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Thesis

For the Master 1 degree of "Biologie integrative: Physiologie et Neurosciences"

An epigenetic approach of long-term-memory in the pond snail *Lymnaea stagnalis*

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To my family, my parents Alain and Christine and my little sis' Melanie, thank you for your support and your love. Thank you for always encouraging me, believing in me and always being there for me.

And lastly, thank you Clara my love, for our everyday talks that kept my moral to a 100%, for your support and patience. I love you!

To all, thank you for this amazing opportunity!

BRIEF PRESENTATION OF THE LAB:

I did my internship in the Lukowiak's lab at the University of Calgary. My supervisor was also the director of the lab, Pr Ken Lukowiak. The lab is specialized in behavioral and electrophysiological studies of an invertebrate model: the pond snail *Lymnaea stagnalis*. The lab is exploring how different stressors can induce either enhancement or suppression of memory (short-term, intermediate or long term memory, respectively STM, ITM and LTM). Thus they discovered that different stressors relevant to memory can induce different responses: crowding and low calcium block LTM whereas exposure to CE, epicatechin or hyperthermia leads to enhance LTM. They also began associating these different stressors and found surprising result doing so. Most of these behavioral studies are supported with electrophysiological data currently done by a post doc, Hiroshi Sunada.

ABSTRACT: Learning and memory are essential characteristics for an accurate adaptive behavior in response to stress, in any organism. According to the Yerkes-Dodson law (1908), a stress – performance relationship exists in the form of a bell curve. Thus too low level or too high level of stress would impair memory formation, whereas the perfect level would induce maximal performances. This experiment consists in exposing the pond snails *Lymnaea stagnalis* to the kairomones of a sympatric predator, a crayfish. We show that exposure to this stressor allows juveniles *Lymnaea* to form long-time memory in a paradigm that usually doesn't produce LTM (0,5h single training session in CE). Furthermore, we also demonstrate that this training paradigm in juvenile snails induces long-lasting alterations in their ability to form memory once they mature as adults. Indeed these snails now have the ability to form LTM in a training paradigm (0,5h single training session in PW) that usually produces only intermediate-term memory (ITM). We hypothesize that these changes are a result of epigenetic modifications occurring during the training paradigm in juveniles. Number of digits: 32 397

KEYWORDS: Long-time memory (LTM), epigenetics, stress, *Lymnaea stagnalis*

RESUME: Apprentissage et mémoire sont des caractéristiques essentielles, possédées par tout organisme, dans le but de savoir répondre de manière appropriée à un stress. En accord avec la loi de Yerkes-Dodson (1908), une relation entre stress et performances existe sous la forme d'une courbe en cloche. Ainsi, un niveau de stress trop faible ou trop élevé va empêcher la formation de la mémoire tandis qu'un niveau parfait va induire une performance maximal. Notre expérience consiste à exposer des escargots d'eau douce, *Lymnaea stagnalis*, aux kairomones d'un prédateur sympatrique, une écrevisse. Nous démontrons que l'exposition à ce stress permet à des jeunes *Lymaea* de former une mémoire à long terme lors d'un protocole qui, normalement, n'en produit pas (une seule session de 0,5h d'entrainement dans du « CE »). De plus, nous démontrons aussi que ce protocole d'entrainement chez des jeunes induit de profonds et durables changements dans leur capacité à former des souvenirs une fois adulte. En effet ces escargots ont dorénavant la capacité de former une MLT lors d'un protocole d'entrainement (une seule session de 0,5h d'entrainement dans de l'eau d'étang artificiel) qui normalement ne produit qu'une mémoire de type intermédiaire. Nous émettons l'hypothèse que ces changements sont le résultat de modifications épigénétiques qui se déroulent lors du protocole d'entrainement des jeunes escargots. Nombre de caractères: 32 397

MOTS CLES: Mémoire à long-terme (MLT), épigénétique, stress, *Lymnaea stagnalis*

INTRODUCTION:

Induction and formation of long term memory involve a great number of steps from signal transduction to gene activation, transcription and translation (for review see: Flavell and Greenberg 2009; Leslie and Nedivi 2011; Loebrich and Nedivi 2009). However far less is known about how these memories fade with time. Indeed if looking at the molecular level as an example, the half-life of a protein ranges from minutes to hours. Moreover we have a constant molecular turnover inside each cell. How to reconcile these facts with life-long lasting memories in humans?

It has been demonstrated in the 1960s and since then, that memory is not a biological property reserved to humans (Kandel, 2001). On the contrary, memory in all its aspects (cellular, behavioral…) is a very old process and thus can be studied in "simpler" animal models like the pond snail *Lymnaea stagnalis*.

One hypothesis relevant to long-lasting memory that is attracting more and more attention is the idea that epigenetic changes play a role in the formation and maintenance of long-term-memory (LTM). Epigenetics is defined by Levenson and Sweatt (2005) as a set of self-perpetuation, post translational modifications of DNA and nuclear proteins that produce lasting alterations in chromatin structure as a direct consequence and lasting alteration in patterns of gene expression as an indirect consequence.

Historically, epigenetics are non-reversible processes occurring during development and inducing definitive cell differentiation (Müller & Leutz, 2001; Orlando & Castellino, 2003). This can be accomplished via two distinct and parallel processes. The first is by post translational modifications of histones: acetylation (governed by Histone ActetylTransferases HATs and Histone DeACetylases HDACs), methylation (Histone MethyTransferase HMT), phosphorylation (multiple kinases and phosphatases) or ubiquitylation . The second process involves DNA methylation (via the DNA MethyTransferase DNMT).

Contrary to embryogenesis epigenetics, it is currently believed that a dynamic (i.e. reversible) form of epigenetic change occurs in the adult nervous system. This dynamic form would be a co-opted form of epigenetics, adapted and used with the purpose of storing memories (for detailed reviews on these epigenetics mechanisms see: Barrett and Wood 2008; Jiang et al. 2008; Lubin et al. 2011).

Some studies have looked at the dynamic of chromatin changes in ischemia and status epilepticus and their importance in regulating neurophysiological changes (Huang *et al.*, 2002; Calderone *et al.*, 2003). However neither association nor correlation was made with LTM at that time.

