated by the transient influx of Ca^{2+} (e.g., through NMDA receptors) and plays a role in subsequent transcriptional and translational processes that involved CREB (Silva et al., 1998; Abel and Lattal, 2001; Hudmon and Schulman, 2002; Wang et al., 2006). Similarly, CaMKII is an highly suitable molecular substrate for LTM storage due to its unique ability to maintain an active auto-phosphorylated state even after the decay of external stimuli (i.e., when Ca^{2+} influx stops) (Hook and Means, 2001). The homologous of CaMKII has been cloned in *Lymnaea* (LymCaMKII) and shared important functional roles in learning and memory (Wan et al., 2010) with its mammalian counterpart. Previous studies identified a critical time window (occurring approximately at 24 hours after training) during which the activation of LymCaMKII was required for the late consolidation of associative memory. In contrast, no evidence was found for a role of the activation of LymCaMKII (or other LymCaM-kinases) in early or intermediate consolidation, for up to 20 hours after training (Wan et al., 2010). Because LymGRINs are only involved in the acquisition of LTM, whereas LymCaMKII participates in the late consolidation phase, there is a dissociation of NMDA receptor function and CaMKII activation between these two different phases of memory formation. This is different from what has been described in NMDA receptor and CaMKII knock-out mice, where the activation of CaMKII resulted from the upstream activation of NMDA receptors (Wang et al., 2003). In this regard, it was hypothesised that the intrinsic activation of LymCaMKII leads to sustained high levels of auto-phosphorylated CaMKII, which the ensuing learning-induced delayed rise in Ca^{2+} from glutamatergic receptors.

Additionally, using CaMKII and NMDA inhibitors it was found that, while memory consolidation depended on both NMDA receptors and CaMKII activation, CaMKII-dependent late memory consolidation did not require the activation of NMDA receptors (Wan et al., 2010). This suggested that the rise of Ca^{2+} was mediated by non-NMDA type voltage-gated calcium channels or intracellular calcium stores in the CGC axon terminals (Kemenes et al., 2006). Actually, it has yet to be

established whether this function has been conserved in other more complex organisms. While LymCaMKII was not involved in late consolidation, LymNO and LymPKA were required for early memory formation (Kemenes et al., 2002, 2006). The complementary roles played by LymCaMKII, LymNO, and LymPKA suggest that the dynamic consolidation phase involves both parallel and sequential activation of different signalling cascades in the different phases of the consolidation of long-term memory.

4.5 Lym-PKA activity induced distinct temporal patterns are correlated with different memory phases

Studies in *Aplysia* first revealed the participation of the cAMP/PKA-signalling pathway in synaptic facilitation and sensitization (Brunelli et al., 1976). Moreover, in both invertebrates and vertebrates the dynamic network of molecular signalling cascades activated by learning, involved highly conserved PKA-mediated mechanisms (Selcher et al. 2002; Roberts and Glanzman 2003; Schwärzel et al. 2007). In *L. stagnalis*, an increase in PKA during the first 10 minutes after training was essential for an early phase of LTM (6 hours). On the other hand, prolonged activation of LymPKA in the late phase of LTM (24 hours) was involved in memory reconsolidation (Michel et al., 2008). Thus, very early PKA-mediated events that are necessary for 6-hour memory formation, are not sufficient for a 24 hours memory trace to form, which depends on more prolonged PKA activity. This finding implies a distinct temporal pattern induced by PKA activity that is related to the formation of different phases of memory, which has been observed in rodents as well (Tronson et al., 2006). Various protein phosphatases regulate the local activity of PKA acting as inhibitory constraints on memory formation. In this regard, it has been suggested that in snails an equilibrium between both kinase and phosphatase activities exists and regulates both memory storage as well as retrieval (Sharma et al., 2003).

4.6 Consequences of LymPACAP learning-induced activation of adenylate cyclase

Studies concerning the role of PKA in the consolidation of memory in *Lymnaea* indicate that, similar to other systems, activation of adenylate cyclase (AC) forms a key step in LTM formation. Interestingly, in the *L. stagnalis* nervous system, the protein homologous to the vertebrate pituitary adenylate cyclase-activating polypeptide, LymPACAP, and its receptors were involved in learning-induced activation of AC (Pirger et al., 2010). In particular, activation of AC by PACAP was necessary for LTM to occur in a food-reward conditioning paradigm. Moreover, the application of PACAP at the beginning of multi-trial conditioning accelerated the formation of transcription-dependent memory. This memory-boosting effect of exogenously applied PACAP was blocked by the PACAP receptor antagonist PACAP6-38 in both single-trial chemical and multi-trial tactile conditioning, suggesting that LymPACAP is released in response to chemical and tactile conditioning (Pirger et al., 2010).

4.7 LymNO-dependent cascade and memory and learning

The nitric oxide (NO)-cGMP signalling system, together with the cAMP system, plays a critical role in the protein synthesis-dependent formation of LTM in many vertebrates and invertebrates (Moroz et al., 1994; Lu et al. 1999; Roberson et al. 1999). In *Lymnaea*, mRNA transcripts from the two related nNOS genes, Lym-nNOS1 and Lym-nNOS2, are expressed in CGCs (Korneev et al., 2005) and there is ample evidence that the consolidation of a memory trace following one-trial chemical conditioning depends on the LymNO-GMP signalling pathway. In fact, 6 hours after chemical conditioning, Lym-nNOS1 was up-regulated and a critical period of sensitivity up to 5 hours after conditioning was observed when blocking this pathway thus preventing the formation of LTM (Kemenes et al., 2002). This is in line with studies in bees and mice, where the NO-cGMP-pathway, together with the PKA-cascade, is necessary in the early post-training phase of memory formation (Müller, 2000). As previously shown in numerous model systems, it was assumed that in *Lymnaea* the LymNO-cGMP cascade (Park et al., 1998; Sadamoto et al., 1998) activates different signalling pathways via LymCREB1 phosphorylation (Lu et al., 1999). In fact, the LymNO-cGMP cascade involves the phosphokinase G (PKG), that, in turn, binds PKG kinase consensus sequences in the kinase inducible domain of LymCREB1, thereby activating LymCREB1 by phosphorylation (Sadamoto et al., 2004). Moreover, in *Lymnaea* NO modulated the strength of serotonergic neurotransmission between CGCs and motoneurons in the feeding system (Straub et al., 2007) which could be involved in conditioning.

