Published in final edited form as:

J Biol Rhythms. 2006 August ; 21(4): 245–255. doi:10.1177/0748730406289890.

Non-Ocular Circadian Oscillators and Photoreceptors Modulate Long Term Memory Formation in *Aplysia*

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Abstract

In *Aplysia californica*, memory formation for long-term sensitization (LTS) and for a more complex type of associative learning, learning that food is inedible (LFI), is modulated by a circadian clock. For both types of learning, formation of long-term memory occurs during the day and significantly less during the night. *Aplysia* eyes contain a well-characterized circadian oscillator that is strongly coupled to the locomotor activity rhythm. Thus, the authors hypothesized that the ocular circadian oscillator was responsible for the circadian modulation of LFI and LTS. To test this hypothesis, they investigated whether the eyes were necessary for circadian modulation of LFI and LTS. Eyeless animals trained during the subjective day and tested 24 h later demonstrated robust long-term memory for both LFI and LTS, while eyeless animals trained and tested during the subjective night showed little or no memory for LFI or LTS. The amplitude of the rhythm of modulation in eyeless animals was similar to that of intact *Aplysia*, suggesting that extraocular circadian oscillators were mainly responsible for the circadian rhythms in long-term memory formation. Next, the authors investigated whether the eyes played a role in photic entrainment for circadian regulation of long-term memory formation. Eyeless animals were exposed to a reversed LD cycle for 7 days and then trained and tested for long-term memory using the LFI paradigm. Eyeless *Aplysia* formed significant long-term memory when trained during the projected shifted day but not during the projected shifted night. Thus, the extraocular circadian oscillator responsible for the rhythmic modulation of long-term memory formation can be entrained by extraocular photoreceptors.

Keywords

Aplysia; biological clock; circadian rhythm; memory; sensitization; feeding; ocular; learning

Biological clocks regulate most physiological and behavioral aspects of organisms, including sleep, locomotor activity, hormone secretion, metabolism, and long-term memory formation as well as many other processes (Fernandez et al., 2003; Bell-Pedersen et al., 2005; Gillette and Sejnowski, 2005). Circadian modulation of memory formation has been demonstrated in both vertebrates and invertebrates (Valentinuzzi et al., 2001; Chaudhury and Colwell, 2002; Fernandez et al., 2003; Lyons et al., 2005; Chaudhury et al., 2005). Robust circadian modulation of long-term memory formation has been shown in *Aplysia* using a simple nonassociative type of learning, long-term sensitization (LTS) (Fernandez et al., 2003), and a more complex type of associative operant learning, learning that a food is inedible (LFI) (Lyons et al., 2005). In both cases, long-term memory formation occurred when animals were trained

during the subjective day while little or no long-term memory formation occurred when animals were trained during the subjective night. For both LTS and LFI, the circadian clock modulates events during the induction and consolidation of long-term memory (at the time of training), rather than events during the recall of memory at the time of testing (Fernandez et al., 2003; Lyons et al., 2005).

The presence of 2 different forms of long-term memory under circadian modulation makes *Aplysia californica* well suited for studying how the circadian clock modulates memory. This study represents a first step toward identifying the circadian system involved in the circadian modulation of memory formation in *Aplysia*. *Aplysia* have a well-characterized circadian oscillator located in neurons at the base of the eyes (Jacklet, 1969b; Jacklet et al., 1972; Luborsky-Moore and Jacklet, 1977; Herman and Strumwasser, 1984). A circadian rhythm in spontaneous compound action potentials can be recorded from eyes in vivo (Block, 1981) or from eyes maintained in vitro (Jacklet, 1969a; Eskin, 1971). The ocular neurons send afferent fibers via the optic nerve throughout the central nervous system of *Aplysia* to the cerebral, pleural, and pedal ganglia and even as far as the abdominal connectives (Olson and Jacklet, 1985). This wide-scale distribution of ocular outputs within the CNS of *Aplysia* suggests a broad functional impact of the eyes on a wide spectrum of behaviors, including a possible role of the ocular circadian oscillator as a “master” circadian clock.

