Interaction between environmental stressors mediated via the same sensory pathway

Sarah Dalesman and Ken Lukowiak*
Department of Physiology and Pharmacology; Hotchkiss Brain Institute; University of Calgary; Calgary, AB Canada

The great pond snail, *Lymnaea stagnalis*, is a calciphile, requiring approximately 20 mg/l dissolved calcium for natural populations to live long and prosper. However, despite population survival we have previously demonstrated that acute exposure for 1 week to low environmental calcium (20 mg/l) acts as a stressor on the snail, blocking long-term memory (LTM) formation. This response to calcium availability is mediated by the snail directly sensing the calcium concentration in its environment using a sensory structure called the osphradium. In addition to sensing the calcium environment, the osphradium also mediates the response to predator kairomones which has an opposite effect on memory i.e., kairomone exposure during training enhances LTM formation. Here we demonstrate how these two stressors (low calcium availability and predator kairomones), that alter memory formation in opposing directions via the same sensory system, interact when experienced simultaneously.

Snail Memory

*Lymnaea stagnalis* is commonly used as a model species to study learning and memory due to the ease with which behavioral traits can be observed and measured, and a relatively simple nervous system allowing the neurons controlling specific behavioral traits to be identified. We used a standard protocol to assess long-term memory (LTM) formation in this species, by operantly conditioning them to reduce aerial respiration in hypoxic conditions. Training involves gently “poking” the snail on the pneumostome (respiratory opening to a basic lung) each time the snail attempts to breath in hypoxia. We then manipulate the conditions under which snails are maintained and trained to assess how different environmental conditions alter the ability of this species to form LTM to reduce aerial respiration attempts. The memory we assess is non-declarative, that is the electrophysiological changes associated with memory formation appear to be formed in the same neurons that are responsible for driving the behavior, i.e., the central pattern generator controlling aerial respiration.

Environmental Stress

The ability of *L. stagnalis* to form LTM is malleable, altered by environmental stressors. Whether memory is enhanced or blocked depends on the nature of the stress and the timing relative to the learning procedure. We were specifically interested here in understanding how environmental factors, such as those that would be experienced by natural populations, act as stressors, altering the ability of *L. stagnalis* to form memory. Predator kairomones enhance the ability of the snail to form LTM, and recent work demonstrated that input from the osphradium is required for the snails to demonstrate this induced memory enhancement. The ability to externally sense predator kairomones was not surprising as a slow moving snail would have to sense a predator’s approach to enable it to perform effective escape behavior. Low calcium (20 mg/l) within the range experienced by natural populations of *L. stagnalis* acts as a...
stressor on the snail, increasing metabolic rate and reducing locomotion,\textsuperscript{11} as well as blocking the ability of this species to form long-term memory.\textsuperscript{12,13} Calcium availability could potentially be sensed via changes in internal calcium homeostasis in addition to sensing external calcium availability; we were therefore surprised that the effect of acute exposure to low environmental calcium availability blocking LTM formation was entirely due to direct sensing of external calcium concentration via the osphradium. When we severed the osphradial nerve connecting the osphradium to the central nervous system (CNS), the snails responded to training in a low calcium environment (20 mg/l) as if they were held in our standard calcium concentration (80 mg/l) by forming LTM, apparently unaware of reduced calcium availability.\textsuperscript{14}

\textbf{Conflicting Information via the Same Sensory System}

Having demonstrated that sensory input from the osphradium is necessary to mediate the effects of both predator kairomones and low calcium availability on LTM formation,\textsuperscript{9,14} we were curious about how the snails would respond if we presented these two disparate environmental stressors in combination. Specifically, would these stressors interact to alter memory formation, or would one signal from the osphradium be blocked by the presence of the other (i.e., can snails only respond to either calcium availability or predator kairomones). To test this we exposed snails to either our standard (80 mg/l) or low (20 mg/l) environmental calcium conditions for 1 week prior to and during training, and either trained them in pond water alone or in the presence of predator kairomones from a crayfish (*Pacifastacus leniusculus*). Training consisted of two half hour training sessions (TR1 and TR2) separated by 1 h; snails were tested for memory in pond water alone either 24 h or 72 h following TR2. Data were analyzed using repeated measures ANOVA in SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Mauchley’s test for sphericity was used to assess homogeneity of variance, and the more conservative Greenhouse-Geisser p values used where assumptions of homogeneity of variance were not met.

In standard control conditions snails formed LTM lasting 24 h but not 72 h, in agreement with previous findings (Fig. 1A; Interaction effect: $F_{2,44} = 11.26$, $p < 0.001$).\textsuperscript{12,13} As found previously for intact snails, following exposure to low calcium conditions for 1 week prior to and during training, LTM formation was blocked, with snails demonstrating learning but not LTM (Fig. 1B; Interaction effect: $F_{2,24} = 5.89$, $p = 0.008$).\textsuperscript{12-14} Similarly, our findings for intact snails held in our standard calcium conditions, but trained in crayfish kairomones, supported previous work demonstrating enhancing effects on LTM formation.\textsuperscript{8} That is, our snails now demonstrate LTM 72 h following training (Fig. 1C; Main effect of training: $F_{1.42,31.29} = 33.90$, $p < 0.001$), at which time the snails trained in control conditions do not exhibit LTM.

