8-1-2014

Genetic disruption of the core circadian clock impairs hippocampus-dependent memory

Sarah M. Wardlaw
University of Washington

Trongha X. Phan
University of Washington

Amit Saraf
University of Washington

Xuanmao Chen
University of New Hampshire, Durham, Xuanmao.Chen@unh.edu

Daniel R. Storm
University of Washington

Comments

This is an article published by Cold Spring Harbor Laboratory Press in Learning & Memory in 2014, available online:

https://dx.doi.org/10.1101/lm.035451.114

Recommended Citation

Wardlaw SM, Phan TX, Saraf A, Chen X, Storm DR. Genetic disruption of the core circadian clock impairs hippocampus-dependent memory. Learning & memory. 2014;21(8):417-23. doi: 10.1101/lm.035451.114. PubMed PMID: 25034823; PubMed Central PMCID: PMC4105720.

Research

Genetic disruption of the core circadian clock impairs hippocampus-dependent memory

Sarah M. Wardlaw,[1] Trongha X. Phan,[1,2] Amit Saraf,[1] Xuanmao Chen,[1] and Daniel R. Storm[1]

[1]*Department of Pharmacology, School of Medicine, University of Washington, Seattle, Washington 98195-7750, USA*

Perturbing the circadian system by electrolytically lesioning the suprachiasmatic nucleus (SCN) or varying the environmental light:dark schedule impairs memory, suggesting that memory depends on the circadian system. We used a genetic approach to evaluate the role of the molecular clock in memory. *Bmal1*$^{-/-}$ mice, which are arrhythmic under constant conditions, were examined for hippocampus-dependent memory, LTP at the Schaffer-collateral synapse, and signal transduction activity in the hippocampus. *Bmal1*$^{-/-}$ mice exhibit impaired contextual fear and spatial memory. Furthermore, LTP in hippocampal slices from *Bmal1*$^{-/-}$ mice is also significantly decreased relative to that from wild-type mice. Activation of $Erk_{1,2}$ MAP kinase (MAPK) during training for contextual fear memory and diurnal oscillation of MAPK activity and cAMP in the hippocampus is also lost in *Bmal1*$^{-/-}$ mice, suggesting that the memory defects are due to reduction of the memory consolidation pathway in the hippocampus. We conclude that critical signaling events in the hippocampus required for memory depend on BMAL1.

Hippocampus-dependent learning and memory is initiated by a calcium influx mediated through NMDA receptors (Morris et al. 1986; Tsien et al. 1996) and leads to the activation of several signaling pathways, including the cAMP and $Erk_{1,2}$ MAP kinase (MAPK) pathways (Adams and Sweatt 2002; Wang and Storm 2003). Activation of CaM-stimulated adenylyl cyclases and MAPK causes stimulation of CRE-mediated transcription (Impey et al. 1998; Athos et al. 2002; Sindreu et al. 2007). Perturbing any of these signaling events disrupts memory consolidation (Bourtchuladze et al. 1994; Atkins et al. 1998; Wong et al. 1999; Athos et al. 2002; Pittenger et al. 2002).

Despite the potential for memories to persist over the lifetime of an individual, the proteins that underlie strengthened synaptic connections degrade. Recurring rounds of transcription and protein synthesis may contribute to the persistence of memories (Bekinschtein et al. 2007; Eckel-Mahan et al. 2008). Specifically, Eckel-Mahan et al. (2008) showed that the cAMP/MAPK/CREB transcriptional pathway undergoes a circadian oscillation in the hippocampus that, when blocked, inhibits the maintenance of contextual fear memory in mice. The mammalian circadian system may provide the signal to coordinate returning rounds of transcription important for memory maintenance.

Previous research has established that time of day can influence learning and memory of fear conditioning under both light:dark entrained and free-running conditions (Chaudhury and Colwell 2002; Eckel-Mahan et al. 2008). Hippocampal LTP also depends on time of day under light:dark entrained conditions (Chaudhury et al. 2005). This time-of-day influence on memory and LTP suggests involvement of the circadian system. Perturbing the circadian system through environmental disruption of lighting conditions or lesioning the master circadian regulator can impair memory (Tapp and Holloway 1981; Devan et al. 2001; Lyons et al. 2006; Ruby et al. 2008; Loh et al. 2010; Phan et al. 2011).