One of the earliest work suggesting an involvement of epigenetic change with LTM came from the Aplysia model system (Guan *et al.*, 2002). They used an *in-vitro* culture of *Aplysia* neurons bathed in a solution in which they applied either serotonin (5-HT) or FMRFamide. They found in the first case that a 5-HT injection induced CREB1 activation which in turn led to expression of C/EBP.
C/EBP or CCAAT enhancer binding protein is one of many family of proteins (CREB, AP-1, Egr and NF-κB) playing a critical role in synaptic plasticity and memory formation (Alberini *et al.*, 1994; Alberini, 2009). C/EBP is a downstream component of the cascade activated after stimulation of NMDA receptor. This cascade is composed of an increased intracellular calcium concentration followed by activation of CAMKK, then CAMK and finally CREB. Then activation of CREB1 will lead to activation of C/EBP. In turn, C/EBP will bind the DNA on a C/EBP binding site and activate the transcription of the late response genes, essential for synaptic plasticity.

On the other hand, with FMRFamide, instead of CREB1, the recruitment of CREB2 occurred which repressed the expression of C/EBP.

C/EBP is thus the common target gene of these 2 proteins. Recruitment of C/EBP also induces recruitment of CREB binding protein (CBP) which in turn acetylates histones around the C/EBP promoter.
Indeed, CPB is in itself an HAT which has the ability to acetylate specific lysine residues of the histone's core and induces alteration in the chromatin structure.
The opposite happens in the presence of FMRFamide, meaning that instead of recruiting a HAT (i.e. CPB), a HDAC is recruited and deacetylate histones around C/EBP promoter.

Furthermore, another experiment (Lee *et al.*, 2001) showed that an overexpression or a prevention of the induction of C/EBP respectively enhanced or inhibited long term facilitation. Thus one of the first links between long time memory and epigenetic mechanisms was established.

This hypothesis was then further investigated and confirmed with the work on mammal fear conditioning. More precisely, the work of (Levenson *et al.*, 2004) looked at the regulation of histone acetylation during memory formation in the hippocampus.
This study was composed of both an *in-vivo* and an *in-vitro* part. The authors stimulated *in-vitro* the NMDAR as well as activated ERK, thus initiating the downstream cascade, and looked at the changes in histone H3 acetylation in the CA1 region of the hippocampus.
ERK is part of the MAP Kinases downstream pathways activated following NMDAR stimulation. It is a pathway parallel to the CaM Kinases pathway seen above but the end-target of both pathways is CREB. Thus it also has a critical role in synaptic plasticity and memory formation.
They found that following stimulation of NMDAR or activation of ERK, an increased acetylation of H3 but not H4 was observed. Furthermore an enhanced induction of LTP was induced in CA1. These results mimicked perfectly what they observed *in-vivo* using simple fear conditioning paradigm in rats. Additionally the rats were injected with drugs modulating the acetylation process and showed differences in memory formation. The uses of deacetylase inhibitors lead to an enhanced memory formation. In the end they showed a direct correlation between acetylation level of H3 and long term memory modulation via chromatin regulation.

At about the same time another study by Weaver et al. illustrated the importance of maternal behavior and its relation with epigenetics. They used female rats' model, separating them in two categories: the mother giving a high maternal care versus the mother giving a low maternal care to their pups. This was measured using the two behaviors: licking and grooming (LG) and arched-back nursing (ABN). They showed that the pups receiving a high maternal care had a decreased CpG methylation on the glucocorticoid receptor (GR) gene promoter (more precisely exon 1_7) in the hippocampus. This difference in methylation occurring during the first week of life, were then associated with a difference in histone acetylation and transcription factor (NGFI-A) binding to the GR receptor. Moreover this difference persists in adults and the rats present an increase resilience to stress.

Then in 2007, another study linked epigenetics with brain-derived neurotrophic factor (BDNF) gene regulation. In rats, as a model for extinction of conditioned fear, Bredy et al. showed that extinction is linked with increased acetylation around the BDNF gene. Indeed they revealed an increased acetylation of H4 on specific exons of the BDNF gene, not only in the hippocampus but

also in the prefrontal cortex. Furthermore the use of an HDAC inhibitor enhanced long term memory for extinction.
A related study using the same paradigm and published the following year (Lubin *et al.*, 2008) showed that DNA methylation on the BDNF gene, in the hippocampus, could modulate its expression as well. Their finding demonstrated that increased demethylation is sufficient to drive an increased expression of the BDNF gene in the hippocampus, *in-vivo.* An increased in BDNF has been proved, in previous studies (Liu *et al.*, 2004), to be critical for memory of contextual fear conditioning paradigm.

Additionally, epigenetic mechanisms have recently been linked to some well-known diseases (Alzheimer disease, autism, depression …)(Lubin *et al.*, 2011). Thus, the study of the mechanisms underlying epigenetics and their consequences appears of utmost importance to understand further these diseases and try to cure them. One potential approache resides in the use of HDACi, like trichrostatin A (TSA), which may show potential as a treatment. However, as of yet, only preliminary data are available on this subject and no real conclusion on the efficacy of that type of treatment can be drawn.

Following these experiments, we ask the question of whether there could be another effective simpler system to study a role for epigenetics in memory.

A specific strain of pond snails, *Lymnea stagnalis*, has been maintained in laboratories since the early 1950's (approximately 250 generations). These snails have not been exposed to any sympatric predator (predator living in the same geographical area) (Orr, Hittel, Lukowiak, *et al.*, 2009) during that time. However they still have the ability to detect kairomones from crayfish (a sympatric predator) and initiate various 'anti-predator' behaviors (Orr *et al.*, 2007, 2010a). This response consists of multiple behaviors: predator avoidance behavior by moving to the surface, decreased righting-response time and cutaneous oxygen consumption, increased shadow-sensitivity, increased exploration and increased breathing time through their pneumostome (primitive lung). Part of that response also consists in both, an increased ability to form long-time-memory (LTM) and an enhanced duration of that memory. It is thought that the presence of

kairomones primes the molecular mechanisms responsible for LTM formation (Orr & Lukowiak, 2008).
Moreover exposure to crayfish kairomones is, at the present date, the only stressful stimuli known to induce a context independent (or context generalized) memory in *Lymnaea* (McComb *et al.*, 2002).

