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Novel interactive effects of darkness and retinoid signaling in the ability to form long-term memory following aversive operant conditioning

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ABSTRACT

The vitamin A metabolite, retinoic acid, is important for memory formation and hippocampal synaptic plasticity in vertebrate species. In our studies in the mollusc *Lymnaea stagnalis*, we have shown that retinoic acid plays a role in memory formation following operant conditioning of the aerial respiratory behaviour. Inhibition of either retinaldehyde dehydrogenase (RALDH) or the retinoid receptors prevents long-term memory (LTM) formation, whereas synthetic retinoid receptor agonists promote memory formation by converting intermediate-term memory (ITM) into LTM. In this study, animals were exposed to constant darkness in order to test whether light-sensitive retinoic acid would promote memory formation. However, we found that exposure to constant darkness alone (in the absence of retinoic acid) enhanced memory formation. To determine whether the memory-promoting effects of darkness could override the memory-inhibiting effects of the retinoid signaling inhibitors, we exposed snails to RALDH inhibitors or a retinoid receptor antagonist in constant darkness. We found that darkness overcame the inhibitory effects of RALDH inhibition, but did not overcome the inhibitory effects of the retinoid receptor antagonist. We also tested whether constant darkness and training affected the mRNA levels of the retinoid metabolic enzymes RALDH and Cyp26, or the mRNA levels of the retinoid receptors, but found no significant effect. Overall, these data demonstrate an interaction between environmental light conditions and the retinoid signaling pathway, which influence long-term memory formation in a mollusc.

1. Introduction

The aerial respiratory behaviour of the mollusc, *Lymnaea stagnalis*, can be operantly conditioned to produce intermediate-term memory (ITM) or long-term memory (LTM), depending on the number of training sessions used (Lukowiak, Adatia, Krygier, & Syed, 2000; Lukowiak et al., 1998). Over the last 20 years, numerous studies have determined how various factors such as age, stress and exposure to toxins can affect the ability of these animals to form long-term memories (reviewed by Lukowiak, Sunada, Teskey, Lukowiak, & Dalesman, 2014; Spencer & Rothwell, 2013). More recently, we have discovered that the vitamin A metabolite, retinoic acid, is important for the formation of long-term memory following operant conditioning of the respiratory behaviour of *Lymnaea* (Rothwell & Spencer, 2014). Studies in vertebrates have also shown that vitamin A and retinoic acid are important in memory formation (Bonnet et al., 2008; Chiang et al., 1998; Cocco et al., 2002; Nomoto et al., 2012; Wietrzych et al., 2005), and disruption of retinoid signaling pathways can have detrimental effects on hippocampal long-term potentiation (LTP) and depression (LTD) (Chiang et al., 1998; Misner et al., 2001; Nomoto et al., 2012).

In both the canonical and reported "ancestral" pathways of retinoic acid synthesis, the final irreversible oxidation of retinal to retinoic acid is carried out by the enzyme retinaldehyde dehydrogenase (RALDH). Retinoic acid enters the nucleus where it binds to retinoid receptors which are members of the nuclear hormone receptor family. There are two classes of retinoid receptors, the retinoid X receptors (RXRs) and retinoic acid receptors (RARs), which bind to retinoic acid response elements (RAREs) in the promoter region of target genes and act as ligand-activated transcription factors to regulate gene expression. Endogenous retinoic acid is then broken down by the enzyme Cyp26. We have previously shown that the CNS (and hemolymph) of *Lymnaea* contains physiologically relevant concentrations of retinoic acid and have previously demonstrated RALDH activity in CNS extracts (Dmetrichuk, Carlone, Jones, Vesprini, & Spencer, 2008). We have also cloned RALDH (Genbank Accession no. FJ539101), Cyp26 (Genbank Accession no. KF669878), an RXR (Carter, Farrar, Carlone, & Spencer, 2010; Genbank Accession no. AY846875) and a putative RAR (Carter, 2011; Genbank Accession no. GU932671) from the *Lymnaea* CNS. Though

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retinoid signaling has apparently been lost in many protostome lineages, it appears to be intact in many molluscan species (Albalat & Cañestro, 2009; Bouton et al., 2005; Simões-Costa, Azambuja, & Xavier-Neto, 2008), including *Lymnaea*.

Previous experiments have shown that pharmacological inhibitors of RALDH and retinoid receptors can prevent LTM formation in *Lymnaea*, while having no effect on either learning or ITM. Furthermore, treatment with retinoid receptor agonists promoted the formation of LTM (Rothwell & Spencer, 2014). In the previous study, only synthetic retinoid agonists were used, so the initial aim of this study was to determine whether exogenous application of the natural ligand, retinoic acid, could also promote memory formation following operant conditioning of the aerial respiratory behaviour. However, a serendipitous discovery that incubation of animals in constant darkness (because of the light-sensitive nature of retinoic acid) enhanced memory formation in these animals, led us to investigate how this memory-enhancing effect of darkness might interact with retinoid signaling. Our main aim was thus to combine two opposing treatments: one memory-enhancing (darkness), the other memory-inhibiting (inhibition of retinoid signaling), to determine which one would prevail.