4.8 LymMAPK and its role in intermediate and long-term memory

The complex molecular signalling cascades activated by behavioural training in *L. stagnalis* also results in the activation of the orthologous of mitogen-activated protein kinase (LymMAPK), as shown in numerous model system and training paradigms (Morris et al., 1986; Tsien et al., 1996; Silva et al. 1998; Müller 2000; Valjent et al., 2001; Sharma and Carew 2004). In *Aplysia*, for example, the translocation of MAPK in the nucleus after stimuli presentation results in long-term facilitation at the sensory motor neuron synapse (Martin et al., 1999). In *Lymnaea*, classical food-reward conditioning training resulted in elevated levels of activated LymMAPK in protein extracts from the cerebral and buccal ganglia and lip tissue surrounding the mouth (Ribeiro et al. 2005), indicating that both the CNS and the peripheral nervous system are involved in memory formation. In addition, in rats, the inhibition of MAPK activity blocked the formation of both ITM and LTM (Rosengger et al., 2010), where fear conditioning was observed as a result of the activation of this cascade (Atkins et al., 1997). Based on these observations, it was hypothesized that in *Lymnaea* LymMAPK was active both during ITM, which only required translation, and during the formation of LTM, providing correlative evidence that for LTM to form, ITM must occur first (Parvez et al., 2006). In light of this, in *Lymnaea*, as in other model systems, LymMAPK and LymPKC-mediated intracellular cascades and play a fundamental role in either signalling, initiating and maintaining processes that alter gene activity and induce new protein synthesis necessary for the formation of memories that persist longer than a few minutes. Remarkably, unlike other factors, after single-trial reward conditioning, LymMAPK was activated not only in response to contingent CS-US application, but also, when the stimuli were applied alone, suggesting that this kinase is necessary but not sufficient for the consolidation of associative LTM (Wan et al., 2010).

Not only, food-reward conditioning selectively increased LymCREB phosphorylation in the same ganglia that expressed LymMAPK to regulate feeding behaviours (Ribeiro et al. 2003), suggesting that phosphorylation of LymCREB in neurons could result in downstream activation of LymMAPK, as is observed in mammals (Thomas and Hugarir 2004).

L. stagnalis studies based on single-trial food-reward classical conditioning have yielded information on a variety of general and specific, molecular and cellular, mechanisms necessary for the consolidation of memory, that involve the regulation of gene expression by transcription factors, such as LymCREB and the activation of LymPKA, LymPKC, and MAPK signalling pathways, as well as LymGRIN receptors and LymCaMKII (Fig. 1). Mounting evidence suggests that these molecular pathways have been highly conserved in learning, both across phylogenetic groups and learning paradigms, involving single- or multi-trials, aversion or reward, operant or classical conditioning. Even if LymPKA, LymNMDA receptors, LymCaMKII, LymCREB, and LymNOS/NO are selectively activated or upregulated, it seems likely that these and other signalling molecules are part of a synergistic effort and together contribute to the memory consolidation process, with none of them alone being sufficient for LTM (Kemenes, 2013) (Fig. 2).

4.9 LymC/EPB synaptic plasticity and memory consolidation

In line with data from *Aplysia*, *Helix* and mammals, LymCREB seems to regulate the expression of the homologous of CCAAT/enhancer binding protein, LymC/EBP, (Alberini et al., 1994; Niehof et al., 1997), which is an immediate-early gene involved in synaptic plasticity necessary for memory consolidation after CTA (Hatakeyama et al., 2006).

In particular, early consolidation of memory after CTA learning in *L. stagnalis* involved not only the rapid synthesis and phosphorylation of LymC/EBP, but also required the rapid breakdown of its mRNA, suggesting that a pool of LymC/EBP mRNA is rapidly translated and degraded after CTA learning. This fast turnover of newly transcribed mRNA was necessary for the prolonged *de novo* synthesis of LymC/EBP, fundamental for the consolidation phase of memory formation (Hatakeyama et al., 2004). These results led to the formation of a general rule stating that effective gene activation by a transcription factor involved in LTM consolidation requires an increase in the amount of the transcription factor itself (Hatakeyama et al., 2006). Although is not yet known which genes act downstream of LymC/EBP activation, based on data from *Aplysia*, *Helix*, and mammals (Alberini, 1994; Niehof et al., 1997; Hatakeyama et al., 2006), it is hypothesized that the expression of LymC/EBP is likely regulated by LymCREB. The potential downstream targets of C/EBP likely are the LymNOS genes. These targets are, in fact, co-localized in B2 motoneurons, and LymNOS genes have three putative LymC/EBP binding sites, which would provide the necessary structural conditions for the interaction of C/EBP with NOs genes in the *Lymnaea* feeding network. In addition, a link has been hypothesized between LymC/EBP and insulin-like growth factor 2 (IGF2), as previously demonstrated in mammals (Alberini et al., 2012). If confirmed, these data would contribute to a better understanding of the role of IGF genes in memory enhancements.

4.10 LymMIP involvement in long-term synaptic enhancement

Another intriguing factor involved in memory formation is the molluscan insulin-related peptide II (MIP-II), that belongs to the insulin superfamily. LymMIP-II was first cloned in *Lymnaea* (Smit et al., 1991; Li et al., 1992) and is expressed in the growth-controlling neuroendocrine light green cells, which are located in the cerebral ganglia (Meester et al., 1992; Smit et al., 1992). Because insulin receptors, including MIP receptors (Roovers et al., 1995), are homologous across *phyla* (Jonas et al., 1996), and ligand-binding sites are well-conserved, the use of an antibody against the extracellular domain of the mammal insulin receptor was assumed to act as an antagonist for MIP receptor (Murakami et al., 2013b). Indeed, injection of the insulin receptor antibody into the snail abdomen before CTA training blocked the memory consolidation process (Murakami et al., 2013a). On the contrary, when partially purified MIPs or bovine insulin were applied to the isolated nervous system of snails, long-term synaptic enhancement was observed at sites thought to play key roles in CTA learning and LTM formation (i.e. CGCs) (Hatakeyama et al., 2013; Murakami et al., 2013a).

Thus, up-regulation of LymMIP-II stimulates neurite formation (Smit et al., 1988; Kits et al., 1990), confirming that one of the physical manifestations of LTM formation is change in the morphology of the synapse during memory formation (Geraerts, 1992), similar to what was observed in *Aplysia* (Bailey and Kandel, 1993), *C. elegans* (Kodama et al., 2002) and rodents (Dou et al., 2005; Ramsey et al., 2005). Because the expression levels of LymMIP-II do not change when LymCREB1 is inhibited (Azami et al., 2006), the upstream transcription factors that regulate the expression of MIP-II do not directly involve LymCREB interaction. Insulin plays an important role in cognitive function across species and numerous human clinical studies suggest a link between type 2 diabetes mellitus, insulin resistance, and cognitive dysfunction (Biessels and Reagan, 2015; Heni et al., 2015; Kim and Feldman, 2015; Mainardi et al., 2015; Tramutola et al., 2018). Consequently, *Lymnaea* as a model system could help to elucidate the involvement of insulin dysregulation and memory impairment.