The suprachiasmatic nucleus (SCN) is the master regulatory clock in mammals. The SCN receives light input from the environment via the retinohypothalamic pathway and synchronizes peripheral oscillators via synaptic connection or diffusible factors (Antle and Silver 2005). A molecular clock comprised of positively and negatively regulated transcriptional/translational loops drives the oscillations in central and peripheral sites with a period of about 24 h (Ko and Takahashi 2006). BMAL1 and CLOCK heterodimerize and bind to E-box elements to promote the transcription of circadian-regulated genes, including the heterodimer's negative regulators, *Period* and *Cryptochrome*. PERIOD and CRYPTOCHROME dimerize and translocate to the nucleus to inhibit the activity of the BMAL1:CLOCK heterodimer. A second loop involving the competitive binding of REV-ERBα and RORα at RORE sites in the *Bmal1* promoter regulates BMAL1 levels.

Only the *Bmal1*$^{-/-}$ mouse is completely arrhythmic in constant conditions and exhibits impaired entrainment to a light:dark cycle (Bunger et al. 2000). Other molecular clock knockout mouse strains retain some rhythmicity due to compensatory functional isoforms and present with subtle or no memory deficits (Garcia et al. 2000; Debruyne et al. 2006; Zueger et al. 2006; Van der Zee et al. 2008). *Bmal1*$^{-/-}$ mice provide an intriguing model to genetically address the role of the molecular clock in memory.

We hypothesize that the molecular clock is required for the diurnal oscillation of signaling activity in the hippocampus and hippocampus-dependent memory. Here, we report that *Bmal1*$^{-/-}$ mice have impaired contextual fear memory, defects in learning and spatial memory, impaired LTP at the Schaffer-collateral synapse, as well as no diurnal change in cAMP and MAPK activity.

[2]Present address: Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
Corresponding author: dstorm@u.washington.edu
Article is online at http://www.learnmem.org/cgi/doi/10.1101/lm.035451.114.

Results

Bmal1$^{-/-}$ mice exhibit impaired hippocampus-dependent learning and memory

Consistent with previous reports (Bunger et al. 2000), *Bmal1*$^{-/-}$ mice are arrhythmic under dark:dark (DD) conditions while *Bmal1*$^{+/+}$ littermates exhibit an expected τ close to 24 h (Fig. 1C,D). *Bmal1*$^{-/-}$ mice also exhibit difficulties entraining to a 12-h light:dark (LD) cycle (Fig. 1A,B), thus all experiments were performed under diurnal conditions.

Bmal1$^{+/+}$ and *Bmal1*$^{-/-}$ mice were trained and tested for contextual fear conditioning from ZT3 (3 h after light onset) to ZT5, the peak of MAPK activity and fear memory (Chaudhury et al. 2002; Eckel-Mahan et al. 2008), and under red light from ZT15 to ZT17, the trough of MAPK activity and fear memory. Mice explored a novel context for 2 min before receiving a 0.7-mA foot shock. *Bmal1*$^{-/-}$ mice froze significantly less than their wild-type littermates when they were tested 24 h after training ($F_{(5,45)} = 31.44$, $P < 0.0001$, one-way ANOVA, Tukey post-hoc test) (Fig. 2A). While *Bmal1*$^{-/-}$ mice did learn to associate the novel context with a foot shock ($P < 0.0001$ and $P < 0.05$, Tukey post-hoc test) (Fig. 2A), their long-term memory for contextual fear conditioning was not as strong as that of *Bmal1*$^{+/+}$ controls. *Bmal1*$^{-/-}$ mice contextual fear memory was comparable to that of *Bmal1*$^{+/+}$ controls when trained and tested during the night (Fig. 2B). Both groups freeze more 30 min and 24 h after training ($F_{(5,41)} = 17.49$, $P < 0.0001$, one-way ANOVA, Tukey post-hoc test). Thus, while *Bmal1*$^{-/-}$ mice are able to learn and remember contextual fear conditioning, the strength of their memory is comparable to *Bmal1*$^{+/+}$ freezing levels during the trough of fear memory.

