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Intermediate-Term Memory is Modulated by the Circadian Clock

Lisa C. Lyons^{*}, Charity L. Green^{*}, and Arnold Eskin

Department of Biology and Biochemistry, University of Houston, Houston, Texas

Abstract

Sensitization of the tail-siphon withdrawal reflex in *Aplysia*, a non-associative form of learning, affords a superb opportunity to investigate the regulation of learning and memory by the circadian clock. The circadian clock has been shown to modulate long-term (LTS), but not short-term sensitization (STS) (Fernandez et al., 2003). However, no previous studies have examined the role of the circadian clock in intermediate-term memory. Noxious stimulation delivered to the side of the animal using a spaced training protocol resulted in canonical intermediate-term sensitization (ITS) dependent upon both MAPK signaling and protein synthesis. We found that ITS exhibited strong rhythms in expression in both light-dark cycles and constant darkness. Animals trained during the (subjective) day demonstrated significantly more intermediate-term memory than animals trained at night. Baseline responses prior to training were not modulated by the circadian clock. Thus, these results indicate that the circadian clock strongly modulates intermediate, as well as long-term memory.

Keywords

Aplysia; biological clock; circadian rhythm; intermediate-term memory; learning; sensitization

Learning, or the change in an animal's behavior due to an environmental stimulus, can be influenced by time of day information via the endogenous circadian clock through multiple mechanisms including time-stamping of the learning event, modulating the robustness of memory formation or disruption of memory due to circadian dysfunction. Recently, the modulatory role of the circadian clock on long-term memory has become more apparent with studies in many systems. In the marine mollusk *Aplysia californica*, the circadian clock modulates long-term, but not short-term, memory for non-associative and associative learning that is dependent upon the time the animals are trained (Fernandez et al. 2003; Lyons et al. 2005). *Aplysia* demonstrate robust long-term memory formation during the day with little long-term memory occurring when animals are trained at night. In contrast to long-term memory, virtually nothing is known about the effect of the circadian clock on intermediate-term memory.

Depending upon the strength and duration of training, sensitization of the tail-siphon withdrawal reflex in *Aplysia* exhibits short-term memory lasting minutes, intermediate-term memory that can be measured 30 – 90 min following training, and long-term memory observed 24 hours or days later. Intermediate-term sensitization (ITS) represents a discrete form of memory that shares many characteristics with long-term sensitization (LTS). ITS induced via spaced tail-shocks to the intact animal results in memory dependent upon MAPK signaling and protein synthesis as is LTS (Ghirardi et al. 1995; Sharma et al. 2003; Sutton and Carew 2000). However, induction of ITS does not depend upon transcription whereas LTS does

Corresponding author: Dr. Lisa C. Lyons, Department of Biological Science, Florida State University, Tallahassee, FL 32306-4295. lyons@bio.fsu.edu.

^{*}Present Address: Department of Biological Science, Florida State University, Tallahassee, Florida.

(Bailey et al. 1992; Castellucci et al. 1986; Ghirardi et al. 1995; Montarolo et al. 1986; Sutton et al. 2001).

Given that circadian modulation of long-term memory in *Aplysia* occurs, in part, through regulation of training-induced activation of pre-synaptic MAPK (Lyons et al. 2006) and the dependence of ITS on MAPK, we hypothesized that ITS in *Aplysia* is modulated by the circadian clock. We used a four trial spaced training protocol to induce ITS in the intact animal (Fig. 1A and Appendix). Sensitization training using electrical shock delivered (during the day) to one side of the animal trained produced unilateral ITS as measured by the change in siphon withdrawal duration (mean $\pm$ SEM: shocked side of animal $65.7\% \pm 8.5$; control (non-shocked) side of animal $-3.8\% \pm 3.9$; $n = 11$ two tailed t test, $t = 6.86$, $P < 0.001$). The protein synthesis inhibitor anisomycin (Sigma-Aldrich; 1 ml of 2mM anisomycin in artificial seawater/100 g body weight injected 30 min prior to training) completely blocked the formation of ITS (anisomycin treated animals (shocked side) = $-26.4\% \pm 8.0$, $n = 3$; ITS for control animals trained at the same time point = $42.7\% \pm 4.2$, $n = 6$; two-tailed t test, $t = 8.63$, $P < 0.01$). ITS also was dependent upon MAPK signaling. Animals treated with the MAP kinase kinase (MEK) inhibitor, PD98059 (Calbiochem; 130 μ l/100 g body weight of 15 mM DMSO stock) or U0126 (Calbiochem; 130 μ l/100 g body weight of 7.5 mM DMSO stock) 30 min prior to training demonstrated significantly less ITS compared to vehicle injected animals (MEK inhibitor treated animals = $-2.1\% \pm 7.1$, $n = 6$; ITS in vehicle treated animals = $33.2\% \pm 10.3$, $n = 6$; two-tailed t test, $t = 2.80$, $P < 0.01$). Treatment with MEK inhibitors did not significantly affect siphon withdrawal duration on the unshocked side of the animal (unshocked side of animal = $-6.6\% \pm 3.3$).