Several different studies on *Aplysia* have shown a strong correlation between an intact ocular circadian system and the circadian locomotor activity rhythm (Strumwasser, 1973; Lickey et al., 1976; Lickey et al., 1983). Locomotor activity becomes largely arrhythmic as a consequence of eye removal (Strumwasser, 1973; Lickey et al., 1977). Furthermore, predawn anticipatory locomotor activity disappears as a result of eye removal (Lickey et al., 1976). Only when the eye is attached to the optic nerve does the phase of the ocular circadian rhythm correspond to the phase of the locomotor rhythm (Lickey et al., 1983). In fact, when the rhythms in compound action potentials of the 2 eyes drift out of phase, the locomotor activity rhythm weakens (Lickey et al., 1983). Despite the strong correlation between the ocular circadian rhythm and the locomotor activity rhythm, there is evidence for the involvement of extraocular circadian oscillators in regulating the locomotor activity rhythm. For example, some animals continue to display weak circadian locomotor activity rhythms after eye removal (Block and Lickey, 1973; Strumwasser, 1973; Lickey et al., 1977). This suggests that while the circadian locomotor activity rhythm in *Aplysia* is primarily regulated by the ocular oscillator, extraocular circadian oscillators could be coupled to locomotor activity. The existence of multiple circadian oscillators distributed throughout the organism has been well established in invertebrates and more recently shown in mammals (Plautz et al., 1997; Whitmore et al., 2000; Welsh et al., 2004).

Although circadian modulation of long-term memory formation in *Aplysia* has been characterized at the behavioral level, the location of the circadian oscillator(s) and the output pathway regulating long-term memory formation has not yet been determined. Since LTS involves the cerebral, pleural, and pedal ganglia and LFI involves the buccal and cerebral ganglia, circadian regulation of long-term memory might be due to a central oscillator with anatomically wide-ranging neuronal connections. Based on the widescale distribution of the ocular nerves throughout the CNS, we hypothesized that the ocular circadian oscillator was responsible for circadian modulation of memory formation in *Aplysia*. Our results show that a circadian rhythm of long-term memory formation persisted in eyeless animals, with robust memory exhibited during the subjective day and significantly less during the night. The rhythm in eyeless animals was similar to the circadian rhythms of long-term memory formation seen in intact *Aplysia* (Fernandez et al., 2003; Lyons et al., 2005). Entraining eyeless *Aplysia* to a reversed LD cycle resulted in the reversal of the circadian rhythm of long-term memory formation of LFI when animals were trained and tested in DD. Thus, extraocular circadian

oscillators are sufficient for modulating learning and memory as well as entraining the rhythm. The results, therefore, indicate the existence of an extraocular circadian oscillator that can be entrained via extraocular photoreceptors and that can modulate long-term memory formation.

MATERIALS AND METHODS

Animal Maintenance

A. californica (Alacrity, Redondo Beach, CA; Charles Hollahan, Santa Barbara, CA) ranging from 120 to 200 g were individually housed in boxes in 15 °C circulating seawater tanks. Animals were fed romaine lettuce every second day until they were fed to satiation. For all experiments, *A. californica* were entrained to 12:12-h LD cycles for at least 5 days prior to being placed into constant dark conditions (DD). For experiments examining entrainment of eyeless animals, eyeless animals were exposed to reverse 12:12-h LD cycles for 7 days prior to DD. Experiments in DD were started on the second day of DD, and the animals were tested 24 h after training. Dim red light was used for manipulations in the dark.

Eye Surgery

Aplysia were cooled on ice for 15 min and then locally anesthetized with 1 to 2 mL of isotonic MgCl₂ injected into the skin between both eyes. After an additional 15 min on ice, the eyes were surgically removed. Removal of the entire eye was visually confirmed. Animals that inked at any time during this procedure or any other procedure prior to training were not used.

Behavioral Training and Testing

A. californica were fed to satiation 2 days following eye surgery with laver seaweed and kept without additional food until the end of the experiment.