4.11 The monoaminergic system of *L. stagnalis* and its role in memory-related changes

Accumulating evidence suggest that when memory scores in CTA are better, the monoamine contents in the nervous system of *Lymnaea* are lower and when the insulin content in the CNS decreases, so do the monoamine contents correlated with higher memory scores. Thus, the ratio of synaptic monoamine concentration is important for memory formation and consolidation while at the same time memory-related changes may occur when the total monoamine contents in the CNS are low (Totani et al., 2019). In this regard, serotonin (5-hydroxytryptamine: 5-HT) plays a key role in the mediation of learning and memory in molluscs (Dyakonova and Sakharov, 2001). The best known example of this is dishabituation and sensitization of the gill withdrawal reflex in *Aplysia* that is mediated by the release of 5-HT from interneurons (Kandel, 2001).

Moreover, 5-HT drives both feeding behaviour and food satiety in *L. stagnalis* (Kemenes et al., 1990; Croll et al., 1999; Yamanaka et al., 2000; Kawai et al., 2011; Dyakonova et al., 2015). CGCs, that are serotonergic, are sensitive to the concentration of glucose in the hemolymph (Dyakonova et al., 2015). As to 5-HT and memory in *Lymnaea*, an injection with a 5-HT receptor antagonist after CTA induced reversible amnesia (Nikitin and Solntseva, 2013).

Also, 5-HT levels are required to be low for learning and LTM to occur. In fact, while a decrease in the ability to learn and remember was observed in snails immersed in 5-HT, an injection with insulin rescued the ability of snails to learn CTA and form LTM (Mita et al., 2014). Finally, the amount of 5-HT released is controlled by a cAMP-PKA-CREB cascade in the CGC (Nakamura et al., 1999; Sadamoto et al., 2011). Together these data, underscore the suitability of *L. stagnalis* as a model to unravel the complexity of the serotonin signalling pathway (Benatti et al., 2017). Furthermore, dopamine (DA) pathways play an essential role in reward systems in both vertebrates and invertebrates. In *L. stagnalis*, DA is involved in LTM consolidation of reward classical conditioning (Kemenes et al., 2011) and consolidation of appetitive conditioning (i.e. sucrose as the US) (Elliott et al., 2011). Similar to dopamine, octopamine, a neurotransmitter first discovered by Erspamer in octopus (Erspamer, 1948), is also thought to be a reward related neurotransmitter and acts in a similar manner as dopamine, mediating feeding behaviour (Elliott and Vehovszky, 2000) and participating in the formation of LTM after aversive food conditioning (i.e. KCl as the US) (Kemenes et al., 2011). Thus, appetitive and aversive food conditioning in *Lymnaea* are mediated by the dopaminergic and octopaminergic system (Kemenes et al., 2011).

5. Memory extinction: when the memory for conditioning is masked by another form of learning

Memory persistence depends in part on the training procedure used, as proven by numerous studies performed in both mammals (human and rodent) and invertebrates (i.e. *Drosophila*, *Aplysia*, *Lymnaea*, *Apis*) that demonstrated that while 'massed-training' and 'spaced training' result in similar behavioural phenotypes, the latter results in a longer-lasting memory (Hovland, 1940; Carew et al., 1972; Hintzman, 1974; Bitterman et al., 1983; Frost et al., 1985; Lukowiak et al., 1998; Sakakibara et al., 1998; Hermitte, 1999; Lukowiak et al., 2000; Commins et al., 2003; Takahashi et al., 2013; Takigami et al., 2014b). In particular, studies using *Lymnaea* indicated that spaced training is more effective than massed training in both classical conditioning (Sakakibara, 2006; Sakakibara, 2008; Takahashi et al., 2013) and operant conditioning (Kobayashi et al., 1998). Moreover, the different behavioural outcome of these training produces reflects the dynamic molecular modifications underlying STM, ITM and LTM formation, respectively (Takigami et al., 2014b).

On the contrary, because memory transience depends from events that interfere after the formation of memory (McGeoch, 1932; Minami and Dallenbach, 1946), manipulation of snails' post-training environment in order to prevent the occurrence of 'interfering events', extends the persistence of memory (Shanga et al., 2003).

The consolidation phase, in fact, requires time, and under some circumstances consolidation related processes appear to be susceptible to a variety of influences, both facilitating and impairing the stabilization of the memory trace (McGaugh, 1966). Interference-based forgetting occurs when new information acquired either before or after a learning event attenuates memory expression (proactive and retroactive interference, respectively) (Dudai, 2004).

Multiple learning events, in fact, often occur in rapid succession, leading to competition between consolidating memories. In this contest, *Lymnaea* offers the opportunity to study the effect of proactive or retroactive interference when the consolidating memory is either in a stable or labile stage. Recently Crossley and colleagues (2019) demonstrated that when new learning takes place during a stable stage, proactive interference only occurs if the two consolidating memories engage the same circuit mechanisms. On the other hand, if different circuits are used, both memories survive.

They also demonstrated that, even if there is some interaction between the memory systems during the acquisition phase of the new memory, the original memory is only vulnerable to interference when it is in a labile state (Crossley et al., 2019). Despite forgetting (Sangha et al., 2005), that is the loss of the learned behaviour (Schacter, 2001), extinction is the gradual loss of a learned behaviour when a reinforcing stimulus was no longer applied (Pavlov, 1927). Previous studies demonstrated that extinction does not result in the destruction of the earlier formed memory, but is thought to be an active process, where the original memory is temporarily occluded by a new memory (Lattal et al., 2006). This process occurs across paradigms and species, passing from *C. elegans* to humans (Myers and Davis, 2002). Extinction in *Lymnaea*, as in other model systems, is not the unlearning of the 'old' memory, on the contrary, it requires new protein synthesis, suggesting that during this process new learning occurs which suppresses, but does not abolish, the memory for previous conditioning (Sangha et al., 2003a). In particular, in *L. stagnalis*, extinction training enhanced the activity of LymGRIN receptors and LymMAPK, both involved in memory formation, implying that changes in the same molecular machinery serves a number of purposes (Rosenegger and Lukowiak, 2010).

These results are consistent with previous studies from rodents, where treatments with NMDA agonists prior to extinction training severely inhibited its acquisition (Cammarota et al., 2005), whereas MAPK was involved in the extinction of conditioned fear (Szapiro et al., 2003) and inhibitory avoidance (Rossato et al., 2006). In this context, *L. stagnalis* represents a good model to determine the differences between the types of memory (extinction and 'original') and more fully understand their mechanisms.