Having established that the ITS training protocol was dependent upon MAPK signaling and protein synthesis, we investigated the role of the circadian clock on ITS. Wild-caught animals were trained at various times throughout the light-dark (LD) cycle. Due to the length of the pre-training baseline assessments, the training procedure and the time necessary for post-testing (Fig. 1A), it was not possible to examine time points less than 6 hours apart for comparison within the same batch of animals. A diurnal rhythm in ITS was observed (Fig. 1B) with animals trained during the day demonstrating significantly greater levels of ITS compared to animals trained during the night. No sensitization was observed on the unstimulated side of the animal (Fig. 1C). To investigate if the rhythm persisted in constant darkness (DD), animals were trained on either the first or second day of DD. As there were no significant differences observed in either the baseline responses or the magnitude of sensitization induced between animals trained on the first day compared to the second day of DD, the data was pooled. Animals trained during the subjective day showed significant intermediate-term memory, whereas animals trained during the late subjective night (CT 21) exhibited no ITS (Fig 2A). Siphon withdrawal duration did not vary with time of day on the unshocked side of the animal (Fig 2B). It should be noted that the magnitude of ITS for animals trained during the subjective day in DD appears lower than when animals were trained during the day in LD cycles. Potentially, the acute effect of light (masking) enhanced the sensitization responses when animals were trained in LD.

Mechanistically, the circadian clock could regulate learning by: 1) regulating sensitivity to sensory input, 2) modulating the induction of ITS or 3) modulating the expression (recall) of ITS. Potentially, the circadian clock modulates sensitization through the animal's response to the shock used to elicit siphon withdrawal. We observed no significant diurnal or circadian differences in siphon withdrawal duration elicited by tail shock in animals prior to sensitization training. Baseline siphon-withdrawal responses before ITS training (measured at 2x threshold current) for animals trained during the day were: ZT 3 = 3.33 ± 0.7 sec, ZT 9 = 2.59 ± 0.4 sec, ZT 15 = 3.20 ± 0.6 sec, ZT 21 = 2.79 ± 0.3 sec; ANOVA $F_{(3,17)} = 0.39$, $P > 0.75$. Baseline siphon-withdrawal responses for animals in DD were: CT 3 = 2.68 ± 0.2 sec, CT 9 = $2.36 \pm$

0.1 sec, CT 15 = 2.43 ± 0.1 sec, CT 21 = 2.26 ± 0.1 sec; ANOVA $F_{(3,24)} = 1.12$, $P > 0.36$. The absence of circadian regulation of the animal's response to electrical shock prior to sensitization training is consistent with previous studies (Fernandez et al. 2003). These results suggest that the circadian clock modulates intermediate-term memory through modulation of induction or recall of ITS. Due to the limited window of expression of ITS, it is not possible to directly test whether the circadian clock modulates the induction or the recall of ITS through behavioral experiments.

Circadian modulation of ITS appears very similar, although not identical, to circadian modulation of LTS (Fernandez et al. 2003). In both cases, animals exhibited significant memory when trained during the subjective day and little or no memory when trained during the late night at CT 21. However, when animals were trained during the early evening at CT 15, differences became apparent between ITS and LTS. At CT 15, the animals exhibited significant ITS not statistically different from the levels observed when animals were trained during the subjective day. In comparison in previous studies on LTS, animals trained at CT 15 expressed lower levels of LTS that significantly differed from the levels of LTS observed for animals trained during the subjective day (Fernandez et al., 2003; Lyons et al., 2006).

The similarity in the phase of the rhythms for ITS and LTS, with peak memory during the day and trough memory in the late night, suggests that the mechanisms underlying circadian modulation of sensitization may involve common mechanisms. During sensitization training, noxious stimulation induces the release of 5-HT from facilitatory neurons triggering a cascade of downstream events in pleural sensory neurons including the production of cAMP, PKA signaling, and the activation of MAPK that result in presynaptic facilitation (reviewed in (Kandel 2001)). In LTS, the circadian clock modulates memory formation at multiple levels including 5-HT release, the activation of MAPK and the induction of transcription (Lyons et al., 2006). Given the commonality of the training procedure between ITS and LTS, it is likely that the circadian clock also modulates ITS through the modulation of 5-HT release. Additionally, due to the shared dependence of ITS and LTS upon MAPK signaling, (Sharma et al. 2003), we hypothesize that the previously observed circadian modulation of MAPK activity following training (Lyons et al. 2006) may underlie, at least in part, the modulation of both ITS and LTS. Whereas the circadian clock may jointly modulate ITS and LTS through 5-HT release and the induction of MAPK, the circadian clock appears to also modulate LTS through additional steps including the induction of transcription (Lyons et al., 2006). Potentially, the differences we observed between modulation of ITS at CT 15 and modulation of LTS at CT 15, or even the variability in ITS between ZT 15 and CT 15, may be explained by the fact that the circadian clock impacts memory formation at multiple steps some of which are involved in ITS and others specific to LTS. It will be interesting in future studies to investigate if the circadian clock modulates PKA activity during memory formation.