LFI—Animals were trained that a food is inedible using a standard protocol (Schwarz and Susswein, 1986; Susswein et al., 1986; Botzer et al., 1998; Katzoff et al., 2002). Briefly, animals were trained by presenting a small piece of laver seaweed wrapped in netting that they could not swallow. *Aplysia* were continuously stimulated on the lips with the netted seaweed until the animals stopped responding. The training session was terminated when the animal failed to respond by taking the netted food into the mouth for 3 consecutive minutes. Two parameters, total response time and the cumulative time the netted food remained in the mouth, were measured during training and testing. Long-term memory was measured 24 h after training as a decrease in the time of these parameters when the animals were again exposed to the netted seaweed. For all behavioral experiments, the person testing the animals was not the same person who trained the animals.

In the reversed-phase entrainment experiments, intact animals and animals in which the eyes had been removed were maintained in the same tank and entrained to LD cycles simultaneously. In addition, the person testing the animals in the reversed-phase experiment was unaware of which animals were eyeless. The coloration of the animal and the small size of the eyes makes it impossible to determine if eyes are present under conditions using dim red light. All reversed-phase experiments were done using the LFI paradigm due to the difficulties associated with performing the necessary procedures and animal maintenance for LTS behavioral testing (parapodectomies and electrode implantation) under reversed LD cycles.

LTS—Animals were trained and tested for long-term sensitization of the siphon-withdrawal reflex, as previously described (Scholz and Byrne, 1987; Cleary et al., 1998; Fernandez et al., 2003). To elicit siphon withdrawal, current from an AC stimulator (1504 Isolated Variable AC Line Supply, Global Specialities, Cheshire, CT) was applied through electrodes implanted in the tail of the animal. Immediately before training, the threshold to elicit siphon withdrawal

was determined separately for each side of the animal using a 20-msec shock. Baseline pretests and posttests were done (5 on each side, 10-min interstimulus interval) at $2 \times$ threshold. For long-term sensitization training, 1 side of the animal was electrically stimulated using four 10-sec blocks of 10 shocks (500 msec, 60 mA delivered at 1 Hz), with each block 30 min apart. Twenty-four hours after LTS training, siphon withdrawal duration was measured. The person measuring pre- and posttraining siphon withdrawal durations was blind to which side of the animal received LTS training.

Statistical Analysis

The data for LFI and LTS are expressed as percentage changes $\pm$ SEM of response times during testing compared to initial response times during training. All comparisons for parametric data were done with the Student *t* test and ANOVA; *p* values of < 0.05 were considered significant. Post hoc analysis was done with the Newmann-Keuls test.

RESULTS

Persistence of Circadian Modulation of Long-Term Memory Formation for LFI in the Absence of the Ocular Circadian Oscillator

The formation of long-term memory for LTS and LFI is modulated by a circadian clock, with robust long-term memory formation occurring when animals are trained during the subjective day and significantly less long-term memory formation occurring when animals are trained during the subjective night (Fernandez et al., 2003; Lyons et al., 2005). We hypothesized that the ocular circadian oscillator was responsible for the circadian modulation of memory formation for LTS and LFI. LFI represents an associative, operant learning paradigm in which the failure of the animal to swallow the netted food acts as a negative reinforcer leading to changes in the animal's behavior. Long-term memory, measured 24 h after training, was represented as a decrease in the time the animal spent responding to the netted food and a decrease in the cumulative time the netted food remained in the mouth. To test whether the eyes were required for the circadian modulation of memory formation for LFI, animals were entrained to 12:12 LD cycles for at least 3 days prior to eye removal, which has been shown sufficient to entrain the *Aplysia* ocular rhythm (Eskin, 1979). After eye removal, animals continued to be maintained on a 12:12-h LD cycle for 5 days and then were switched to constant darkness and trained with netted seaweed on the second day of DD at CT3, CT9, CT15, and CT21. Testing occurred 24 h later on the third day of DD. Eyeless *Aplysia* showed robust long-term memory formation for LFI when trained during the subjective day and significantly less when animals were trained during the subjective night (Figs. 1 A₁ and A₂), as measured by decreases in both the total response time (Fig. 1A₁; $F_{3,25} = 9.077$; $p < 0.001$) and the total time netted food was kept in the mouth (Fig. 1A₂; $F_{3,25} = 6.984$; $p < 0.005$). These results suggest the involvement of an extraocular circadian clock in modulating long-term memory formation for LFI. Moreover, the rhythm of circadian modulation of long-term memory formation in eyeless animals was as robust as that observed in intact animals (Lyons et al., 2005).