It is well known that circadian rhythms affect memory formation in a number of different diurnal species, including the mollusc *Aplysia* (Lyons, 2011; Lyons, Rawashdeh, Katzoff, Susswein, & Eskin, 2005; Lyons, Sol Collado, Khabour, Green, & Eskin, 2006), with animals learning better during the day than at night. *Lymnaea* are more active in the daytime and have also demonstrated more effective acquisition of a conditioned behaviour in the morning than in the afternoon (Wagatsuma et al., 2004). Many of these studies have used constant darkness in their protocols, yet to our knowledge, there has been no previous evidence that such conditions might promote LTM formation. Interestingly, it has also been shown that photoperiod can affect retinoid metabolism and affect levels of both RALDH and Cyp26 (Helfer et al., 2012), though no studies have previously reported the effects of constant darkness on retinoid enzyme levels. In this study, we first demonstrated that the memory-enhancing effect of constant darkness was able to override any inhibitory effects of RALDH inhibition on LTM formation, but was not able to override the inhibitory effects of a retinoid receptor antagonist. Using real-time quantitative PCR, we then determined whether constant darkness (with and without training), affected the mRNA levels of either RALDH or Cyp26, as well as the retinoid receptors, in the *Lymnaea* CNS.

2. Materials and methods

2.1. Animals

L. stagnalis (originally obtained from stocks at the Vrije University, Amsterdam) were bred in the laboratory environment and maintained in dechlorinated water at room temperature on a 12:12 h fixed light–dark cycle. *Lymnaea* were fed a combination of romaine lettuce leaves and NutraFin Max Spirulina fish food (Hagen). Adult animals ranging in shell length from 22 to 30 mm were used for all experimental procedures. Prior to the initiation of any experimental procedures, *Lymnaea* were labeled with coloured markings on their shells and were permitted to freely perform aerial respiration.

2.2. Chemicals

All-*trans* retinoic acid (atRA) was obtained from Sigma–Aldrich and prepared in 100% EtOH, and then diluted in water to a final concentration of 10^{-6} M (0.01% EtOH). Vehicle control experiments were performed with 0.01% EtOH. The RALDH inhibitors, citral and 4-diethylaminobenzaldehyde (DEAB) were obtained from Sigma–Aldrich and stock solutions were prepared in 70% EtOH. Citral was diluted to a final concentration of 50 μM (0.35% EtOH) and DEAB was diluted to a final concentration of 100 μM (0.35% EtOH) in water. Vehicle control experiments were performed with 0.35% EtOH. The RXR pan-antagonist, HX531, was a generous gift from Dr. H. Kagechika (University of Tokyo, Japan) and was prepared in 100% DMSO. HX531 was diluted to a final concentration of 10^{-6} M in water. Vehicle control experiments were run in 0.01% DMSO.

2.3. Operant conditioning of aerial respiration

The aerial respiratory behaviour of *Lymnaea* was operantly conditioned as previously described (Khan & Spencer, 2009; Lukowiak, Ringseis, Spencer, Wildering, & Syed, 1996; Lukowiak et al., 2000; Rothwell & Spencer, 2014). Animals were trained in a 1 L test beaker containing 800 mL of hypoxic water (water bubbled with 100% N_2 gas for 20 min prior to and during all training sessions and memory tests) in order to encourage the performance of aerial respiration over cutaneous respiration (Lukowiak et al., 1996). *Lymnaea* were given 30 min to acclimate to the hypoxic environment before session 1 (S1) (unless otherwise stated) and 10 min before all other training sessions and the memory test. Snails were gently propelled to the bottom of the test beaker to mark the initiation of each training session and memory test. All training sessions were 45 min in duration with a 1 h consolidation period between sessions. Operantly conditioned animals received a tactile stimulus to the open pneumostome each time aerial respiration was attempted during each training session. This stimulus was sufficient to induce immediate pneumostome closure without inducing the whole body withdrawal response. Yoked control animals received the same number of tactile stimuli as the operantly conditioned animals, but the tactile stimulus was applied to the closed pneumostome each time the animal to which it was yoked opened its pneumostome. Thus in the yoked control group, stimulus application was not contingent upon pneumostome opening. The number of attempted pneumostome openings was recorded for each operantly conditioned animal, while the number of pneumostome openings was recorded for the yoked control groups. Snails were placed in eumoxic home tanks or incubation beakers between training or testing sessions.

To produce intermediate-term memory (ITM) lasting 2 h, two 45 min training sessions (S1 and S2) were administered, with a 1 h consolidation period between sessions. The memory test (MT) was administered 2 h after S2. A separate group of animals were also tested 24 h after S2 to test for long-term memory (LTM).

To produce LTM lasting 24 h, four 45 min training sessions (S1 to S4) were administered with 1 h consolidation periods between training sessions. A MT was conducted 24 h after the final training session and the conditioned animals were again stimulated on the open pneumostome during the MT. LTM was also assessed using 30 min freely-breathing pre-test and post-test sessions before and after the training procedure respectively. During these freely-breathing sessions, the number of pneumostome openings was recorded for each snail and the number of pneumostome openings observed during the post-test was compared to the behaviour observed during the pre-test.

2.4. Procedure for exposure to darkness

Animals were randomly assigned to either a 'darkness' group or to a control group. The darkness group was maintained in constant darkness prior to S1, between all training sessions, and until the MT, whereas the animals in the control group were maintained on the normal 12:12 h light–dark cycle. To assess the influence of darkness