Critical role of the circadian clock in memory formation: lessons from *Aplysia*

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Unraveling the complexities of learning and the formation of memory requires identification of the cellular and molecular processes through which neural plasticity arises as well as recognition of the conditions or factors through which those processes are modulated. With its relatively simple nervous system, the marine mollusk *Aplysia californica* has proven an outstanding model system for studies of memory formation and identification of the molecular mechanisms underlying learned behaviors, including classical and operant associative learning paradigms and non-associative behaviors. *In vivo* behavioral studies in *Aplysia* have significantly furthered our understanding of how the endogenous circadian clock modulates memory formation. Sensitization of the tail-siphon withdrawal reflex represents a defensive non-associative learned behavior for which the circadian clock strongly modulates intermediate and long-term memory formation. Likewise, *Aplysia* exhibit circadian rhythms in long-term memory, but not short-term memory, for an operant associative learning paradigm. This review focuses on circadian modulation of intermediate and long-term memory and the putative mechanisms through which this modulation occurs. Additionally, potential functions and the adaptive advantages of time of day pressure on memory formation are considered. The influence of the circadian clock on learning and memory crosses distant phylogeny highlighting the evolutionary importance of the circadian clock on metabolic, physiological, and behavioral processes. Thus, studies in a simple invertebrate model system have and will continue to provide critical mechanistic insights to complementary processes in higher organisms.

Keywords: learning and memory, circadian rhythms, *Aplysia*, sensitization, operant learning

APLYSIA AS A MODEL FOR CIRCADIAN RESEARCH

Although less familiar to current students of circadian biology, *Aplysia californica* represents one of the earliest models used to systematically study the circadian clock. Members of the genus *Aplysia*, frequently referred to as sea hares due to the appearance of the upward projecting rhinophores, are marine mollusks from the family Aplysiidae (class: Gastropoda, order: Opisthobranchia). While the fossil record for *Aplysia* species is limited, the genus *Akera*, a sister taxon to Aplysiidae representing the primitive sea hare, appears in the fossil record as early as 165 mya (reviewed in Medina and Walsh, 2000; Medina et al., 2001). *Aplysia* species likely diverged considerably later, perhaps as recently as 25 mya during the Miocene period (reviewed in Medina and Walsh, 2000; Medina et al., 2001). *Aplysia* are found throughout the world generally in warm waters with the diurnal *A. californica* (northeast Pacific), nocturnal *A. fasciata* (Mediterranean) and *A. kurodai* (northwestern Pacific), commonly used in neuroscience. *Aplysia* are hermaphroditic animals, although not self-fertilizing, that budget their time for reproductive, feeding, and exploratory activities (Susswein et al., 1983; Carefoot, 1989; Ziv et al., 1991a,b). Feeding on algae, *Aplysia* inhabit the photic zone, primarily the intertidal and sub-littoral zones commonly at depths less than 20 m (Kandel, 1979), and are influenced by daily light–dark cycles. *Aplysia* exhibit robust circadian rhythms in locomotor activity

(Strumwasser, 1973) and feeding behavior (Kupfermann, 1974; Levenson et al., 1999).

Groundbreaking research determined that the isolated *Aplysia* eye (<1 mm in size) contained all necessary components of a circadian system: entrainment, oscillator, and outputs (Jacklet, 1969; Eskin, 1971). Isolated eyes demonstrate free-running circadian rhythms in optic nerve impulses that can be entrained and phase-shifted (Jacklet, 1969; Eskin, 1971). Ocular circadian rhythms can also be recorded *in vivo* (Block, 1981). Numerous studies in *Aplysia* outlined the necessity of transcription (Raju et al., 1991; Koumenis et al., 1996) and translation (Rothman and Strumwasser, 1976, 1977; Jacklet, 1977; Lotshaw and Jacklet, 1986; Yeung and Eskin, 1987) for circadian oscillator function. Early studies using the isolated eye system identified second messenger signaling and the effectiveness of neurotransmitters in phase-shifting the oscillator (Corrent and Eskin, 1982; Corrent et al., 1982; Eskin et al., 1982; Eskin and Takahashi, 1983; Colwell et al., 1992) as well as the necessity of protein synthesis for phase-shifts (Eskin et al., 1984; Raju et al., 1990).