Furthermore, it has been demonstrated that different types of snails collected in multiple geographical regions only respond to sympatric predators (no response to allopatric predator) (Orr, Hittel, & Lukowiak, 2009; Orr, Hittel, Lukowiak, *et al.*, 2009). This suggested a form of genetic memory arising from a co-evolution of both species and that memory is conserved along generations.

Taking into consideration the previous studies both on epigenetics and in *Lymnaea,* we hypothesize that training in the presence of kairomones from a crayfish have the potential to induce epigenetic changes in the snails. Thus our purpose is to try to develop an invertebrate model of epigenetics induced by a predator odor.

MATERIEL AND METHODS:

Animals

Lymnaea stagnalis used in this study were breed and reared in the lab. They were originally derived from the stocks maintained at the *Vrije University* in Amsterdam. The ancestors of these snails were obtained from canals in a polder located near Utrecht in the early 1950s. They haven't been exposed to any naturally occurring predator since that time. Shell length was measured from the apex of the spiral to the distal prominence of the apertural opening. Shell length for juveniles was 15 ± 2 mm (mean 15,6 mm) and for adults 23±3 mm (mean 21,1 mm). Snails were maintained in aquaria (PW = deionized water + 80mg/L CaSO4 + 0,26g/L instant ocean), fed romaine lettuce *ad libidum* and at room temperature (≈20°C) until training.

Crayfish effluent

A crayfish of the genus *Cambrus* was housed in a 110L aquarium and fed a mix diet of lettuce

and snails *ad libidum*. In order to create CE water (crayfish effluent) (Orr *et al.*, 2010a) a 2L container was filled with PW (pond-water = standard housing solution) and the crayfish was transferred inside for a 1h period.

Respiration

Lymnaea stagnalis are bimodal breathers. They have the ability to exchange oxygen through their skin as well as emerging from the surface of the water and opening a breathing tube, the pneumostome, which leads to a primitive lung. For that reason it is possible to make the water hypoxic without harming them.

Operant conditioning

Juveniles *Lymnaea* were transferred from their rearing aquaria to a housing aquaria at least 3 days before being individually marked (number on their shell) which in turn was at least 48h before the experiment. Animals were placed into a 1L beaker filled with 500mL CE water (see above for CE water). Prior to the experiment, pure N2 was strongly bubbled into the water in order to make it hypoxic. N2 was then continuously bubbled at a slower rate throughout the experiment (no disruption of the water surface), to maintain hypoxia without disturbing the snails. Before each session, animals were given a 10 min acclimatization period in which they could freely open their pneumostome. After this period, the snails were gently pushed under water to convey the start of the training. Every time an animal began to open its pneumostome, a gentle tactile stimulus was applied on it with the help of a sharpened wooden stick. The stimulus was strong enough to induce the rapid closure of the pneumostome but at the same time gentle enough to do that without inducing a full body withdrawal response (=when the snail retract completely inside the shell) (Inoue *et al.*, 1996). A one training session paradigm of 30min was followed (Orr *et al.*, 2010a). Then, 24h later, the snails received the same training in pond water (which in that case is called memory test MT).

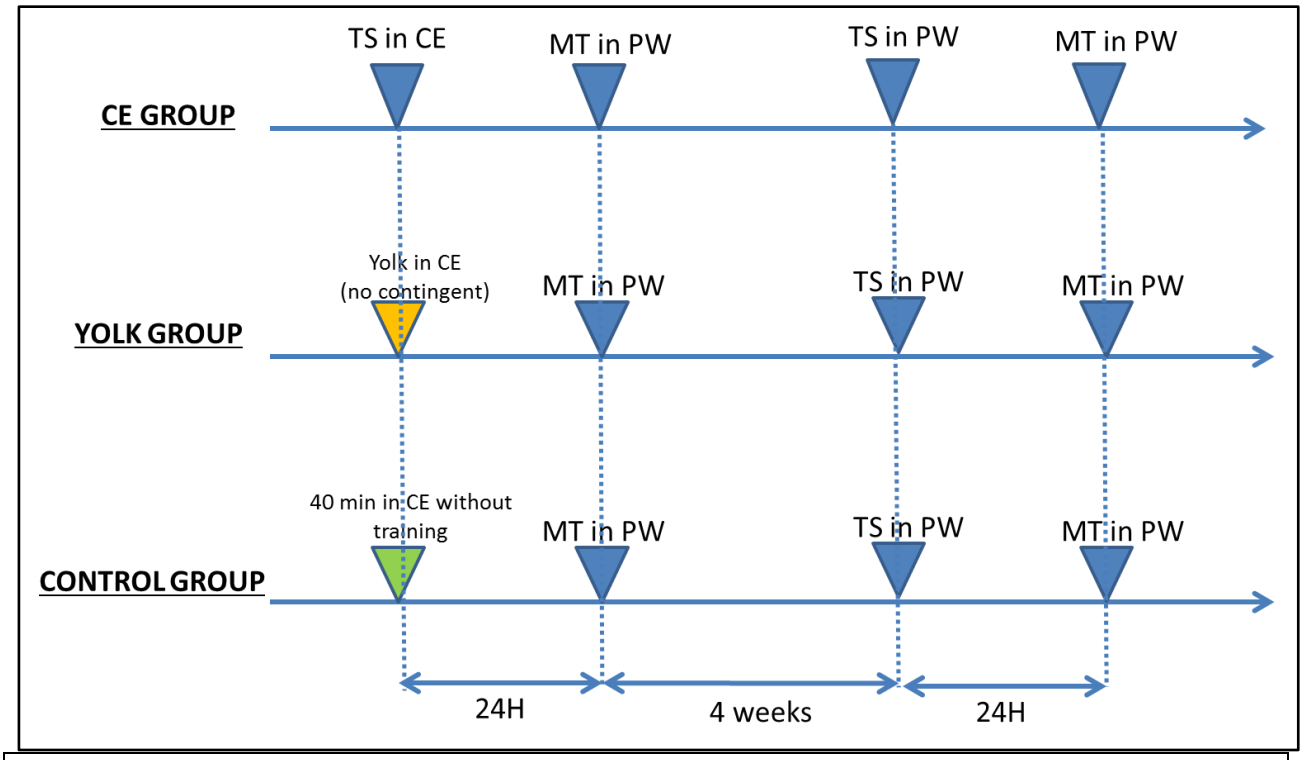


Figure 1: Course of the experimental protocol, group by group. Training session and memory test paradigm consist of 10 min acclimatation and 30 min "poking" the pneumostome as soon as it opens. Yolk group presents no contingency "poking" as their first training: the temporal pattern of the TS of the CE group is reproduced. Control group is only put in CE for 40 min as their first training. Between each session (either 24h or 4 weeks), the snails were returned to the housing tank. TS = training session, MT = memory test, PW = pond-water, CE= crayfish effluent.