The circadian rhythm of long-term memory formation observed in eyeless *Aplysia* (Figs. 1 A₁ and A₂) could be a result of a circadian rhythm in the feeding behavior rather than a rhythm in long-term memory formation. In *Aplysia*, feeding behavior is regulated by the circadian clock (Kupferman, 1974; Levenson et al., 1999). We therefore analyzed the total response time and total mouth time during training on the second day of DD in eyeless *Aplysia* and compared the mean training times obtained at CT3, CT9, CT15, and CT21. The data revealed no circadian rhythm in training responses under the interactive learning conditions used in our experiment (Figs. 1 B₁ and B₂). There was no significant difference when comparing training responses during the subjective day (CT3, CT9) with training responses during the subjective night (CT15, CT21) for either the total response times (Fig. 1B₁; $F_{3,25} = 0.241$; $p > 0.86$) or the total

mouth times (Fig. 1B₂; $F_{3, 25} = 1.67$; $p > 0.199$). These results suggest that circadian rhythms in feeding behavior during training were not responsible for the circadian rhythm of memory formation. This result is similar to that obtained using animals with eyes (Lyons et al., 2005).

The Rhythm in Memory Formation Is Due to Circadian Modulation Rather Than Factors Associated with Length of Time in DD

Memory formation could potentially be affected by a change in the animals' motivation due to stress, age, or health. Thus, we investigated whether extended time without food or extended time in DD was responsible for the rhythm in memory formation we observed. We compared the performance times during training of naive eyeless *Aplysia* on the second and third days of DD at 2 time points (CT3, CT15) (Figs. 2 A and B). Naive animals trained on the second day of DD displayed the same basal response times as naive animals trained on the third day of DD for both total response time (Fig. 2A₁; $t = 0.552$; $p > 0.5$) and total mouth time (Fig. 2A₂; $t = 0.755$; $p > 0.46$). Furthermore, the basal response times of naive animals at CT15 on the third day of DD were similar to basal response times during training observed on the second day of DD for total response time (Fig. 2B₁; $t = 1.36$; $p > 0.2$) and total mouth time (Fig. 2B₂; $t = 1.85$; $p > 0.09$). Therefore, extended time in DD by itself was not responsible for increasing responses of LFI-trained eyeless animals at CT15 or decreasing responses of trained eyeless animals at CT3. These results establish that the rhythms in the modulation of memory formation we observed (Figs. 1A₁ and A₂) were not due to factors associated with length of time in DD or extended time without food but were due to differences associated with training and testing the animals at different circadian times.

The Extraocular Circadian Oscillator Modulating Long-Term Memory Formation for LFI Can Be Entrained Via Extraocular Photoreceptors

The results presented so far indicate that the ocular circadian clock was not necessary for the circadian modulation of long-term memory formation of LFI; however, ocular photoreceptors potentially could entrain extraocular oscillators. The ocular photoreceptors have processes within the eye and send processes via the optic nerve into the CNS of *Aplysia* (Olson and Jacklet, 1985). Alternatively, *Aplysia* have extraocular photoreceptors that may be involved in entrainment of the extraocular circadian system (Eskin, 1971; Block and Smith, 1972; Lukowiak and Jacklet, 1972).