When we combined stressors, such that snails were held in low calcium concentration and also trained in predator kairomones (produced in low calcium water), we demonstrate an interactive effect between these two stressors. That is, snails were able to form LTM (i.e., they was not blocked as in low calcium alone), but the memory they formed lasted only 24 h, not 72 h (i.e., not enhanced as seen in predator kairomones alone; Fig. 1D;...
Interaction effect: $F_{2,46} = 11.69, p < 0.001$). The memory phenotype in response to exposure to combined stressors is not significantly different from snails held and trained in control conditions throughout. Further work is required to assess whether this cancelling out effect on behavioral phenotype occurs at the level of sensory input (i.e., electrophysiological activity in the osphradial nerve is identical to control conditions), or whether it is occurring within the CNS in the processing of sensory input from the osphradium. We suspect that the latter is more likely, and this should be testable within our model system.

Previous work demonstrated that in standard calcium conditions memory retention following training in predator kairomones can last as long as 8 d, compared with only one day in control conditions. This duration is very similar to the length of time juvenile *L. stagnalis* retain information about increased predation risk. Therefore, this memory enhancement in the presence of predation threat may be integral in allowing a snail to accurately assess predator identification and predation risk. The effect of low calcium availability in reducing LTM retention, even in the presence of predator kairomones, is likely to significantly alter the ability of this species to demonstrate behavioral plasticity in the face of variable predation threat.

Acknowledgments

Alberta Innovates—Health Solutions provides an AHFMR postdoctoral fellowship for S.D. and Canadian Institutes for Health Research supports K.L. and the laboratory.

References

1. Benjamin PR, Kemenes G. Behavioral and circuit analysis of learning and memory in mollusks. In: Byrne JH, Ed. Learning and Memory: A Comprehensive Reference. Oxford: Academic Press 2008; 587-604.  
2. Benjamin PR, Starks K, Kemenes G. A systems approach to the cellular analysis of associative learning in the pond snail *Lymnaea*. Learn Mem 2000; 7:124-31; PMID:10837501; DOI:10.1101/lm.7.3.124.

3. Lukowiak K, Sangha S, Scheibenstock A, Parvez K, McComb C, Rosenegger D, et al. A molluscan model system in the search for the engraver. J Physiol Paris 2003; 97:69-76; PMID:14706692.

4. Parvez K, Rosenegger D, Orr M, Martens K, Lukowiak K. Canadian association of neurosciences review: Learning at a snail's pace. Can J Neurol Sci 2006; 33:347-56; PMID:17168159.

5. Lukowiak K, Ringseis E, Spencer G, Wildering W, Syed N. Operant conditioning of aerial respiratory behaviour in *Lymnaea stagnalis*. J Exp Biol 1996; 199:683-91; PMID:9318425.

6. Lukowiak K, Martens K, Rosenegger D, Browning K, de Caigny P, Orr M. The perception of stress alters adaptive behaviours in *Lymnaea stagnalis*. J Exp Biol 2008; 211:1747-56; PMID:18490390; DOI:10.1242/jeb.014886.

7. Lukowiak K, Orr M, de Caigny P, Lukowiak KS, Rosinegger D, Han JI, et al. Ecologically relevant stressors modify long-term memory formation in a model system. Behav Brain Res 2010; 214:18-24; PMID:20478338; DOI:10.1016/j.bbr.2010.05.011.

8. Orr MV, Lukowiak K. Electrophysiological and behavioral evidence demonstrating that predator detection alters adaptive behaviors in the snail *Lymnaea*. J Neurosci 2008; 28:2726-34; PMID:18337402; DOI:10.1523/JNEUROSCI.5132-07.2008.

9. Il-Han J, Janes T, Lukowiak K. The role of serotonin in the enhancement of long-term memory resulting from predator detection in *Lymnaea*. J Exp Biol 2010; 213:3603-14; PMID:20952608; DOI:10.1242/jeb.048256.

10. Boycott AE. The habitats of fresh-water Mollusca in Britain. J Anim Ecol 1936; 5:116-86; DOI:10.2307/1096.

11. Dalesman S, Lukowiak K. Effect of acute exposure to low environmental calcium alters respiration and locomotion of *Lymnaea stagnalis* (L.). J Exp Biol 2010; 213:1471-6; PMID:20408031; DOI:10.1242/jeb.040493.

12. Dalesman S, Braun MH, Lukowiak K. Low environmental calcium blocks long term memory formation in a pulmonate snail. Neurobiol Learn Mem 2011; 95:393-405; PMID:21130174; DOI:10.1016/j.nlm.2010.11.017.

13. Knezevic B, Dalesman S, Karnik V, Byzitter J, Lukowiak K. Low external environmental calcium levels prevent forgetting in *Lymnaea*. J Exp Biol 2011; 214:2118-24; PMID:21613529; DOI:10.1242/jeb.054635.

14. Dalesman S, Karnik V, Lukowiak K. Sensory mediation of memory blocking stressors in the pond snail, *Lymnaea stagnalis*. J Exp Biol 2011; 214:2528-35; PMID:21753046; DOI:10.1242/jeb.058024.

15. Sangha S, Scheibenstock A, McComb C, Lukowiak K. Intermediate and long-term memories of associative learning are differentially affected by transcription versus translation blockers in *Lymnaea*. J Exp Biol 2003; 206:1665-13; PMID:12682092; DOI:10.1242/jeb.03301.

16. Dalesman S, Rundle SD, Coleman RA, Cotton PA. Cue association and antipredator behaviour in a pulmonate snail, *Lymnaea stagnalis*. Anirr Behav 2006; 71:789-97; DOI:10.1016/j.anbehav.2005.05.028.