Animals were put to rest in housing aquaria for 4 weeks after the end of this first phase of the experiment. During that time they matured from juvenile to adult state (McComb *et al.*, 2005). The same operant conditioning paradigm was used again on these -now- adults *Lymnaea,* except that the training water was not CE water but simple pond water for both training and memory test sessions (figure 1).

Definition of memory

Memory was considered present when the number of attempted pneumostome openings in the memory test was significantly less than the attempted number of opening during the training session (Orr *et al.*, 2010a)

Yoked control

Snails that received a yoked training were treated in an identical manner to the operant conditioned animals. The only difference was that for these snails, the tactile stimuli were not contingent with the pneumostome openings. That is, the times of attempted pneumostome openings of the operant conditioned snails were recorded and reproduced in the same exact temporal manner with the yoked snails (but in that case there is no contingency with pneumostome openings) (Lukowiak *et al.*, 1996, 2000, 2003).

These snails were given a 30min yoked training session followed 24h later by a memory test (this time with contingency between pneumostome openings and tactile stimuli) (figure 1).

Control

Snails called control were only exposed to CE (but not trained) for 40 min which is the equivalent time of exposure of the acclimatization plus training session for both operant conditioned and yoked control snails. They were then tested for memory 24h later in the same way described above (figure 1).

Learner versus non learner

Juvenile snails' ability to learn was individually looked at and was considered as learning a 20% decreased of the attempted number of pneumostome openings during the MT compared to the TS (according to the assignment of grades, M Orr et al. 2009).

Statistics

The analysis of the data, was performed using one way ANOVA with Prism software on Macintosh. The outliers were excluded from the results using the empirical rule (95,5% of the data are contained within a 2*SD range, the others are judged outliers). For the first part of the experiment an ANOVA followed by a Tukey *post-hoc* test for multiple comparisons was realized. For the second part, after 4 weeks, paired t-tests were performed to analyze the different groups (for reasons that will be described later on). Additionally a one-way ANOVA was performed between the CE groups' data, before and after 4 weeks. Lastly to compare the "learner" and "non-learner" groups, the first part of the experiment was analyzed using one-way ANOVA and the second part using t-tests followed by a *post hoc* Wilconxin t-test.

RESULTS:

As explained previously (see materiel and methods) these experiments can be subdivided in two distinct parts: first the training, yoked or just exposed to CE, and second the training in pond water (PW) after reaching adulthood some 4 weeks later. The primary purpose of these experiments is to test the hypothesis that operant conditioning training in juvenile snails will cause long-lasting changes in the ability of snails to form long-term memory (LTM) as an adult.

First, the snails were either trained or yoked for 30 min in CE or exposed to CE for 40 min (control). Then they were tested for LTM in PW 24h later. The results are presented in figure 2 and demonstrate that the snails trained in CE (CE group) exhibited LTM. That is, the number of attempted pneumostome openings was significantly less than in the TS (TS = 21,43±1,12 N = 42; MT= 16,05±0,99 N = 42; one-way ANOVA p = 0,0006).

Moreover neither control groups, yoked or CE-exposure only, showed any decrease in the number of attempted pneumostome openings in their MT compared to TS of the operant conditioning group (MT Yolk = 21,66±1,13 N = 35; MT Control = 21,65±1,31 N = 40; one way ANOVA) and both were significantly different from the MT of the CE group (one way ANOVA).

These data indicate that our results are similar to previous work (Orr *et al.*, 2010b). Indeed, in both reports, LTM at 24h was only observed when the training of juvenile snails was done in CE. The only differences between reports are the number of attempted pneumostome openings; but this is most likely due to differences in the experimenter and how strongly or weakly they apply the tactile stimulus to the pneumostome as it begins to open. However, both reports show that CE enhances the ability of juvenile snails to form LTM.

In addition, preliminary data obtained by another experimenter in the Lukowiak lab, using juvenile snails born and raised in the same conditions as those in this report, found that *Lymnaea* trained in PW on average exhibited 14,73±1,17 and 14,95±0,88 attempted pneumostome openings in TS and MT respectively, which are similar values to what I obtained.