To investigate whether the eyes are necessary to entrain the extraocular circadian oscillator involved in modulating the circadian rhythm of long-term memory formation, both intact and eyeless *Aplysia* were reentrained to reversed LD cycles for 7 days. Animals were then trained for LFI on the second day of DD and tested 24 h later (Figs. 3 A and B). Both eyeless and intact *Aplysia* showed robust long-term memory formation during the shifted subjective day (CT3, CT9) and no long-term memory formation when animals were trained and tested during the shifted subjective night (CT15, CT21). Phase-shifted eyeless *Aplysia* showed a significant rhythm in the changes in total response time (Fig. 3A₁; $F_{3, 27} = 10.02$; $p < 0.001$) as well as changes in total time the food remained in the mouth (Fig. 3A₂; $F_{3, 27} = 4.53$; $p < 0.05$). Phase-shifted intact *Aplysia* also showed strong circadian modulation in long-term memory formation, as seen by changes in total response time (Fig. 3B₁; $t = 6.238$; $p < 0.005$) and changes in total time the food was retained in the mouth (Fig. 3B₂; $t = 2.5$; $p < 0.05$). The eyeless animals appeared to reentrain as completely as intact animals since ratios of the responses at CT3/CT15 were similar between intact and eyeless animals, and the variances in the amount of long-term memory of intact and eyeless animals were similar at CT3 and CT15. These results strongly indicate that eyeless animals were entrained to the reversed LD cycle and demonstrate that the extraocular circadian oscillator involved in the circadian modulation of long-term memory formation can receive photic input for entrainment from extraocular photoreceptors.

Persistence of the Circadian Rhythm in Long-Term Sensitization in the Absence of the Ocular Circadian Oscillator

Our results (Fig. 1) suggest that the ocular circadian clock is not the major oscillator involved in the modulation of long-term memory formation of an associative form of learning, LFI. However, this does not preclude the ocular circadian clock from a role in the circadian modulation of other forms of long-term memory formation such as LTS of the siphon withdrawal reflex, a nonassociative form of learning. In LTS, a noxious stimulus presented to 1 side of the animal leads to an increase in reflex withdrawal of the siphon and tail when the stimulated side of the animal is tested (Byrne, 1987; Cleary et al., 1991; Fernandez et al., 2003). Memory formation by LFI and LTS involves 2 separate neuronal circuits that are not only separated spatially but differ in the type of neurons involved with respect to the nature of neurotransmitters (Cleary et al., 1998; Elliott and Susswein, 2002; Bristol et al., 2004; Cropper et al., 2004). Thus, we compared the circadian role of the ocular clock on LTS with its role in LFI.

Aplysia exhibit diurnal and circadian modulation of memory formation of LTS, with significant LTS occurring when the animals are trained and tested during the subjective day and less LTS occurring when the animals are trained and tested during the subjective night (Fernandez et al., 2003). Eyeless *Aplysia* trained for LTS exhibited a diurnal rhythm with significantly greater LTS when the animals were trained and tested during the day as compared to animals trained and tested during the night (Fig. 4A₁; $t = 2.811$; $p < 0.05$). Long-term sensitization was unilateral, with no sensitization observed on the control side of the animal (Fig. 4A₂; $t = 0.228$; $p > 0.82$). Pretraining baseline responses for siphon withdrawal (siphon withdrawal duration at ZT9: 4.75 ± 0.29 sec, ZT21: 3.74 ± 0.55 sec; $t = 1.75$; $p > 0.08$) and the threshold to elicit siphon withdrawal (mean threshold at ZT9: 2.53 ± 0.219 mA, ZT21: 1.91 ± 0.22 mA; $t = 1.92$; $p > 0.07$) were not rhythmic, suggesting that the rhythm in LTS was not due to light-regulated expression of the behavior.