What is the significance of circadian modulation of memory? Although the answer remains unknown, insight may be gained by examining modulation of memory across species. When memory formation (as opposed to recall) is regulated by the circadian clock, peak long-term memory seems to be coordinated with the animal's activity period as seen in *Aplysia*, cockroach, mice and zebrafish (Decker et al. 2007; Fernandez et al. 2003; Lyons et al. 2005; Rawashdeh et al. 2007; Valentinuzzi et al. 2001). Given the similarity between ITS and LTS, circadian modulation of intermediate term memory may be shaped by similar selective pressures that affect modulation of long-term memory. Modulation of short-term memory may be more dependent upon the type of learning or behavior assayed. For example, circadian modulation of short-term memory occurs in cockroaches (Decker et al. 2007), but not *Aplysia*. The work presented here provides a framework for future studies investigating the function of circadian modulation of memory as well as the molecular mechanisms through which the circadian clock modulates intermediate-term and other forms of memory.

Appendix

Appendix

Aplysia (100-150 g; Charles Hollahan, Santa Barbara, CA; Alacrity, Redondo Beach, CA) were housed in artificial seawater at 15°C with a 12:12 h light:dark (LD) cycle. Sensitization training was performed using electrical stimulation as the noxious stimulus as previously described for LTS with pre-training procedures including parapodectomies, feeding to satiation and electrode implantation (Fernandez et al. 2003; Lyons et al. 2006). Briefly, *in vivo* ITS training consisted of four 10 sec blocks of 10 shocks (500 msec, 60 mA shocks given at 1 Hz) delivered via a hand-held electrode to one side of the body wall with 30 min intervals between blocks (Fig. 1A). Siphon-withdrawal was elicited by a low current test shock via wire electrodes implanted in the animal's tail. Pre-training and post-training siphon withdrawal durations were measured using one 20 msec shock at 2x threshold current (interstimulus interval 10 min). The duration of withdrawal was measured from the time the siphon initially contracted to the first sign of siphon relaxation. The stimulus used to elicit pre-training siphon withdrawal did not cause habituation. Post-training measurements of siphon withdrawal duration were made for each side of the animal starting at 30 minutes following training with an interstimulus interval of 10 min. Siphon withdrawal duration measurements did not vary significantly between 30 - 60 minutes after training. Statistical analysis was performed using ANOVA with Tukey post-hoc analyses. P values less than 0.05 were considered significant.

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Figure 1. Diurnal Modulation of Intermediate-Term Sensitization

A) Training schematic for ITS. Training consisted of four 10 sec blocks of 10 shocks (500 msec, 60 mA shocks, 1 Hz) with 30 min intervals between blocks of shocks. Pretests measuring siphon withdrawal duration began 30 min prior to training with 4 measurements 10 min apart, while posttests for ITS were given 30 – 60 min following training. B) Animals were entrained to 12:12 h LD cycles, and subjected to training at either ZT 3, 9, 15 or 21 ($n = 4$ to 6 for each). Testing occurred 30 min - 1 h post training. ITS varied significantly depending on when training was administered during the LD cycle (ANOVA $F_{(3,17)} = 8.37$, $P < 0.01$) with significantly greater amounts of ITS observed during the day. Tukey post-hoc analyses revealed significant differences ($P < 0.05$) between ZT 3 v. 15, ZT 9 v. 15 and ZT 9 v. 21. C) Training produced unilateral ITS. No differences were observed on the untrained side of the animal (ANOVA $F_{(3,15)} = 0.23$, $P > 0.8$).

Figure 2. Circadian Modulation of Intermediate-Term Sensitization

A) Animals were entrained to LD cycles, transferred to DD, and subjected to training at either CT 3, 9, 15 or 21 ($n = 6$ to 8 for each). ITS exhibited robust circadian rhythms in expression (ANOVA $F_{(3,25)} = 5.11$, $P < 0.01$) with significantly greater amounts of sensitization observed for animals trained during the subjective day compared to animals trained at CT 21. Tukey post-hoc analyses revealed significant differences ($P < 0.05$) between CT 3 v. 21 and CT 9 v. 21. B) No circadian differences were observed on the untrained side of the animal (ANOVA $F_{(3,21)} = 1.23$ $P > 0.3$).