Despite a half-century of circadian research in *Aplysia*, the molecular circuitry of the *Aplysia* circadian system has not been fully elucidated. *The Aplysia* central nervous system consists of about 20,000 neurons organized into discrete ganglia. Circadian oscillatory neurons localizing to the base of the eye likely serve as central pacemakers with the eyes also serving in primary

photoentrainment. Ocular oscillators send afferent fibers via the optic nerve to central nervous system ganglia excluding the buccal ganglia (Herman and Strumwasser, 1984; Olson and Jacklet, 1985). Extraocular photoreceptors present in rhinophores and anterior tentacles can affect circadian locomotor activity (Block and Lickey, 1973; Roberts and Block, 1982). The cerebral ganglion also contains photoreceptors and may serve as a point of convergence for photic information (Eskin, 1971; Block and Smith, 1973; Roberts and Block, 1982). As the molecular components of the oscillator remain unknown, potentially peripheral circadian oscillators contain light sensitive molecules that function in entrainment as observed in *Drosophila* and zebrafish (Plautz et al., 1997; Whitmore et al., 2000).

In the intact animal, ocular circadian oscillatory neurons strongly influence circadian locomotor activity with removal of the eyes resulting in arrhythmicity in most animals (Strumwasser, 1973; Lickey et al., 1976, 1977, 1983). However, the eyes are not strictly necessary for circadian activity as some eyeless animals continue to demonstrate rhythmic locomotor activity (Block and Lickey, 1973; Strumwasser, 1973; Lickey et al., 1977) suggesting the existence of extraocular pacemakers in addition to extraocular photoreceptors. Little progress has been made in identifying pacemakers in central nervous system ganglia. Although the abdominal ganglion is unnecessary for circadian activity rhythms (Strumwasser et al., 1972), early research described abdominal neuron R15 as a candidate for a circadian pacemaker (Strumwasser, 1965) entrained by light–dark cycles (Lickey, 1969) with diurnal and circadian rhythms in spiking activity (Audesirk and Strumwasser, 1975; Woodson and Schlapfer, 1979). However, sustained firing rhythms appear absent under prolonged constant conditions (Lickey et al., 1976). The cerebral ganglion appears a likely location for central nervous system circadian pacemaker neurons with its integration of multiple photic inputs and numerous connections to other ganglia (Weiss et al., 1978; Rosen et al., 1991; Wright et al., 1995). Moreover, cerebral ganglion neurons send efferent connections to ocular pacemaker neurons (Olson and Jacklet, 1985; Takahashi et al., 1989) potentially providing a mechanism for central nervous system feedback.

Although the components of the core circadian oscillator in *Aplysia* remain unknown, the circadian gene *period* has been cloned in the closely related mollusk *Bulla* (Constance et al., 2002). Unusually *bPer* mRNA and protein levels were shown to cycle only in light–dark cycles in retinal pacemakers with no oscillations observed in constant conditions (Constance et al., 2002). The only identified *Aplysia* gene for which rhythmic expression has been shown is the immediate early gene *ApC/EBP*, a leucine β-Zip transcription factor, which shows significantly higher mRNA and protein levels during the night (Hattar et al., 2002; Lyons et al., 2006b). Whilst ApC/EBP appears unlikely to function in the core oscillator, circadian rhythms in protein abundance in cerebral and pleural ganglia indicate that the circadian clock impacts the central nervous system.

CIRCADIAN MODULATION OF LEARNING AND MEMORY

The circadian clock imparts a powerful adaptive advantage to organisms through the coordination of metabolic and physiological events in anticipation of regular environmental occurrences.