Since the rhythm in LTS observed in LD conditions could be due to an indirect effect of light not regulated by the circadian clock, experiments were performed in constant conditions. When eyeless *Aplysia* were trained in constant darkness on the second day of DD for LTS, a large difference in memory formation for LTS was observed when animals were trained during the subjective day compared to the subjective night. Significantly greater LTS occurred when animals were trained and tested during the subjective day at CT9 (~3-fold difference) compared to animals trained and tested during the subjective night at CT21 (Fig. 4B₁; $t = 4.47$; $p < 0.005$). As previously observed for intact animals in DD (Fernandez et al., 2003), the absence of significant differences between the mean siphon withdrawal thresholds at CT9 (2.55 ± 0.7 mA) and CT21 (2.59 ± 0.41 mA) ($t = 0.05$; $p > 0.96$) and the absence of a rhythm in the mean pretraining baseline siphon withdrawal durations between CT9 (3.5 ± 0.12 sec) and CT21 (3.75 ± 0.23 sec) ($t = 0.931$; $p > 0.355$) suggest that the circadian rhythm of LTS in eyeless *Aplysia* was due to a circadian modulation of long-term memory formation rather than a circadian regulation of the baseline behavior of siphon withdrawal. The diurnal and circadian rhythms of LTS in eyeless animals appear similar to the circadian rhythms of LTS observed in intact animals, indicating that long-term memory formation for LTS is modulated by an extraocular circadian oscillator. It remains to be established whether an extraocular photoreceptor is involved in entrainment of the rhythm of LTS as it is for LFI.

DISCUSSION

A. californica have a circadian oscillator with an entrainment pathway located in the eyes (Jacklet, 1969b; Eskin, 1971). Yet the functional role of the eyes as a circadian pacemaker in *A. californica* has not been well established. The existing evidence suggests that the circadian clock in the eyes plays a major role in the circadian modulation of locomotor activity in

Aplysia (Strumwasser, 1973; Lickey et al., 1977; Lickey et al., 1983). However, extraocular circadian oscillators also appear to contribute to the modulation of locomotor activity in *Aplysia* (Block and Lickey, 1973; Strumwasser, 1973; Lickey et al., 1977). Given the robust rhythms in the eye and the widespread projections of outputs from the eye, we investigated whether the eyes acted as the circadian pacemaker for the circadian modulation of long-term memory formation.

Long-term memory formation for 2 very different types of learning, LFI and LTS, occurred during the subjective day but not the subjective night in eyeless *Aplysia* (Figs. 1 and 4). Rhythms of memory formation in eyeless animals were very similar to the rhythms of modulation in intact *Aplysia*. Therefore, circadian oscillators exist outside of the eyes and are sufficient for the circadian modulation of long-term memory formation of LFI and LTS.

The eyes also were not necessary for the entrainment of the circadian rhythm of long-term memory formation of LFI (Fig. 3). Memory formation for LFI in eyeless *Aplysia* that were reentrained to a reversed LD cycle occurred during the subjective day of the shifted LD cycles. Therefore, both extraocular photoreceptors and extraocular circadian oscillators appear to be mainly responsible for the circadian modulation of long-term memory formation in *Aplysia*. In fact, our results suggest that the ocular oscillators may contribute little if at all to the modulation of long-term memory formation. These results do not rule out the possibility that normally, the eyes may contribute a small amount to the entrainment or free running of the rhythm of modulation of memory formation. For example, the phase of the entrained rhythm of memory formation, the rate of entrainment, or the free-running period could all be modified to some degree by the presence of the ocular oscillator.

Given that the ocular oscillator is not responsible for the circadian modulation of long-term memory formation, a reasonable location for an extraocular circadian oscillator(s) modulating memory formation of LFI and LTS is the cerebral ganglion. The location and function of neurons in the cerebral ganglion suggests a broad role in the regulation of a wide spectrum of behaviors, including LTS and LFI. The processes of facilitatory neurons in the cerebral ganglion spread to distant parts of the CNS, such as the buccal, pleural, pedal, and abdominal ganglia, and are part of various neuronal circuits (Weiss et al., 1978; Rosen et al., 1991; Wright et al., 1995). For example, the serotonergic CB1 and MCC neurons in the cerebral ganglion are major modulatory neurons that have been shown to facilitate motor processes such as those involved in the siphon withdrawal reflex and *Aplysia* feeding behavior (Rosen et al., 1989; Rosen et al., 1991; Wright et al., 1995; Marinesco et al., 2004). Furthermore, the cerebral ganglion represents an anatomical convergence point of light input pathways from photoreceptors located in the eyes, rhinophores (Eskin, 1971), and anterior tentacles. In addition, cells of the cerebral ganglion (Block and Smith, 1972) and of the body wall of the mantle have also been shown to be photosensitive (Lukowiak and Jacklet, 1972). These photoreceptors could provide photic inputs for entrainment of extraocular oscillators. The presence of modulatory neurons in the cerebral ganglion that can facilitate various behaviors, as well as the presence of light input pathways that converge from multiple sources into the cerebral ganglion, makes it a good candidate for the presence of a circadian oscillator regulating various aspects of the behavior of *Aplysia*.