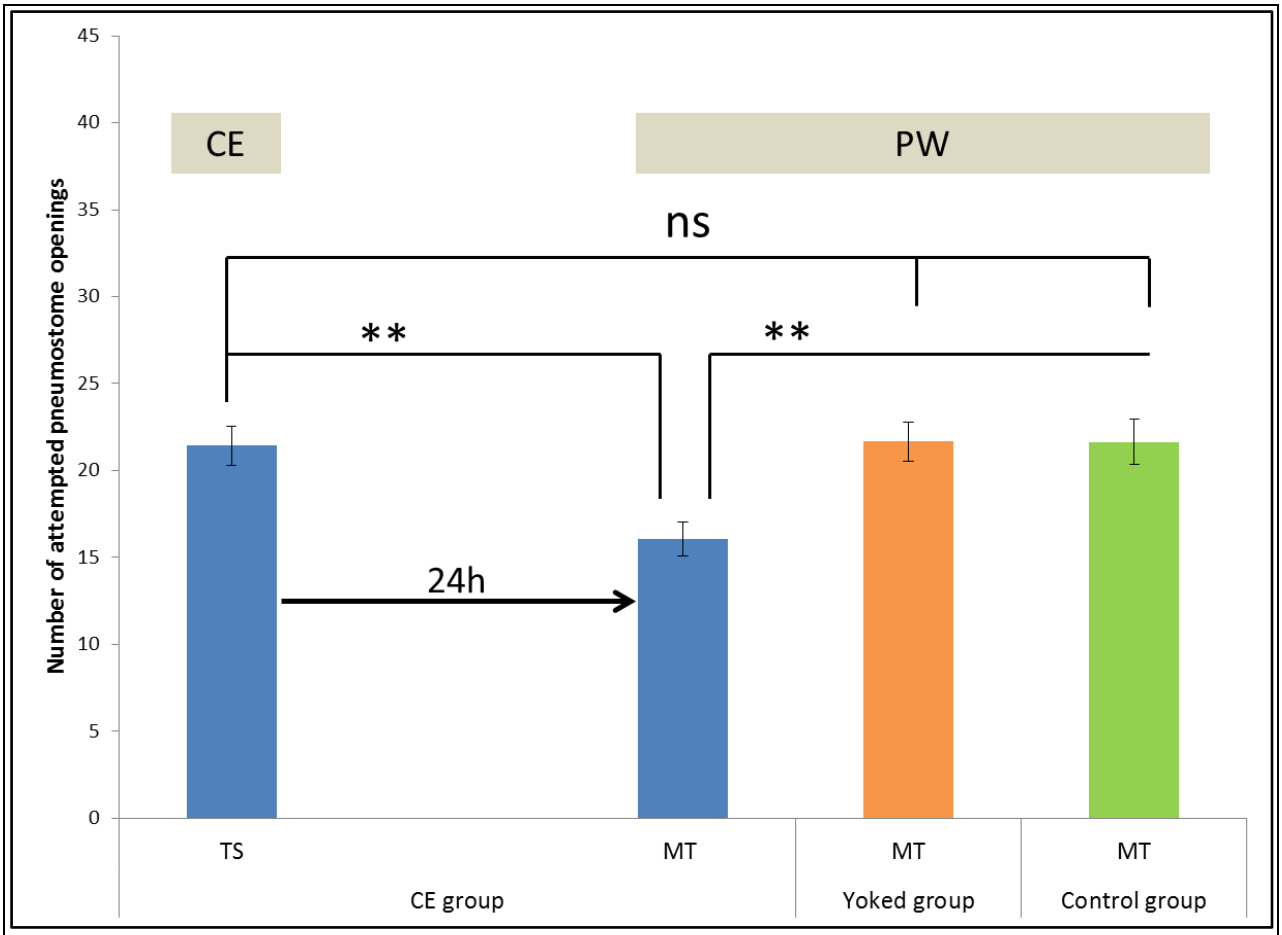


Figure 2: One 0,5h training session paradigm in CE supported with both yoked and control groups as controls for the presence of LTM. The snails trained in CE (N=42) exhibited LTM at 24h after one training session. Neither yoked nor control groups (respectively N=35 and 40) showed any LTM. The grey squares above represent the environment the snails were trained or tested in (CE = crayfish effluent, PW = pond water). Errors bars represent SEM. TS = training session, MT = memory test. ** $p < 0,01$.

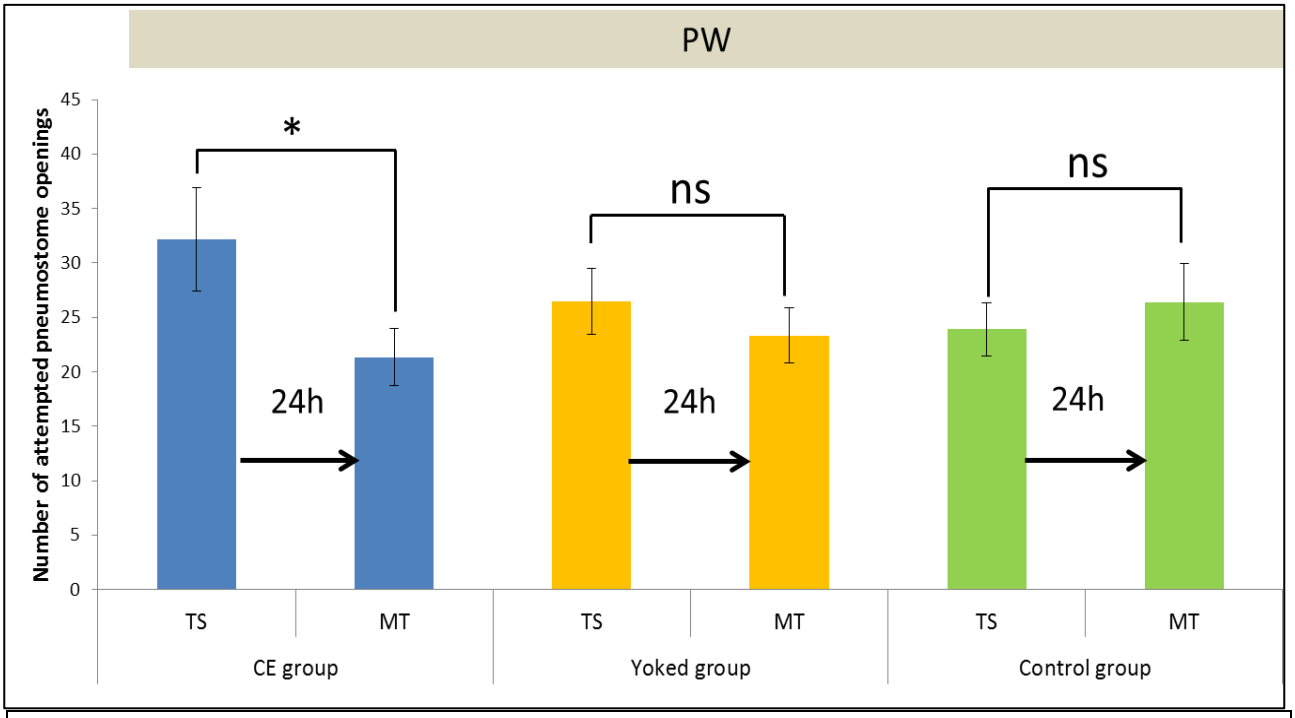
In the second series of experiments, operant conditioning in PW only occurred some 4 weeks later, when snails are now considered as adults. The snails were all trained following the same paradigm: a 0,5h training session in PW followed 24h later by a MT also in PW (figure 1).

These data are presented in figure 3. Clearly two things stand out.