Aplysia represents an excellent system for investigating circadian modulation of memory given the simplicity of its neuronal organization combined with numerous *in vivo* and *in vitro* learning paradigms that have made *Aplysia* a favorite model organism for neuroscientists. The most well-studied learning paradigms concentrate on defensive withdrawal reflexes using non-associative sensitization and habituation, and classical conditioning of withdrawal reflexes (reviewed in Bailey et al., 2008). In addition to defensive reflexes, the neural circuitry of the feeding system exhibits a high degree of plasticity and is frequently studied using operant and classical conditioning paradigms (reviewed in Elliott and Susswein, 2002; Baxter and Byrne, 2006).

Initial research in *Aplysia* examining circadian modulation of memory investigated sensitization of the siphon-withdrawal reflex, non-associative learning in which application of a noxious stimulus enhances subsequent responses to a mild stimulus. In light–dark cycles and constant conditions, animals exhibited robust long-term memory when training was administered during the (subjective) day (Fernandez et al., 2003; Lyons et al., 2006b). However, when training was performed at night, little long-term sensitization was observed. Potentially, circadian regulation of memory formation occurs through gating sensory perception during initial reception or transmission of sensory stimuli. Pre-training baseline responses in the threshold stimulus necessary to elicit siphon withdrawal and withdrawal duration were not circadianly regulated suggesting that the circadian clock modulated memory rather than sensory perception. In later studies, diurnal and circadian rhythms also were observed for intermediate-term sensitization (Lyons et al., 2008), a form of memory dependent upon protein synthesis but not transcription (Sutton et al., 2001; Sutton and Carew, 2002).

Circadian modulation of memory has also been investigated using a more complex operant learning paradigm, learning that food is inedible (LFI). In this paradigm, the animal is presented with netted seaweed that cannot be swallowed to which the animal responds with repeated cycles of biting, swallowing attempts, and rejection of the netted food (Susswein and Schwarz, 1983; Schwarz and Susswein, 1986). An association is formed between the failure of swallowing attempts for feeding and the specific seaweed (Susswein et al., 1986; Michel et al., 2011a). As with sensitization, long-term LFI memory appears strongly regulated by the circadian clock. Animals that are trained during the (subjective) day demonstrate robust memory 24 h later, whereas animals trained at night display virtually no long-term memory with responses similar to naïve animals (Lyons et al., 2005, 2006a). Thus, the circadian clock strongly modulates non-associative and associative long-term memory in *Aplysia*.

For long-term sensitization and LFI memory, circadian modulation targets memory formation rather than recall. Animals trained during the day demonstrated robust memory even when tested 30–36 h later during the following night (Fernandez et al., 2003; Lyons et al., 2005). However, animals trained during the night showed little long-term memory at any time point. These results stand in contrast to hippocampal dependent contextual fear conditioning in which the strength of memory recall appears to be regulated by the circadian clock (Chaudhury and Colwell, 2002).

Where are the oscillators responsible for modulation of memory? As with locomotor activity, pacemaker neurons located in the eyes do not appear necessary for entrainment or maintenance of circadian modulation of memory in *Aplysia*. Eyeless animals exhibit circadian rhythms in long-term memory for sensitization and LFI (Lyons et al., 2006a). Furthermore, animals re-entrained after eye removal to a new light–dark cycle exhibited significant circadian rhythms in long-term memory in phase with the re-entrained cycle (Lyons et al., 2006a). These experiments support the hypothesis that central nervous system ganglia contain independent circadian oscillators that can be entrained through extraocular photoreceptors. While the neurons responsible for memory may not be oscillators themselves, mechanisms exist through which hypothetical pacemaker cells located in the cerebral ganglion could modulate the activity of neurons involved in sensitization and LFI memory circuits.

POSSIBLE MECHANISMS OF MODULATION

Learning and memory can be diagrammed linearly with sensory perception, learning, memory formation, and recall occurring sequentially. Memory formation encompasses all molecular and cellular changes necessary to maintain learned behavior (Sweatt, 2010) and can be subdivided into induction, molecular consolidation involving transcription and translation, and maintenance. For *Aplysia*, it appears that memory formation rather than sensory perception or recall is the primary process modulated by the circadian clock as time of training appears to be the determinant factor. However, short-term memory for sensitization and LFI is independent of the circadian cycle (Fernandez et al., 2003; Lyons et al., 2005) suggesting that early steps common to short, intermediate, and long-term memory may not be primary targets for circadian regulation.