As a result of these studies, it is clear that the circadian system has a major impact on memory formation through the action of extraocular circadian oscillators and extraocular photoreceptors in *Aplysia*. In our experiments, it was not possible to monitor locomotor activity rhythms in conjunction with the studies on learning and memory, so it remains unknown whether the extraocular oscillators that modulate memory formation also modulate locomotor activity. It would be extremely interesting to determine the contribution of individual circadian oscillators by monitoring multiple circadian outputs. We do not know whether the same or

different extraocular circadian oscillators are responsible for the modulation of long-term memory for LFI and LTS. Since the neuronal circuits for LFI and LTS are quite different from each other, we might expect an extraocular circadian oscillator to modulate memory formation through humoral factors or that circadian modulation of memory formation of different behaviors occurs through different extraocular circadian oscillators. Determining the mechanism through which the circadian system regulates events involved in long-term memory formation and the specific circadian oscillators involved in modulation of memory in *Aplysia* will help in understanding how the circadian system regulates multiple outputs. Such studies in *Aplysia* will be aided by the considerable knowledge available on the neuronal and molecular circuits responsible for LTS.

ACKNOWLEDGMENTS

We thank Ms. Marta Nunez-Regeiro, Mr. Raymond Fernandez, and Ms. Charity Green for technical assistance and Dr. Omar Khabour for helpful discussions and comments. This work was supported by National Institute of Neurological Disorders and Stroke Grant NS050589.

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Figure 1.

Circadian modulation of long-term memory formation of learning that a food is inedible (LFI) in eyeless *Aplysia*. Eyeless *Aplysia californica* were placed in constant darkness for 2 days; trained for LFI by exposing the animals to netted food at CT3, CT9, CT15, or CT21 ($n = 7, 8, 6, \text{ and } 8$, respectively); and tested 24 h later. Long-term memory formation was measured as a decrease in both total response time and the cumulative time the netted food remained in the mouth. Gray bars represent experiments done in the subjective day (projected time in DD based on the previous LD cycle), while filled black bars represent experiments done during the subjective night. Error bars are SEM. (A₁) Twenty-four hours after training, eyeless *Aplysia* displayed a significant decrease in total response time during the subjective day, while no change in total response time occurred during the subjective night ($F_{3,25} = 9.077$; $p < 0.001$; Newmann-Keuls post hoc analysis $p < 0.01$ for CT3 v. CT15, CT3 v. CT21, CT9 v. CT15, and CT9 v. CT21). (A₂) Twenty-four hours after training, eyeless *Aplysia* showed a significant change also in the total time netted food remained in the mouth during the subjective day, while no change in total time that netted food remained in the animals' mouths occurred during the subjective night ($F_{3,25} = 6.984$; $p < 0.005$; Newmann-Keuls post hoc analysis $p < 0.01$ for CT3 v. CT15 and CT9 v. CT15, $p < 0.05$ for CT3 v. CT21 and CT9 v. CT21). (B₁) Eyeless *Aplysia* displayed no circadian rhythm in baseline measurements of total response time on the 2nd day of DD ($F_{3,25} = 0.241$; $p > 0.86$). (B₂) In addition, eyeless *Aplysia* displayed no circadian

rhythm in total time that netted food remained in the mouth on the second day of DD ($F_{3, 25} = 1.67; p > 0.199$).

Figure 2.

