Neurobiology of Learning and Memory 136 (2016) 34–46

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Extending the duration of long-term memories: Interactions between environmental darkness and retinoid signaling

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ARTICLE INFO

Article history:
Received 16 March 2016
Revised 30 August 2016
Accepted 15 September 2016
Available online 16 September 2016

Keywords:
Retinoic acid
Invertebrate learning
Operant conditioning
Electrophysiology
Lymnaea stagnalis

ABSTRACT

Retinoid signaling plays an important role in hippocampal-dependent vertebrate memories. However, we have previously demonstrated that retinoids are also involved in the formation of long-term implicit memory following operant conditioning of the invertebrate mollusc *Lymnaea stagnalis*. Furthermore, we have discovered an interaction between environmental light/dark conditions and retinoid signaling and the ability of both to convert intermediate-term memory into long-term memory. In this study, we extend these findings to show that retinoid receptor agonists and environmental darkness can both also extend the duration of long-term memory. Interestingly, exposure to constant environmental darkness significantly increased the expression of retinoid receptors in the adult central nervous system, as well as induced specific changes in a key neuron mediating the conditioned behaviour. These studies not only shed more light on how retinoids influence memory formation, but also further link environmental light conditions to the retinoid signaling pathway.

1. Introduction

In order to survive, a species must adapt to ever-changing environmental conditions, which is the basis of learning and memory. The mollusc *Lymnaea stagnalis* is a model organism for studying learning and memory, as its aerial respiratory behaviour can be operantly conditioned and this behaviour is controlled by a well-characterized neuronal network (Syed, Bulloch, & Lukowiak, 1990; Syed, Harrison, & Winlow, 1991; Syed & Winlow, 1991). Changes within this network, particularly the neuron that triggers the behaviour, right pedal dorsal 1 (RPeD1), have been identified and associated with memory formation (Scheibenstock, Krygier, Haque, Syed, & Lukowiak, 2002; Spencer, Kazmi, Syed, & Lukowiak, 2002; Spencer, Syed, & Lukowiak, 1999). Memory formation following operant conditioning of respiration in *Lymnaea* can be influenced by a number of external factors, such as predator detection (Dalesman, Rundle, & Lukowiak, 2011; Orr, Hittel, & Lukowiak, 2009, 2010; Orr & Lukowiak, 2008) and temperature (Teskey, Lukowiak, Riaz, Dalesman, & Lukowiak, 2012), both of which can promote memory. In addition, various chemical signals, such as serotonin (Lukowiak et al., 2014) and methamphetamine (Kennedy et al., 2010) can also enhance memory formation in this mollusc.

In vertebrates, retinoid signaling in the adult brain is important for various types of learning and memory, such as novel object recognition (Wietrzych et al., 2005) and spatial working memory (Chiang et al., 1998). We have recently shown that retinoid signaling is also required for the formation of implicit memory in *Lymnaea* (Rothwell & Spencer, 2014). Retinoic acid (RA), the active metabolite of vitamin A, acts by binding to nuclear retinoid receptors, of which there are two classes, the retinoid X receptors (RXRs) and the retinoic acid receptors (RARs). Both an RXR (Carter, Farrar, Carlone, & Spencer, 2010) and a putative RAR (Carter et al., 2015) have been cloned from the central nervous system (CNS) of *Lymnaea*, and the CNS contains both all-*trans* and 9-*cis* isomers of RA (Dmetrichuk, Carlone, Jones, Vesprini, & Spencer, 2008).

Our previous research has shown that when retinoid signaling is pharmacologically impaired (either by preventing RA synthesis or with retinoid receptor antagonists), *Lymnaea* fail to form long-term memory (LTM) following operant conditioning. However, pharmacological stimulation of retinoid pathways using receptor agonists, converts an intermediate-term memory (ITM) into LTM (Rothwell & Spencer, 2014), thus enhancing memory formation in *Lymnaea*. An additional finding of these studies (with light-sensitive RA), is that exposure to constant darkness also promotes LTM formation (from ITM) in this mollusc (Rothwell, Simmons,

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http://dx.doi.org/10.1016/j.nlm.2016.09.008
1074-7427/

Peters, & Spencer, 2014). Interestingly, we found that constant darkness overcomes memory inhibition resulting from impaired RA synthesis, but not from retinoid receptor blockade with an antagonist. These previous findings strongly suggest an interaction between retinoid signaling and environmental light/dark conditions, which together can influence memory formation.

In this study, we aimed to determine whether retinoid receptor agonists and darkness can not only convert ITM into LTM (Rothwell & Spencer, 2014; Rothwell et al., 2014), but also whether they can extend the duration of LTM. We demonstrate here that all synthetic retinoid receptor agonists studied do indeed extend LTM duration, and that a change in light conditions to constant darkness has the same effect. We also provide evidence that such exposure to constant darkness changes the expression of retinoid receptors in the adult CNS, as well as induces changes in the activity of a key network neuron (RPeD1) underlying this conditioned behaviour in *Lymnaea*.