What are potential mechanisms through which the circadian clock modulates memory formation? Sensitization and LFI memory involve distinct neural and molecular circuits (Cleary et al., 1998; Elliott and Susswein, 2002; Bristol et al., 2004; Cropper et al., 2004; Bailey and Kandel, 2008). During sensitization, serotonin release by cerebral ganglion facilitatory neurons initiates a sequence of events resulting in pre-synaptic facilitation of pleural sensory neurons and post-synaptic changes in pedal motor neurons (Villareal et al., 2007; Bailey et al., 2008; Cai et al., 2008). Early induction steps include amplification of adenylyl cyclase activity, increased cAMP levels and PKA activation (Kandel, 2001; Reissner et al., 2006). Sensitization training induces serotonin release into the hemolymph with increased training resulting in greater levels of serotonin (Marinesco and Carew, 2002; Marinesco et al., 2004). Serotonin release following sensitization training is circadianly regulated (Lyons et al., 2006b) providing one mechanism through which the circadian clock modulates intermediate and long-term sensitization. Although serotonin induced activation of cAMP–PKA signaling is common to short and long-term memory, potentially circadian regulation of persistent PKA activity and subsequent CREB-dependent transcription occurs for long-term memory. During long-term facilitation, an *in vitro* correlate for sensitization, persistent PKA activity and CREB activation induce necessary expression of *ApC/EBP* (Alberini et al., 1994; Muller and Carew, 1998). The circadian clock regulates training-induced

ApC/EBP expression with sensitization training during the day, but not the night, resulting in multi-fold increases in protein abundance (Lyons et al., 2006b). Persistent PKA activation also is necessary for long-term LFI memory and LFI training results in increased *ApC/EBP* mRNA and protein abundance in the buccal ganglia (Levitan et al., 2008; Michel et al., 2011a). Potentially, circadian modulation of LFI memory also occurs through regulation of the cAMP–PKA–CREB pathway.

There appear to be distinct processes in separate neuronal clusters modulated by the circadian clock during sensitization. Circadian modulation of sensitization also occurs downstream of serotonin release from facilitatory neurons. In experiments in which *in vivo* sensitization was induced using serotonin, animals exhibited long-term sensitization with a robust circadian rhythm similar in phase and amplitude to previous studies (Lyons et al., 2006b). This suggests that circadian modulation occurs either pre-synaptically or post-synaptically through a mechanism independent of rhythmic serotonin release.

The MAPK signaling cascade appears a likely target for circadian modulation in sensitization and potentially for LFI memory as MAPK also acts as an integrator of neuronal inputs. Intermediate and long-term sensitization, but not short-term memory, are dependent upon training-induced MAPK activation and prolonged MAPK signaling (Martin et al., 1997; Michael et al., 1998; Sharma et al., 2003; Sharma and Carew, 2004). Overall basal MAPK activity in pleural and cerebral ganglia does not appear to be rhythmically regulated. Following sensitization training during the (subjective) day, but not the night, levels of phospho-MAPK are greatly increased in pleural ganglia (Lyons et al., 2006b). While the circadian induction of MAPK activation has not been examined for LFI memory, a prolonged MAPK signaling phase in the buccal ganglia appears necessary for long-term, but not short-term, LFI memory (Michel et al., 2011b). The dual circadian modulation of learning-induced activation of MAPK and *ApC/EBP* expression may be sufficient to explain circadian modulation of long-term sensitization and LFI memory.

Just as the mechanisms involved in memory formation occur through a network of interconnected signaling pathways, it seems unlikely that circadian modulation of memory occurs through simple linear connections. More realistically, circadian modulation affects a network of signaling pathways resulting in dynamic regulation of behavior. Although the extent of circadian regulation of gene expression or signaling has not been investigated in *Aplysia*, genome wide expression studies in other organisms suggests that a considerable portion of the genome is under circadian regulation (reviewed in Doherty and Kay, 2010). Given the disparate anatomical and molecular circuitry for sensitization and LFI memory, humoral factors may also serve as a means for circadian regulation of multiple neural circuits.