6. Key-molecular factors involved in stress-induced memory block: beyond the Yerkes–Dodson/Hebb law

A large body of evidence from humans and rodents affirms that stress has complex influences on memory performance, with both negative and positive consequences depending on the nature and the "intensity" of the stressor (Baldi and Bucherelli, 2005; Lupien et al., 2007; Sandi and Pinelo-Nava, 2007). According to the 'Yerkes–Dodson Law', too much or too little stress obstructs LTM formation, while 'just the right amount' enhances LTM (Yerkes and Dodson, 1908). Moreover, single-acute versus repetitive-chronic stressors influence memory in distinct ways (Sandi and Loscertales, 1999; Byrne et al., 2014). Many studies provide evidence that opposing effects are induced by stress during the phases of consolidation (generally facilitating) and retrieval (generally impairing) of information (Roosendaal, 2002). That is, stress can be defined as a state that requires dynamic physiological, psychological or behavioral readjustment or modification in order to maintain allostatic load of the organism low, which would help the organism to sustain a 'neuronal cost' (in terms of gene activity and new protein synthesis) to form LTM to 'relevant' events (Lukowiak et al., 2014). It is also important to consider that stress only facilitates learning and memory when experienced in the same context and around the time of the event that needs to be remembered (Joëls et al., 2006). The effects of stress on learning and memory could produce contradictory results: the same stimulus may be perceived as a stressor by one organism but not by another, or may be perceived as a stressor only at certain times in the same organism.

From the literature, disagreement emerges regarding the different effects of stress on memory function (including facilitating, impairing, or the lack of effects). In this regard, *L. stagnalis* represents a compelling integrative model to understand how stress affects memory formation (Benjamin et al., 2000; Otsuka et al., 2013; Takahashi et al., 2013; Lukowiak et al., 2014; Sunada et al., 2014).

Using ecologically relevant stressors that snails are likely to encounter in their natural environment, it is possible to study how learning and memory formation are modified by stressors (Lukowiak et al., 2010). *Lymnaea*, in order to live long and prosper, require adequate quantity of food and a balanced source of calcium, necessary to grow the shell and to detect predators (Dalesman and Lukowiak, 2010). Consequently, restriction of food and/or calcium are considered to be environmental stressors. At the same time, because crowding increases competition for resources, it is considered a social stressor. There is ample evidence that some stressors (e.g. predator detection) lead to enhanced memory formation (Orr and Lukowiak, 2008), whereas other stressors (e.g. crowding) lead to suppression of memory formation (de Caigny and Lukowiak, 2008). Not only, when stressors are experienced in combination, the outcome results in unpredictable consequences on snails' ability to learn and form memory and cannot be predicted based on the impact of the stressors on memory formation when the stressors are presented individually (Huges et al. 2017).

6.1 Enhancement of LTM formation and the initiation of HSPs production in *L. stagnalis*

The exposition of *Lymnaea* for a brief period to heat (1 h at 30°C) before operant conditioning training not only enhances the formation of LTM (Teskey et al., 2012), but also increases the synthesis of two heat shock proteins (HSPs): HSP40 and HSP70 (Foster et al., 2015). Studies from rodents indicated that the flavonoid quercetin blocks the effects on memory formation of those stressors that act via HSPs (Mohammadi et al., 2014) by altering the expression levels of CREB (Costa-Mattioli et al., 2009). Future studies using *Lymnaea* could help to better comprehend the link between the enhancement of LTM formation and the start of HSPs production (Sunada et al., 2016).

6.2 The cooling-induced modification on ITM and LTM

Even if cooling can be considered a more naturally event, in particular circumstance, it can be used as stressful stimulus. Numerous studies established that brief periods of hypothermia after conditioning training interfere with memory consolidation in both *Lymnaea* and in other species because of a reduction in protein synthesis (Sekiguchi et al., 1994; Xia et al., 1998; Sangha et al., 2003d). In fact, it seems that cooling interferes with the metabolic processes necessary for memory formation (Fulton et al., 2008; Takahashi et al., 2014). In this regard, Sangha and colleagues (2003) demonstrated that cooling the snails for 1 hour immediately after training blocked ITM or LTM, whereas delaying the same cold-block by 10 or 15 minutes allowed for the formation of ITM and LTM, respectively (Sangha et al., 2003d). These data imply that cooling-sensitive processes required during the establishment of ITM and LTM operate through a brief time-window immediately following learning. Furthermore, it seems that the processes leading to stabilization of the learned behaviour, once started, are not broken down by cooling (Sangha et al., 2003d). Moreover, Takahashi and colleagues (2013) showed that prolonging the cold-block up to 180 minutes following training inhibited memory formation as well, suggesting that there are two critical periods for LTM formation. In fact, the application of the cold-block immediately after training

interferes with the macromolecular protein synthesis required for memory consolidation, whereas the cooling effect observed 180 minutes after training alters a second round of protein synthesis occurring following memory formation (Takahashi et al., 2013). Finally, exposure of snails to 4°C for 8 days once LTM is consolidated, resulted in disruption of the events downstream memory formation that are responsible for forgetting. In this way, LTM that normally persisted for 2 days was extended for at least 8 days (Sangha et al., 2003d). These data are consistent with the hypothesis that forgetting is an active process due to the learning and remembering of interfering events. If confirmed, it should be possible to disrupt the process of forgetting (i.e., block the new consolidation process for 'interfering events') by using cooling. In this context, *L. stagnalis* represents a good model to study the time-window required for ITM and LTM using reversibly cold-block induced amnesia which, in turn, is a non-toxic and easily reversible procedure, that can be applied and removed for discrete amounts of time (Takahashi et al., 2013). Moreover, cooling can be utilized to study how loss of memory may be prevented.

6.3 DNA methylation in memory persistence in relation to longer-term stressors or environmental changes

Considering that the interval between stress experienced by snails and the enhancing effect of this stressor on the formation of LTM is in the order of days, epigenetic mechanisms have been hypothesized to play a key role. In this regard, epigenetic changes, such as DNA methylation, have emerged as common mechanisms involved in memory formation across species (Zovkic et al., 2013). In particular, DNA methylation was involved in olfactory LTM in honeybees (Biergans et al., 2012) as well as in long-term potentiation at sensory-motor synapses in *Aplysia* (Rajasethupathy et al., 2012). Studies from rodents suggest that DNA methylation represents a dynamic state, that can be strongly influenced by various environmental manipulations, including exposure to stress (Chertkow-Deutsher et al., 2010). Stressful stimuli, in fact, alter DNA methylation state which, in turn, is the result of upstream events, including increased glutamate and neuropeptide transmission and enhanced activation of transcription factors (Stankiewicz et al., 2013). In this context, the enhancement of LTM or the length of its persistence in stressed snails required DNA methylation (Lukowiak et al., 2014). In fact, treatment with a DNA methylation blocker (5-Aza-2'-deoxycytidine (5-AZA)) 1 hour before exposing snails to a memory-promoting stressor, altered the persistence of LTM (Lukowiak et al., 2014; Sunada et al., 2016). Interestingly, in the absence of stress-enhanced memory, 5-AZA was not active (Lukowiak et al., 2014). Because drugs of abuse, such as cocaine and methamphetamines, activate stress pathways (Moldow and Fischman, 1987), it has been hypothesized that intense memories characteristic of post-traumatic stress disorder (PTSD) and drug addiction, may be resistant to forgetting because memories appear to be stabilized by epigenetic changes (Carter et al., 2006; Kennedy et al., 2010; Debiec, 2012; Nestler, 2014; Schmidt et al., 2013). Therefore, *Lymnaea* represents a good model to study the involvement of DNA methylation in memory persistence related to longer-term stressors or environmental changes, while at the same time contributes to elucidate the role of epigenetic changes in memory impairments (Carter et al., 2006).