2. Materials and methods

2.1. Animals

Lymnaea stagnalis were bred in the laboratory environment and maintained at room temperature on a fixed 12:12 h light-dark cycle in aerated dechlorinated water supplemented with Instant Ocean salts (Aquarium Systems, Ohio, USA). The snails were fed a combination of romaine lettuce and NutraFin Max Spirulina fish food (Hagen) and permitted to freely perform aerial respiration prior to the initiation of any experimental procedures. All behavioural experiments utilized adult *Lymnaea* ranging in shell length from 22 to 28 mm. Individual snails were identified using coloured markings applied to their shells at least 24 h prior to training.

2.2. Chemicals

The pan-RXR agonist PA024 was a gift from Dr. H. Kagechika (University of Tokyo, Japan). The retinoid receptor agonists Ch55 and SR 11237, as well as the synthetic retinoid EC23 were obtained from Tocris Bioscience (Bristol, U.K.). All agonists were prepared in 100% DMSO and subsequently diluted in dechlorinated water to a concentration of 10^{-6} M (in 0.01% DMSO), as used previously (Rothwell & Spencer, 2014). Vehicle control experiments used 0.01% DMSO. Animals were bathed in the agonist solutions prior to training (Rothwell & Spencer, 2014; Rothwell et al., 2014). The DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine (5-AZA; Sigma-Aldrich) was prepared in sterile *Lymnaea* saline to a final concentration of 87 μM (Lukowiak et al., 2014; Sunada et al., 2016). Control experiments used only sterile *Lymnaea* saline.

2.3. Retinoid receptor agonist incubations

Lymnaea were incubated in 200 mL of aerated retinoid receptor agonist solution in 400 mL beakers for 24 h before the first training session (S1). Snails were only removed from the incubation beakers during the training sessions and memory test (MT). Animals were randomly assigned to one of five groups and incubated in 200 mL of either (i) DMSO (0.01%; vehicle control), (ii) the RXR pan-agonist PA024 (10^{-6} M), (iii) the RXR agonist SR11237 (10^{-6} M), (iv) the synthetic retinoid EC23 (10^{-6} M), or (v) the RARα/β agonist Ch55 (10^{-6} M). Following the 24 h of incubation, the animals were operantly conditioned as described below.

2.4. Incubations in constant darkness

Animals were randomly assigned to either a 'constant darkness' or 'light' control group, and maintained in 200 mL of aerated water in 400 mL beakers at room temperature. The 'darkness' group was maintained in constant darkness, in a separate room, for 48 h before the initiation of training and returned to the dark between training sessions and until the MT. The 'light' control animals were maintained on the normal 12:12 h light-dark cycle of the laboratory. All training sessions and MTs were conducted in the light and at room temperature, unless otherwise stated, and training commenced in the mornings.

2.5. Injection procedure

Animals were randomly assigned to one of three groups and received either: (i) no injection, (ii) a saline injection, or (iii) a 5-AZA injection (87 μM; Lukowiak et al., 2014; Sunada et al., 2016). Animals were anesthetized on ice for 20 min and 100 μL of 5-AZA or saline was injected into the hemocoel, through the foot. For some experiments, these injections were carried out before the retinoid receptor agonist or darkness incubation. For a different series of experiments, these injections were administered following the treatments, but prior to training. In this case, the animals were given at least one hour to recover from the injections before being operantly conditioned.

2.6. Operant conditioning procedures

The aerial respiratory behaviour of *Lymnaea* was operantly conditioned as previously described (Khan & Spencer, 2009; Lukowiak, Adatia, Krygier, & Syed, 2000; Lukowiak, Ringseis, Spencer, Wildering, & Syed, 1996; Rothwell & Spencer, 2014). Training and testing was conducted in a hypoxic environment, to encourage the performance of aerial respiration over cutaneous respiration (Lukowiak et al., 1996). This hypoxic environment was created by vigorously bubbling 800 mL of water in the 'test beaker' with 100% N_2 gas for 20 min prior to (and at a slower rate during) each training session and memory test. Animals were permitted to acclimate to the hypoxic environment prior to each training session or memory test. Following this acclimation period, animals were gently propelled to the bottom of the 'test beaker' to signify the beginning of each session.

Snails were given four 45 min training sessions (S1, S2, S3, S4) separated by a one hour period for consolidation. During each training session, a tactile stimulus was immediately applied to the open pneumostome of an operantly conditioned animal each time aerial respiration was attempted. This stimulus induced immediate pneumostome closure without initiating the full body withdrawal response. Yoked control animals received the same number of tactile stimuli as conditioned animals, but the application of this stimulus was not dependent upon pneumostome opening. That is, yoked controls received a punishing tactile stimulus to the closed pneumostome each time the animal to which it was yoked performed aerial respiration.

The number of attempted pneumostome openings was recorded for the operantly conditioned animals, while the number of pneumostome openings was recorded for the yoked controls. Snails were returned to eumoxic incubation beakers (or home tanks) between the training and testing sessions and permitted to freely perform aerial respiration. A memory test (MT) was conducted at various times after the final training session (S4) depending on the specific experimental procedure.