FUNCTION OF CIRCADIAN MODULATION OF MEMORY

From an evolutionary perspective, if no function or selective advantage to circadian modulation of memory existed then one would predict considerable laxity in regulation. The observance of circadian influences on memory across phylogeny and apparent multiple levels of regulation as observed in *Aplysia* suggest specific functions for circadian modulation. Alternatively,

the synchronized phase regulation of memory could occur as a byproduct of broader circadian regulation of cellular and metabolic processes.

Given the relatively small number of neurons in the central nervous system in *Aplysia*, wide-ranging circadian regulation of transcription, translation, or kinase signaling pathways seems feasible. Potentially, regulation of memory occurs alongside functional regulation of other processes and behaviors. If cellular processes for feeding or locomotion are circadianly regulated and these same signaling pathways or neurons are involved in learning, memory formation may be coincidentally modulated. Several lines of evidence suggest that circadian modulation of memory is not a consequence of general circadian regulation, although this does not preclude functional coordination of behavior and memory through cooperative regulatory mechanisms. (1) Signaling pathways involved in memory do not appear to exhibit widespread basal circadian activity, at least for MAPK activity in whole ganglia. If circadian regulation of memory was due to coincident regulation of MAPK signaling, one would expect basal MAPK activity levels to be circadianly regulated. (2) Multiple neuron types and distinct pathways appear targeted by circadian regulation during sensitization. (3) Circadian dysfunction induces decrements in memory formation in animal models and humans (Cho et al., 2000; Cho, 2001; Ruby et al., 2008; Loh et al., 2010). Thus, circadian modulation of memory appears to represent purposeful regulation that may be phase coordinated with behavioral activities as for *A. californica*. One would expect a nocturnal *Aplysia* species to demonstrate greater memory during the night. This is indeed the case as observed for long-term LFI memory in the nocturnal *A. fasciata* (Lyons et al., 2005).

Intermediate and long-term memory exact a metabolic energy cost through requirements for persistent kinase activity, protein synthesis, and gene expression. Coordination of memory formation with an animal's activity allows for the orchestrated circadian downregulation of neuronal activity during periods of rest. Sleep has been postulated as functioning in synaptic homeostasis by reducing synaptic strength to baseline levels enabling energy savings (Tononi and Cirelli, 2006; Hanlon et al., 2011).

Sleep dependent reductions in synaptic markers, synaptic strength, synaptic branching, and gene expression were recently shown in *Drosophila* (Cirelli et al., 2005; Gilestro et al., 2009; Bushey et al., 2011). While *A. californica* sleep has not been examined, circadian interactions between regulation and the homeostatic need for rest are likely. Intermediate and long-term memory formation at night with associated energy demands would be contraindicated with the synaptic homeostasis hypothesis. However, the coordination of intermediate and long-term memory formation with behavioral activities does not preclude the animal's response to immediate crises or dire situations. Short-term memory in *Aplysia* is not circadianly regulated allowing for increased defenses in response to predators or differentiation between edible and inedible foods to meet an immediate feeding need.

FUTURE DIRECTIONS

Across phylogeny most studies of circadian modulation of memory employ negatively reinforced learning paradigms such as fear conditioning (Valentinuzzi et al., 2001; Chaudhury and Colwell, 2002), conditioned taste aversion (Manrique et al., 2004; Gomez-Serrano et al., 2009), conditioned place avoidance (Rawashdeh et al., 2007), and olfactory conditioning (Lyons and Roman, 2009). Negatively reinforced paradigms produce robust learning with a greater dynamic range in memory strength possible compared to positively reinforced paradigms allowing for increased sensitivity in quantifying modulation. Understanding the function(s) of circadian modulation of memory will require a broader perspective with additional research investigating positively reinforced learning paradigms. The detailed neurocircuitry and reductionist approaches available in *Aplysia* suggest future research will provide continuing mechanistic insight into circadian modulation of memory validating the usefulness of simple invertebrate models for understanding evolutionarily conserved system interactions.

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