6.4 Neuro-modulatory role of the endocannabinoid system in how stress modifies LTM formation

The mechanisms by which various stressors affect memory formation are not entirely clear. In this contest, the endocannabinoid system and, in particular cannabinoid receptors (CBs), seems to play a key neuro-modulatory role in how stress modifies LTM formation (Campolongo et al., 2009; Atsak et al., 2012; Tan et al., 2014). In mammals, in fact, the activation of the endocannabinoid system not only enhanced the effects of a stressor on adaptive behaviours (Morena and Campolongo, 2014; Goodman and Packard, 2015), but it also suppressed the formation of working memory and LTM. On the contrary, both forms of memory were enhanced when the endocannabinoid system was inhibited. Because the endocannabinoid system is phylogenetically ancient (McPartland, 2004) and well conserved among species, Sunada and colleagues (2017) hypothesized that in *Lymnaea* putative cannabinoid receptors (LymCBRs) are involved in learning and memory formation in stressful conditions (Sunada et al., 2017). Indeed, *L. stagnalis*, expressed two G-protein-coupled neuronal receptor genes, which encoded proteins closely related to well characterized vertebrate CBRs (Sunada et al., 2017). Injecting a mammalian CBR agonist (i.e. WIN 55) in snails, mimicked the traumatic event of exposure to severe stressor and rendered snails unable to learn and form memories for up to one week after the traumatic event. On the contrary, injection of a mammalian CBR antagonist (i.e. AM 251) before snails received the traumatic stimulus reduced the effect on learning and memory. Injection of the same antagonist into untrained and not traumatized snails enhanced their ability to form LTM (Sunada et al., 2017). Evidence from the literature also supports these data. Previous research in rodents, in fact, indicated that blocking of CBRs enhanced spatial and associative memory (Terranova et al., 1996; Robinson et al., 2008). While the effects of WIN 55 was prolonged and maintained for at least 4 days, the effect of other stressors on LTM formation persisted for only a few hours (Orr and Lukowiak, 2008). Consequently, it was hypothesized that WIN 55 causes a state of extreme fear that is incompatible with learning and memory, as demonstrated in mammals, where the endocannabinoid system plays a key role in the neuronal regulation of anxiety and responses to fear (Ruehle et al., 2012). Future research could benefit from our simple model system and use it to better understand how the endocannabinoid system is involved in the acquisition of learning and memory. At the same time, gaining better comprehension as to how behaviorally relevant stressors may alter LTM formation and/or its persistence focusing on simple systems may lead us to understand how to treat 'memory disorders' such as phobias, PTSD and substance abuse more effectively (Dębiec, 2012; Agren, 2014).

7. Necessity knows no law: when the conditioned stimulus sucrose becomes a source of energy

It is well-established that different stress states resulting from different durations of food deprivation alter the ability of snails to express LTM. According to the Yerkes–Dodson/Hebb law, while some degree of a food-deprivation-induced stressed state must exist for the CTA to successfully occur, the length of food deprivation alters learning and LTM formation (Ito et al., 2015). In particular, food deprivation for 1 day resulted in optimal learning and memory, whereas food deprivation for 5 days before training resulted in little or no learning and memory (Sugai et al., 2007; Mita et al., 2014). Because memory formation is energetically expensive (Barnard et al., 2006; Burns et al., 2011), if energy intake is too much restricted, LTM formation should be impaired in order to conserve energy. Thus, snails are hypothesized to learn and form LTM, but in an overly stressed state associated with prolonged food deprivation, their ability to express the LTM phenotype should be suppressed, suggesting that hunger triumphs over the memory not to respond to the CS. That is, starvation generates a conflict between memory formation versus the desire or necessity to eat (Ito et al., 2015). Moreover, the context-specificity of memory expression (Haney and Lukowiak, 2001) played an important role in the lack of LTM observed in 5-day severely food-deprived snails. In fact, LTM is only observed in 5-day deprived snails when tested for LTM following *ad libitum* access to food for additional 7 days while in a 1-day food-deprived state (day 13 snails). In contrast, snails did not express the memory phenotype if they had recovered from 5-days of food deprivation. These data support the fact that 1 day of starvation after refeeding reinstalls the optimal context in which snails memorize (Ito et al., 2015). Based on the observation that during CTA training there was an up-regulation of LymMIP-II (Azami et al., 2006), it has been hypothesized that sucrose on the lips, which represents the CS, induced an insulin spike, that, in turn, modulated the neural circuit underlying CTA-LTM (Murakami et al., 2013b). In addition, considering that an injection of insulin to 1-day food-deprived snails improved learning and CTA memory (Mita et al., 2014), it is likely that the occurrence of an insulin spike correlates with the acquisition and retention of associative learning. Finally, previous studies indicated that 5 days food-deprived snails trained in the presence of a food smell, no longer learned nor formed memory (Lukowiak et al., 2014), which stresses the importance of the ‘state’ of the organism and how and when it perceives a stimulus as a stressor. Thus, the expression of memory is both context and state-dependent and may only be expressed following the resolution of the conflict between the homeostatic drive to eat versus having a memory of learning under what conditions not to eat (Mita et al., 2014).

8. *L. stagnalis* as a model for age-associated memory decline

Evidence is accumulating affirming that aging affects memory. However, the role of molecular dysregulation in age-associated memory deficits is not well understood. Learning and memory impairments are a common and evolutionarily conserved feature of the normal aging brain (Burke and Barnes, 2006; Luck et al., 2010).