First the juvenile snails trained in CE have obtained the capability to form LTM even after only a single 0,5h training session. That is they showed a significant decreased number of attempted

pneumostome openings in the MT compared with TS (TS = 32,17 ± 4,74 and MT = 21,33 ± 2,62 , p= 0,0474 , paired t-test). Moreover, neither juvenile snails that received yoked control training, nor those just exposed to CE, gained the capability to form LTM following a single training session. That is, both cohorts showed no significant decreased in the number of attempted pneumostome openings between their respective TS and MT (Yoked: TS = 26,5 ± 3,02 and MT = 23,33 ± 2,50 , paired t-test p=0,4064; Control: TS = 23,9 ± 2,45 and MT = 26,4 ± 3,54 , paired t-test p = 0,4869).

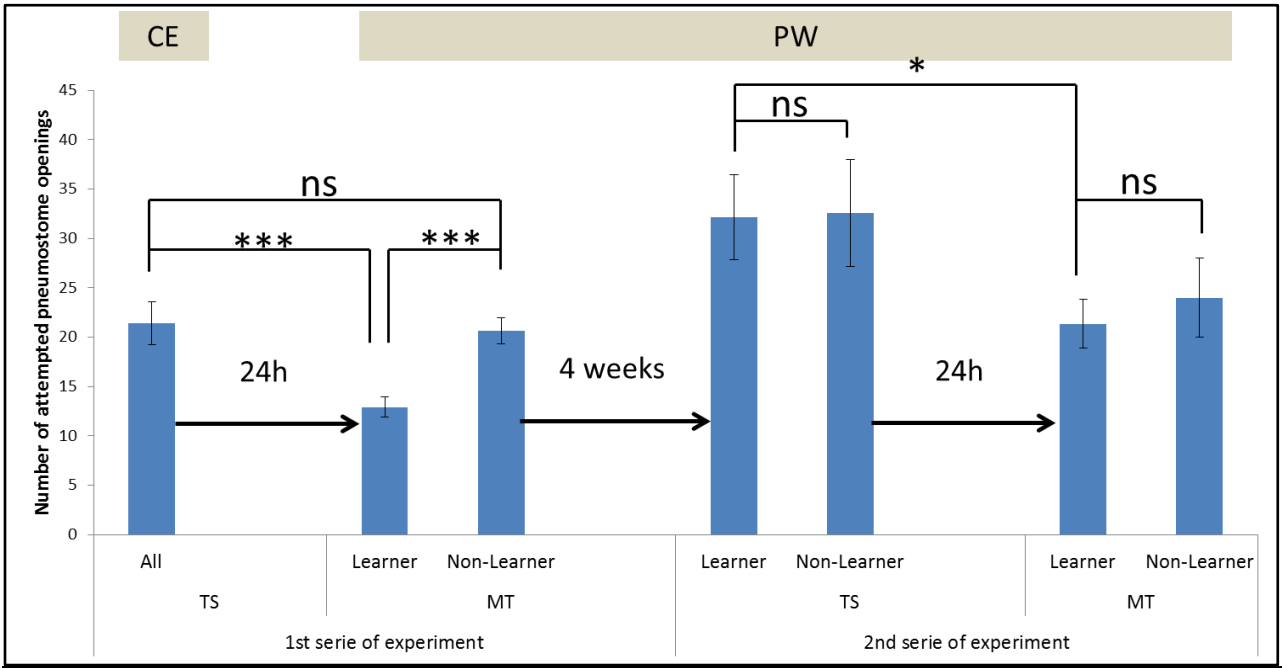
The second thing to notice here is, for the CE group and only this group, an increased attempted number of pneumostome openings during this TS compared with the TS four weeks before. This difference is statistically different when a one-way ANOVA is performed on the CE group data



<u>Figure 3</u>: One 0,5h training session paradigm for all 3 groups (CE group, Yoked group and Control group) in standard PW. Only the snails that were previously trained in CE show both a decrease number of pneumostome openings in the MT compared to the TS and increased number in the TS compared with the TS four weeks before (N = 12). Neither yoked (N = 12) nor control (N = 10) groups showed any decrease in numbers. The grey squares above represent the environment the snails were trained or tested in (PW = pond water). Errors bars represent SEM. TS = training session, MT = memory test. *p<0,05

from the 2 parts of this experiment: p <0,0001. For that reason it was impossible to apply a one-way ANOVA as a statistical test to compare all groups and instead resort to the use of paired t-tests.

Next an interesting result emerged from dissociating the snails that learned in the first half of this experiment, from those that didn't (see corresponding section in materiel and methods to see how they were grouped). Using this distinction and then looking at their learning after 4 weeks, no significant differences were observed between the two subgroups (figure 4). That is in the first training paradigm the number of attempted pneumostome openings for the "learner" group was



<u>Figure 4</u>: Training paradigm of the entire experiment subdividing "learner" from "non learner". The first part of the experiment allows a separation of the snails that learned in CE from those that didn't ("learner" N = 25, "non-learner" N = 17). These snails when tested 4 weeks later all showed a decreased number of pneumostome openings during the MT (however significant only in case of the learner groups) ("learner" N = 12, "non-learner" N = 7). The grey squares above represent the environment the snails were trained or tested in (CE = crayfish effluent, PW = pond water). Errors bars represent SEM. *p<0,05; *** p<0,001. TS = training session, MT = memory test.

significantly different between the TS (21,43 ± 2,18) and the MT (12,92 ± 1,01)(one way ANOVA: p< 0,0001), whereas no differences were observed between the TS and the MT (20,65 ± 1,34) for the "non-learner" group (one-way ANOVA). After 4 weeks we observed no significant differences between the TSs of the two subgroups (learner: TS = 32,167 ± 4,3 ; non-learner: TS 32,57 ± 5,44) and also no significant difference between the MTs (learner: MT = 21,33 ± 2,47 ; non-learner: MT = 24 ± 3,98). We also see a significant difference between the TS and the MT of the learner group (t-test, p=0,0474). However no significant difference was observed between the TS and the MT of the non-learner group (Wilcoxon test, p = 0,1563).

DISCUSSION :

This experiment showed that in juvenile *Lymnaea*, a 0,5h training session in CE results in a 24h LTM. It is known that this memory extends up to 8 days when adults are trained in the same way (Orr & Lukowiak, 2008). However here we did not investigate if it was also the case in juveniles. It is also important to remember that the Dutch juvenile *Lymnaea* do not ordinarily exhibit LTM (McComb *et al.*, 2005).