Extended time in DD was not responsible for the modulation of learning that a food is inedible (LFI) in eyeless *Aplysia*. (A₁) Eyeless *Aplysia* showed no significant difference between total response times of naive animals trained on the 2nd and 3rd days of DD ($n = 7, 6$, respectively) at CT3 ($t = 0.552$; $p > 0.5$). (A₂) Eyeless animals also did not demonstrate a significant difference between the total time netted food remained in the animals' mouths when trained on the 2nd or 3rd day of DD ($n = 7, 6$, respectively) at CT3 ($t = 0.755$; $p > 0.46$). (B₁) Eyeless *Aplysia* showed no significant difference between total response times of animals trained at CT15 on the 2nd or 3rd day of DD ($n = 6, 5$, respectively) ($t = 1.36$; $p > 0.2$). (B₂) Eyeless animals also did not demonstrate a significant difference in total time netted food remained in

the animals' mouths when trained on the 2nd or 3rd day of DD ($n = 6, 5$, respectively) at CT15 ($t = 1.85$; $p > 0.09$).

Figure 3.

Extraocular photoreceptors can entrain the extraocular circadian oscillators. *Aplysia californica* entrained to 12:12-h LD cycles had their eyes removed and were reentrained to a reversed LD cycle for 7 days. Eyeless *Aplysia* were trained that a food was inedible on the 2nd day of DD at CT3, CT9, CT15, and CT21 ($n = 7, 8, 8, \text{ and } 8$, respectively) and tested 24 h later. Gray bars represent subjective day (projected time in DD based on the reversed LD cycle), while black bars represent subjective night. (A₁) Phase-shifted eyeless *Aplysia* displayed a significant decrease in total response time 24 h after training during the subjective day, while no significant decrease in total response time was observed during the subjective night ($F_{3, 27} = 10.02$; $p < 0.01$; Newmann-Keuls post hoc analysis $p < 0.01$ for CT3 v. CT15 and

CT3 v. CT21, $p < 0.001$ for CT9 v. CT15 and $p < 0.01$ for CT9 v. CT21). (A₂) Phase-shifted eyeless *Aplysia* displayed a significant decrease in total time netted food remained in the animals' mouths 24 h after training during the subjective day, while no significant decrease in total mouth time was observed during the subjective night ($F_{3, 27} = 4.53$; $p < 0.05$; Newmann-Keuls post hoc analysis $p < 0.05$ for CT3 v. CT15 and CT3 v. CT21, CT9 v. CT15, and CT9 v. CT21). (B₁) Phase-shifted intact *Aplysia* demonstrated a significant decrease in total response time 24 h after training during the subjective day at CT3 ($n = 6$), while no significant decrease in total response time occurred during the subjective night at CT15 ($n = 5$; $t = 6.238$; $p < 0.005$). (B₂) Phase-shifted intact *Aplysia* showed a significant decrease in total time the netted food remained in the mouth when trained during the subjective day (CT3; $n = 6$) but not a decrease when trained during the subjective night ($n = 5$; $t = 2.5$; $p < 0.05$).

Figure 4.

Diurnal and circadian modulation of long-term sensitization in eyeless *Aplysia*. (A₁) Eyeless animals were trained for longterm sensitization (LTS) at ZT9 during the day ($n = 4$) or ZT21 during the night ($n = 3$). Eyeless animals trained during the day showed significantly increased LTS compared with eyeless animals trained during the night 24 h after training ($t = 2.811$; $p < 0.05$). White bars represent measurements done in the light; black bars represent experiments in the dark. Error bars are SEM. (A₂) LTS was unilateral as no sensitization occurred on the unstimulated side ($t = 0.228$; $p > 0.82$). (B₁) Eyeless *Aplysia* trained for LTS during the subjective day of DD at CT9 ($n = 4$) or CT21 ($n = 6$) and then tested 24 h later expressed significantly increased LTS at CT9 compared to CT21 ($t = 4.47$; $p < 0.005$). Gray bars represent

experiments in the subjective day. Black-filled bars represent experiments in the subjective night. (B₂) No LTS was observed when the unstimulated control side was tested ($t = 1.737$; $p > 0.12$).