8.1 Age-associated memory decline and oxidative stress

The (neuro)biological foundations of the natural decline in plasticity are not completely understood but appear to involve a progressive alteration of neuronal excitability resulting from an impaired activity in glutamate receptor subunits, such as glutamate ionotropic receptor AMPA type subunit 2 and NMDA type subunit 2B (Disterhoft and Oh, 2006; Hermann et al., 2007; Kashiya et al 2009), together with a shift in the mechanisms that regulate synaptic plasticity, including Ca^{2+} channel function and Ca^{2+} -dependent processes (Fukaya et al., 2007). Moreover, there is extensive evidence for a role of oxidative stress, and particularly lipidic peroxidation, as a key factor in aging and age-associated neural impairment (Harman, 1956; Dröge and Schipper, 2007; Sultana and Butterfield, 2010). Lipid peroxidation results in loss of membrane polyunsaturated fatty acids and oxidized phospholipids as polar species contributing to increased membrane rigidity (Farooqui and Horrocks, 1998). Alterations in the neural membrane phospholipid components, in turn, not only influence crucial intra- and inter-cellular signalling, but also alter many physical properties of the membrane, such as fluidity, phase permeability, bilayer thickness and lateral domains (Horrocks and Farooqui, 2004). Polyunsaturated fatty acids (PUFAs) are released from membrane phospholipids by a number of enzymic mechanisms involving the receptor-mediated stimulation of phospholipase A2 (PLA₂) and phospholipase C/diacylglycerol lipase pathways (Farooqui and Horrocks, 1998; Niki, 1990). Thus, PLA₂ represents a key factor in age-associated decline in neuronal excitability and the related impairment in activity-dependent forms of learning and memory formation (Niki, 1990). Similar results, obtained in different model systems, suggest that a decline of electrical activity/excitability and synaptic functions, associated with lipid peroxidation, are conserved characteristic of neuronal aging (Arundell et al., 2006; Disterhoft and Oh, 2007; Hermann et al., 2007; Spiteller, 2010; Watson et al., 2012). In this regards, Watson and colleagues (2012) demonstrated that PLA₂-dependent free fatty acids (FFAs) release was significantly enhanced in CNS of old snails, and that experimental oxidative stress raised PLA₂-dependent FFAs release in CNS of younger snails to the level observed in older brains. Moreover, both experimentally induced and naturally senescent phenotypes were fully reversed by blocking the activity of PLA₂. This suggests a central role of lipid metabolism, particularly oxidative stress-induced activation of PLA₂, in the process of neuronal aging and age-associated learning and memory impairment in *L. stagnalis* (Watson et al., 2012). Similarly, evidence regarding PLA₂-mediated excision of fatty acids and fatty acid metabolism in the genesis of age-related cognitive impairment has been observed in mammals including humans as well (Adibhatla and Hatcher, 2008; Darios et al., 2007; Sanchez-Mejia and Mucke, 2010). In this regard, numerous studies from mammals showed that PUFAs deficiency markedly affects neurotransmission, ion channel activities and synaptic plasticity (Yehuda et al., 2002).

On the other hand, prolonged PUFAs supplementation in the diet restored membrane fluidity and calcium homeostasis in the brain, improved electrophysiological parameters (i.e. hippocampal long-term potentiation) and learning ability in aged rats and, not least, enhanced cognitive function in humans with memory deficits (Kotani et al., 2003; Kotani et al., 2006; Fukaya et al., 2007; Kashiya et al., 2009). Moreover, studies from senescent rats demonstrated that the dysregulation of calcium-dependent neuronal processes could be restored to the state observed in young rats with prolonged PUFA-supplementation (Fukaya et al., 2007). These parallelisms between species that are phylogenetically distant lead to converging evidence which postulates that lipid peroxidation-dependent PLA2 activity is a fundamental, evolutionary conserved aspect of neuronal aging and a cause of age-associated changes in neuronal signalling and cognitive decline in the normal aging process of brain.

8.2 The role of PKA/CREB1 and PACAP38 in age-associated memory decline

As illustrated before, the conserved activation of adenylate cyclase by LymPACAP and the consequent activation of the LymPKA-LymCREB1 molecular cascade are fundamental during the formation of LTM (Sadamoto et al., 2004).

Previous studies indicated that the exogenous application of PACAP before training rescued memory impairments in old snails, suggesting that the inactivation of this target represents an useful tool for the study of age-associated memory impairment, that, in turn, seems to be only suspended but not irreversibly extinguished (Watson et al., 2010). Along a similar vein, treatment with insulin-like growth factor-1 (IGF-1), which in vertebrates activates PACAP type I receptors (Delcourt et al., 2007), increased memory formation in aged snails (Pirger et al., 2014), suggesting that IGF-1 may exert a comparable memory-boosting effect on aged snails as PACAP. Based on these observations, the PKA-CREB1 pathway, together with PA2 and IGF-1, have been proposed as targets for therapeutic interventions for age-related memory loss (Pirger et al., 2014). In this contest, due to the evolutionarily conserved nature of these polypeptides and their established role in memory and synaptic plasticity in snails (Kemenes et al., 2013), *Lymnaea* should be considered as an excellent model in which to conduct drug discovery studies.

9. Limitations of molecular analysis of the nervous system of *L. stagnalis*

The purpose of this review was to reassume the data and illustrate that *L. stagnalis* relies on a set of core molecules required for learning and memory in a similar way as observed in a number of other species, ranging from invertebrates to mammals. Thanks to the simplicity and accessibility of neuronal circuits, *Lymnaea* has provided important insights into the fundamental cellular and synaptic elements required for establishing conserved cognitive functions, such as memory and learning. However, a serious drawback in the molecular analysis of the nervous system of *L. stagnalis* is the lack of large-scale genomic or neuronal transcriptome information. In fact, although its genome has been sequenced, gene characterization has not yet been performed. This turns out to be an important obstacle for the use of this model in comparative molecular studies (Feng et al., 2009). The molecular information available has been obtained by cloning of partial cDNA sequences, together with *in situ* hybridization and immunohistochemistry. Thus, an important scientific puzzle to solve in the near future is the characterization of the gene networks that play central roles in the functioning of the CNS in *L. stagnalis*. A pre-requisite for such exploration is the knowledge of gene sequences, which can be used to monitor when, where, and how particular genes are expressed. Investment in such a research effort, will not only add a new experimental model to the limited number of invertebrate models already used in translational neuroscience research.

10. New directions of neuroscience translational research using *L. stagnalis*

Understanding how biological aging processes affect the brain and how they contribute to the onset and progress of age-associated neurodegenerative diseases is a central research goal in neuroscience. In this context, *Lymnaea* provides a powerful model system to learn more about the cellular and molecular details of memory processes. Unlike *D. melanogaster* and *C. elegans*, the most common and best characterized invertebrate models (Yamaguchi and Yoshida, 2018; Möller et al., 2018), *Lymnaea* has a relative long life span (lasting approximately 9–12 months) which offers a powerful new tool to study age-related modifications involving genetic, molecular, and cellular mechanisms, which usually take time to manifest their full effects (Nestler et al., 2010; Tascetta et al., 2015). This last factor is of particular interest in studies on neuro-aging, chronic human pathologies, especially neurodegenerative diseases such as Alzheimer's, Parkinson's, and chronic psychiatric diseases such as major depression, schizophrenia or bipolar disorder.