It has also been demonstrated several times that in adults, a training paradigm of a single 0,5h training session with the Dutch lab-bred snails in PW induces ITM but no LTM (Lukowiak *et al.*, 1998, 2008; Parvez *et al.*, 2005, 2006; Orr & Lukowiak, 2008).

The data I obtained showed that a training paradigm of this type (0,5h TS in CE) in juveniles causes long-lasting changes in their ability to form LTM in PW as adults. This is a totally new finding. As such a single 0,5h training session in PW is now enough to induce a 24h long-term memory.

According to our hypothesis, epigenetic mechanisms may be the underlying mechanism of this phenomenon.

In a previous paper, (Il-Han *et al.*, 2010) showed that a serotoninergic pathway is activated when the snails detect a predator (or in the case of the lab, CE). Using serotonin antagonist (mianserin) the authors managed to block LTM formation. On the other hand when injected with serotonin (5-HT), the snails showed an enhanced ability to form LTM.

The authors postulated that CE exposure enhances the consolidation process necessary for LTM via a serotoninergic pathway that ultimately leads to altered gene activity in neurons essential for memory. It is a strong possibility that in parallel to the activation of the late genes required for the synaptic plasticity and indispensable to form LTM, other proteins are recruited. Their role would be to modify the chromatin structure around genes coding for proteins playing a role in LTM.

Indeed in the mammalian hippocampus, 5-HT binding with its G-protein-coupled receptor lead to activation of an intracellular cascade: increased cAMP, recruitement of PKA and then the MAP kinases. This leads to recruitment of CREB and CBP which induce histone acetylation.
It is entirely possible that this mechanism has been conserved through evolution and that one of its primitive forms is found in *Lymnaea.*

Furthermore it is known that in *Lymnaea* a particular neuron RPeD1 (right pedal dorsal 1), a member of the CPG (central pattern generator) underlying aerial respiration, is known to be both necessary and sufficient for LTM (Syed *et al.*, 1990, 1992; Winlow & Syed, 1992). It has also been shown that LTM at the behavioral level is correlated, at the electrophysiological level, with decrease in the number of spikes and number of burst, decrease in the burst length and decrease in the number of spikes per burst.

Thus could it be possible that epigenetic mechanisms induced result in the alteration of the genes coding the proteins involved in these characteristics: ion channels, enzymes …

Furthermore, the “learner” versus “non-learner” distinction enables to see that even if the snails do not show LTM on a behavioral level after the training in CE, they still show a decreased number of pneumostome openings after 4 weeks. I think that the non-significant statistic result between the TS and the MT is due to the very low number of animals considered in this data. Moreover, as demonstrated in a previous paper (Orr & Lukowiak, 2008), it is possible to have alterations on the electrophysiological level (RPeD1) that are not reflected on the behavioral level. Thus the priming mechanism of LTM by CE is enough to induce the cascade discussed above.

However at that point, we are not able to conclude with certainty if epigenetics mechanisms are responsible for the data just observed. Several complementary experiments need to be done and are ongoing in our laboratory, before any definitive conclusion can be drawn.

One of these experiments consists in using 5-aza-2 deoxycytidine, a DNMT (DNA MethylTransferase) inhibitor. Preliminary data from our lab tends to show that the use of 5-AZA blocks LTM.
Thus it could mean that contrary to what we hypothesized above, the serotoninergic pathway is not the main player of these epigenetics mechanisms or it could also mean that we have more than one modification of the epigenome during this task. Weaver et al. have already proved this possible when they studied epigenetics in maternal behavior.

The second set of experiment consist of mating the snails used in this experiment and see if the ability of the snails to form LTM after a single 0,5 training session in PW is maintained across generations. A positive result would prove that genetic memory in in fact an epigenetic memory associated with a certain epigenome. This experiment is also ongoing in our laboratory and the first results should be available in a few months.

REFERENCES:

1. Alberini, C.M. (2009) Transcription factors in long-term memory and synaptic plasticity. *Physiol. rev.*, 89, 121–145.

2. Alberini, C.M., Ghirardi, M., Metz, R., & Kandel, E.R. (1994) C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in Aplysia. *Cell*, 76, 1099–1114.

3. Barrett, R.M. & Wood, M. (2008) Beyond transcription factors: the role of chromatin modifying enzymes in regulating transcription required for memory. *Learn. memory (Co. Spr. Har., N.Y.)*, 15, 460–467.

4. Bredy, T.W., Wu, H., Crego, C., Zellhoefer, J., Sun, Y.E., & Barad, M. (2007) Histone modifications around individual BDNF gene promoters in prefrontal cortex are associated with extinction of conditioned fear. *Learn. memory*, 14, 268–276.

5. Calderone, A., Jover, T., Noh, K., Tanaka, H., Yokota, H., Lin, Y., Grooms, S.Y., Regis, R., Bennett, M.V.L., & Zukin, R.S. (2003) Ischemic insults derepress the gene silencer REST in neurons destined to die. *J. Neurosci.*23, 2112–2121.

6. Flavell, S.W. & Greenberg, M.E. (2008) Expression and Plasticity of the Nervous System. *Annu. rev. Neurosci.*, 31, 563–590.

7. Guan, Z., Giustetto, M., Lomvardas, S., Kim, J.-H., Miniaci, M.C., Schwartz, J.H., Thanos, D., & Kandel, E.R. (2002) Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. *Cell*, 111, 483–493.

8. Huang, Y., Doherty, J.J., & Dingledine, R. (2002) Altered histone acetylation at glutamate receptor 2 and brain-derived neurotrophic factor genes is an early event triggered by status epilepticus. *J. Neurosci.*, 22, 8422–8428.

9. Il-Han, J., Janes, T., & Lukowiak, K. (2010) The role of serotonin in the enhancement of long-term memory resulting from predator detection in Lymnaea. *J. exp. biol.*, 213, 3603–3614.

10. Inoue, T., Takasaki, M., Lukowiak, K., & Syed, N. (1996) Inhibition of the respiratory pattern-generating neurons by an identified whole-body withdrawal interneuron of Lymnaea stagnalis. *J. exp. biol.*, 199, 1887–1898.

11. Jiang, Y., Langley, B., Lubin, F.D., Renthal, W., Wood, M. a, Yasui, D.H., Kumar, A., Nestler, E.J., Akbarian, S., & Beckel-Mitchener, A.C. (2008) Epigenetics in the nervous system. *J. Neurosci.*, 28, 11753–11759.