As previously illustrated, *L. stagnalis* embodies a useful tool for translation-oriented research aimed at developing new therapeutic approaches to age-associated memory dysfunction. In particular, PA2, PACAP38, and IGF-1 and their related biochemical cascades, represent fascinating “memory rejuvenating” agents and their (genetic) characterization could help to elucidate some of the mechanisms underlying cognitive decline in the aging human brain.

At the same time, *Lymnaea* represents an excellent model for the comprehension of the causal mechanisms of memory extinction. Identifying the molecular substrates of extinction could promote the development of pharmacological treatments for psychiatric disorders, such as fear disorders, and substance addiction in humans.

However, because similar molecular processes contribute to the development and persistence of both memory consolidation and extinction, pharmacological interventions designed to facilitate extinction should be explored carefully.

In addition, *Lymnaea* as model system provides an outstanding platform to investigate the crosstalk between neuronal metabolism (energy) and the formation of memory and how such mechanisms are altered during aging and neurodegenerative disorders. As explained, insulin and IGF-1 modulate aspects of plasticity in the CNS of *Lymnaea* and enhance learning abilities in older learning-impaired snails (Murakami et al., 2013; Pirger et al., 2014).

These findings echo very well with the growing evidence suggesting a the role of ILPs and insulin resistance in aging (Alcedo et al., 2013).

The *Lymnaea* research platform we portrayed in this review will also be a great tool to investigate fundamental mechanisms of stress-mediated memory impairments.

In this regard, stressors or bioactive agents that cause enhancement of LTM formation in *Lymnaea* have been hypothesized to act via putative CBrs. If confirmed, these studies will help to elucidate the role of the endocannabinoid system in learning and in memory in mammals including humans.

Moreover, because epigenetic changes, such as DNA methylation, are emerging as a common mechanism in synaptic plasticity and memory formation across species (Zovkic et al., 2013) and are involved in the maintenance of memories in PTSD and drug addiction, research using *Lymnaea* will add to this growing area of research and will further highlight the common mechanisms of memory formation in vertebrates and invertebrates species.

Beside investigating molecular and cellular aspects of numerous human diseases, *Lymnaea* could also open interesting perspectives concerning both the validation of the mechanism of action of existing drugs and the preclinical studies of drugs discovery (Tascedda et al., 2015). Given the high costs and the long time needed to identify and develop new drugs, a faster and less expensive system of drug discovery is both necessary and urgent. Therefore, *Lymnaea*, represents a versatile platform for the screening of new compound, the identification of innovative drug targets and for the deciphering of mechanisms underlying drug action. Snails, in fact, are aquatic invertebrates with an open circulatory system, allowing the use of membrane-permeant drugs that can be easily absorbed, to unravel the complexity of various signalling pathways and to provide new insights on how drugs and molecules can modulate different neuronal functions and behaviours (Benatti et al., 2017). At present, very few pharmacological studies using *Lymnaea* as model system are available up today. Benatti and colleagues (2017), for example, evaluated the transcriptional effects of a serotonergic stimulation on selected targets involved in 5-HT signalling and neurotransmission in the CNS of *Lymnaea*. They treated chronically (48 hours) or acutely (6 hours) snails with the immediate precursor of serotonin (5-hydroxy tryptophan - 5-HTP), with fluoxetine (FLX) or with a combination of the two compounds. They demonstrated that transcription of Lym-CREB1 was strongly induced following chronic, but not acute, exposure to 5-HTP. This pivotal study suggested that *Lymnaea* could significantly contribute to finding novel functions of known drugs or molecular targets and for the identification of new drugs and their validation.

Not least, *Lymnaea* represents a valid model for neuro-engineering research. Counting on the fact that many neurons in the CNS of *Lymnaea* have large somata, the formation of high-quality neuron/semiconductor interfaces is facilitated (Birmingham et al., 2004). Thus, in the last years, considerable efforts have been made to develop techniques through which individual neurons can be noninvasively coupled to electronic microstructures of a semiconductor substrate, such as capacitors for stimulation and transistors for recording (Fromherz et al., 1991, 1995; Zeck and Fromherz, 2001; Bonifazi and Fromherz, 2002). Using this approach, Kaul and co-workers (2004) interfaced the pre- and postsynaptic neuron of an excitatory chemical synapse of *Lymnaea* by a silicon chip (Kaul et al., 2004). In this way, stimulating the presynaptic cell with a chip capacitor and recording the activity of the postsynaptic cell with a transistor, they enhanced the strength of the soma-soma synapse by repetitive capacitor stimulation (Kaul et al., 2004).

Moreover, Zeck and Fromherz (2001) demonstrated that isolated *Lymnaea* neurons can be anchored to a semiconductor chip and neurite growth cones can be guided along specified pathways (Zeck and Fromherz, 2001). Using this approach, it would be possible to reconstruct chemical synapses between specific neurons to form neuroelectronic circuits that exhibit various forms of synaptic plasticity and specific neuro-modulatory properties, adding different degree of flexibility to an already dynamic circuit (Birmingham et al., 2004).

Thus, studies of *Lymnaea* neural networks on silicon chips not only are promoting our understanding of the long-term dynamics and plasticity of relatively simple neural circuits, but are also providing the basis for reliable interfaces for new neuro-devices for humans (Birmingham et al., 2004).

On the basis of these considerations, although animal models can never summarize the full phenotype of a human clinical disorder, in particular neurological ones, *L. stagnalis* offers a new important and innovative tool for neuroscientists (Tascedda et al., 2015), representing a remarkable model system in which to study the genetic and molecular basis of human CNS physiology and pathology (Benatti et al., 2017). We think that this model allows for a better vantage point from which innovative therapies and technology can be developed in order to treat a range of neurological and neurodegenerative disorders, and that the ultimate success of neuroscientists engaged in translational research will depend on collecting and improving the quality and quantity of knowledge to “translate” (Alberts, 2010) obtained in invertebrates and in more complex organisms, until arriving at human beings. Because research on invertebrate models represents the shortest and most efficient tool to study and treat human diseases, *L. stagnalis* offers a model to bridge the gap between traditional *in vitro* and preclinical animal assays, and to move from pond to the bench.

11. Conclusions

In conclusion, what have we learn from *L. stagnalis* to date?

- 7) A variety of general and specific, molecular and cellular, mechanisms necessary for the consolidation of memory in snails have been highly conserved in learning, both across phylogenetic groups and learning paradigms, involving single- or multi-trials, aversion or reward, operant or classical conditioning;
- 8) similarly, the model contributes to define more clearly in what conditions we form memory in the most optimal way, “teaching” us the importance of context in which we eat, age, form memories and consolidate them;
- 9) *Lymnaea* allows us to move beyond simple homology, representing a valid and reliable model in which to study the genetic and molecular basis of human CNS physiology and pathology

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Figure Captions

Fig. 1 – Schematic representation of the molecular mechanisms implicated in memory formation in *Lymnaea*. LymCREB-driven transcription results downstream of:

- the activation of AMPc by LymPACAP, which, in turn, mediates almost all its actions through Lym-PKA and the subsequent activation of LymMAPK;
- the entrance of Ca²⁺ through NMDARs, that activates directly or indirectly numerous protein kinases, including PKC and LymCaMKII, together with LymNOS. LymNOS, for its part, promotes the synthesis of LymNO, which regulates LymCREB activation, acting via LymPKG.

After phosphorylation LymCREB1 initiated a cascade of altered gene activity and new protein synthesis, necessary for synaptic enhancement in memory consolidation, acting via LymC/EBP and IGF1. In contrast, LymCREB2 inhibited the function of LymCREB1 and the ratio of activator/repressor LymCREBs has been proposed to act as a “molecular switch” in determining whether LTM is formed. Even if LymPKA, LymNMDA receptors, LymCaMKII, LymCREB, and LymNOS/NO are selectively activated or upregulated, it seems likely that these and other signalling molecules are part of a synergistic effort and together contribute to the memory consolidation process, with none of them alone being sufficient for LTM

Adapted from Kemenes, 2013

Fig. 2 – Different time windows of the key molecular targets necessary for memory in snails, in the 0h to 6h (acquisition/early consolidation) and 24hr time window (late consolidation).

During the acquisition phase of memory LymPACAP, LymPKA, LymNO, LymGRIN, LymCaMKII and LymMAPK are activated. During the first hours of memory formation and consolidation (defined as early and intermediate term consolidation phase) the activation of LymPKA, LymNO, LymCamKII, LymMAPK, LymPKA and LymCREB occurs, together with the transcription and the synthesis of new mRNA and proteins respectively. LymPKA is also activated during the intermediate consolidation phase (5-6 hr), when the synthesis of new proteins occurs. Finally, in the last phase of memory consolidation, LymCaMKII is expressed.

Adapted from Kemenes, 2013

TABLE 1 Abbreviations of used Acronyms

AC	adenylate cyclase
AMPC	the homologous of Cyclic Adenosine Monophosphate
LymPACAP	the homologous of pituitary adenylate cyclase-activating polypeptide
LymPKA	the homologous of protein kinase A
LymCaKMII	the homologous of calcium calmodulin-dependent protein kinase II
LymMAPK	the homologous of mitogen-associated protein kinase
LymGRIN	the homologous of ionotropic glutamatergic NMDA receptors
LymNOS	the homologous of nitric oxide synthase
LymNO	the homologous of nitric oxide
LymPKG	the homologous of protein kinase G
LymCREB	the homologous of cAMP response element-binding protein
LymC/EBP	the homologous of CCAAT/enhancer binding protein
Lym-IGF2	the homologous of insulin growth factor 2
MIP II	Molluscan insulin-related peptide
5-HT	5-hydroxytryptamine - serotonin
DA	Dopamine
HSPs	Heat shock protein
LymCBrs	The homologous of cannabinoid receptors
PLA ₂	Phospholipase A ₂
FFAs	Free fatty acids
PUFAs	Poly-unsaturated fatty acids
CNS	Central nervous system
STM	Short-term memory
ITM	Intermediate-term memory
LTM	Long-term memory
CS	Conditioned stimulus
US	Unconditioned stimulus
CTA	Conditioned taste aversion
RPed1	Right pedal dorsal 1 interneuron
CGCs	Cerebral giant cells
PTSD	Post-traumatic stress disorder

FIGURE 1.TIF

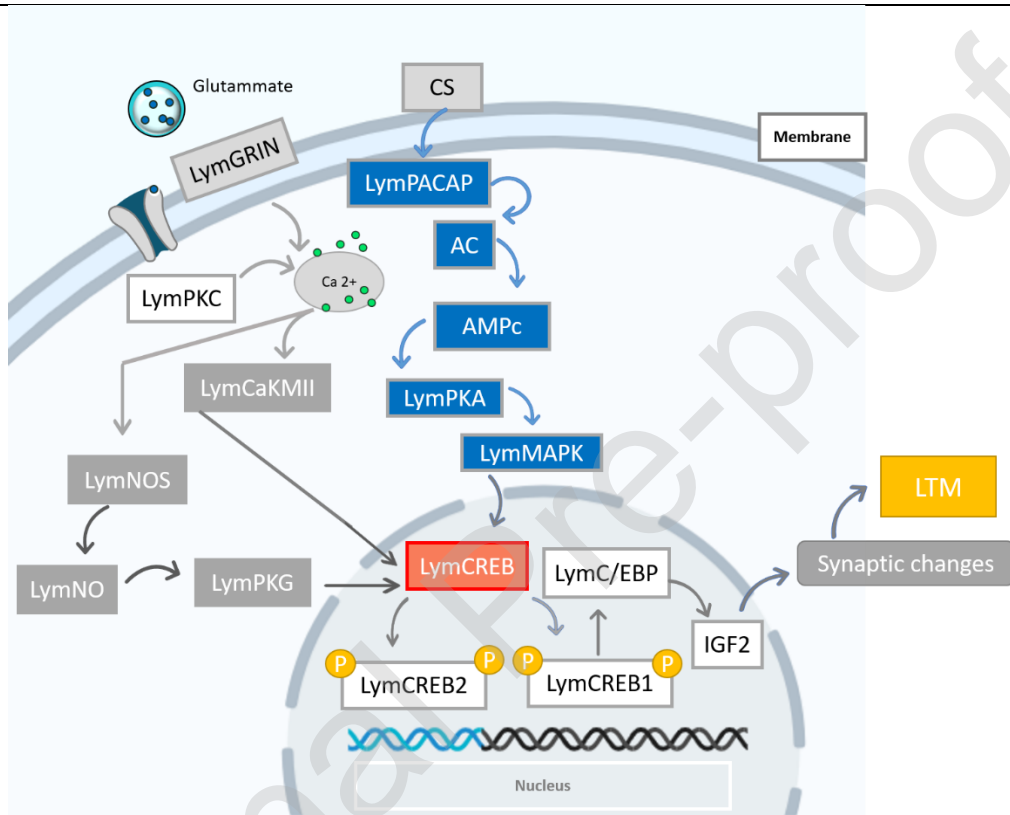


FIGURE 2.TIF