12. Kandel, E.R. (2001) The molecular biology of memory storage: a dialog between genes and synapses. *Bioscience rep.*, 21, 565–611.

13. Lee, J.-A., Kim, H.-K., Kim, K.-H., Han, J.-H., Lee, Y.-S., Lim, C.-S., Chang, D.-J., Kubo, T., & Kaang, B.-K. (2001) Overexpression of and RNA interference with the CCAAT enhancer-binding protein on long-term facilitation of Aplysia sensory to motor synapses. *Learn. Memory*, 8, 220–226.

14. Leslie, J.H. & Nedivi, E. (2011) Activity-regulated genes as mediators of neural circuit plasticity. *Prog. neurosci.*, 94, 223–237.

15. Levenson, J., O'Riordan, K., Brown, K., Trinh, M., Molfese, D., & Sweatt, J. (2004) Regulation of histone acetylation during memory formation in the hippocampus. *J. Biol. Chem.*, 279, 40545–40559.

16. Levenson, J. & Sweatt, J. (2005) Epigenetic mechanisms in memory formation. *Nat. Rev. Neurosci.*, 6, 108–118.

17. Liu, I.Y.C., Lyons, W.E., Mamounas, L. a, & Thompson, R.F. (2004) Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning. *J. Neurosci.*, 24, 7958–7963.

18. Loebrich, S. & Nedivi, E. (2009) The Function of Activity-Regulated Genes in the Nervous System. *Physiol. Rev.*, 89, 1079–1103.

19. Lubin, F.D., Gupta, S., Parrish, R.R., Grissom, N.M., & Davis, R.L. (2011) Epigenetic mechanisms: critical contributors to long-term memory formation. *Neuroscientist*, 17, 616–632.

20. Lubin, F.D., Roth, T.L., & Sweatt, J.D. (2008) Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. *J. Neurosci.*, 28, 10576–10586.

21. Lukowiak, K., Adatia, N., Krygier, D., & Syed, N. (2000) Operant conditioning in Lymnaea: evidence for intermediate- and long-term memory. *Learn. memory (Co. Spr. Har., N.Y.)*, 7, 140–150.

22. Lukowiak, K., Cotter, R., & Westly, J. (1998) Long-term memory of an operantly conditioned respiratory behaviour pattern in Lymnaea stagnalis. *J. Exp. Biol.*, 201, 877–882.

23. Lukowiak, K., Martens, K., Rosenegger, D., Browning, K., De Caigny, P., & Orr, M. (2008) The perception of stress alters adaptive behaviours in Lymnaea stagnalis. *J. exp. biol.,* 211, 1747–1756.

24. Lukowiak, K., Ringseis, E., Spencer, G., Wildering, W., & Syed, N. (1996) Operant conditioning of aerial respiratory behaviour in Lymnaea stagnalis. *J. Exp.Biol.*, 199, 683–691.

25. Lukowiak, K., Sangha, S., Scheibenstock, A., Parvez, K., McComb, C., Rosenegger, D., Varshney, N., & Sadamoto, H. (2003) A molluscan model system in the search for the engram. *J physiol., Paris*, 97, 69–76.

26. McComb, C., Sangha, S., Qadry, S., Yue, J., Scheibenstock, A., & Lukowiak, K. (2002) Context extinction and associative learning in Lymnaea. *Neurobiol. learn. mem.*, 78, 23–34.

27. McComb, C., Varshney, N., & Lukowiak, K. (2005) Juvenile Lymnaea ventilate, learn and remember differently than do adult Lymnaea. *J. Exp. biol.*, 208, 1459–1467.

28. Müller, C. & Leutz, (2001) Chromatin remodeling in development and differentiation. *Curr. opin.genetics dev.*, 11, 167–174.

29. Orlando, V. & Castellino, V. Pietro (2003) and Control of Cell Identity. *Cell*, 112, 599–606.

30. Orr, M., Hittel, K., Lukowiak, K.S., Han, J., & Lukowiak, K. (2009) Differences in LTM-forming capability between geographically different strains of Alberta Lymnaea stagnalis are maintained whether they are trained in the lab or in the wild. *J. Exp. Biol.*, 212, 3911–3918.

31. Orr, M. V, El-Bekai, M., Lui, M., Watson, K., & Lukowiak, K. (2007) Predator detection in Lymnaea stagnalis. *J. Exp. biol.*, 210, 4150–4158.

32. Orr, M. V, Hittel, K., & Lukowiak, K. (2009) "Different strokes for different folks": geographically isolated strains of Lymnaea stagnalis only respond to sympatric predators and have different memory forming capabilities. *J. Exp. Biol*, 212, 2237–2247.

33. Orr, M. V, Hittel, K., & Lukowiak, K. (2010a) Predator detection enables juvenile Lymnaea to form long-term memory. *J. Exp. Biol*, 213, 301–307.

34. Orr, M. V, Hittel, K., & Lukowiak, K. (2010b) Predator detection enables juvenile Lymnaea to form long-term memory. *J. Exp. Biol*,, 213, 301–307.

35. Orr, M. V & Lukowiak, K. (2008) Electrophysiological and behavioral evidence demonstrating that predator detection alters adaptive behaviors in the snail Lymnaea. *J. Neurosci.,*28, 2726–2734.

36. Parvez, K., Moisseev, V., & Lukowiak, K. (2006) A context-specific single contingent-reinforcing stimulus boosts intermediate-term memory into long-term memory. *Eur. J. neurosci.*, 24, 606–616.

37. Parvez, K., Stewart, O., Sangha, S., & Lukowiak, K. (2005) Boosting intermediate-term into long-term memory. *J. Exp. Biol*, 208, 1525–1536.

38. Syed, N., Bulloch, A., & Lukowiak, K. (1992) The respiratory central pattern generator (CPG) of Lymnaea reconstructed in vitro. *Acta.Biol.Hung.*, 43, 409–419.

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

40. Weaver, I.C.G., Cervoni, N., Champagne, F. a, D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., & Meaney, M.J. (2004) Epigenetic programming by maternal behavior. *Nat. Neurosci.*, 7, 847–854.

41. Winlow, W. & Syed, N. (1992) The respiratory central pattern generator of Lymnaea. *Acta. Biol. Hung.*, 43, 399–408.