In the Morris water maze, *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice were trained to navigate to a hidden platform for 8 d from ZT4 to ZT8. Throughout this training, *Bmal1*$^{-/-}$ mice consistently took longer to find the hidden platform compared to their wild-type littermates (Bonferroni post-hoc test) (Fig. 2C). Two-way ANOVA revealed a significant effect of genotype ($F_{(1,98)} = 34.25$, $P < 0.001$) and day of training ($F_{(7,98)} = 9.043$, $P < 0.001$). When the platform was removed during the probe trial, *Bmal1*$^{+/+}$ littermates spent significantly more time in the quadrant where the platform was during training compared to *Bmal1*$^{-/-}$ mice, $45.4 \pm 3.9\%$ vs. $20.3 \pm 5.0\%$ of probe trial ($F_{(3,52)} = 4.080$, $P < 0.01$, two-way ANOVA with Bonferroni post-hoc test) (Fig. 2D). Furthermore, *Bmal1*$^{+/+}$ mice crossed the platform's previous location more frequently than *Bmal1*$^{-/-}$ mice, 3.9 ± 0.8 vs. 0.8 ± 0.4 crossings ($t_{(13)} = 2.964$, $P = 0.0110$, two-tailed unpaired *t*-test).

While *Bmal1*$^{-/-}$ mice showed impairment in contextual fear conditioning and the Morris water maze, they demonstrated no deficit in the novel object recognition task. Mice were exposed to object A for 5 min, three times. Both *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice spent more time exploring novel object B 30 min after training ($F_{(3,22)} = 18.12$, $P < 0.001$, one-way ANOVA, Tukey post-hoc test) (Fig. 2E). In fact, *Bmal1*$^{-/-}$ mice even spent more time interacting with a novel object 24 h after exploring the original object ($P < 0.001$, Tukey post-hoc test) (Fig. 2E).

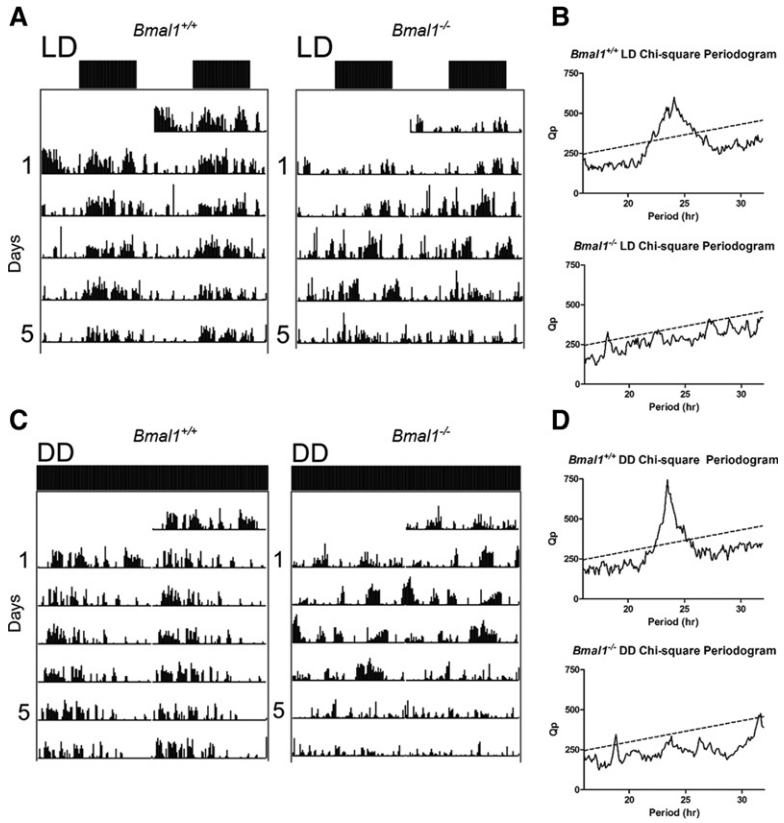

Figure 1. *Bmal1*$^{-/-}$ mice locomotor activity. Mice were monitored in 12-h LD conditions for 1 wk before exposed to DD conditions for 2 wk. Analysis of DD conditions is restricted to the second week of monitoring. (*A*) Representative *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ double-plotted actograms in 12-h LD conditions. Each line represents 48 h. (*B*) *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ χ-square periodograms. *Bmal1*$^{+/+}$ periodogram reveals $\tau = 1445$ min, or 24.1 h. *Bmal1*$^{-/-}$ periodogram does not reveal a period of 24 h. For the one peak that does cross the line of significance, $\tau = 1085$ min, or 18.1 h. (*C*) Representative *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ double-plotted actograms in DD conditions. (*D*) *Bmal1*$^{+/+}$ periodogram reveals $\tau = 1410$ min, or 23.5 h. *Bmal1*$^{-/-}$ periodogram does not reveal a period of 24 h. For the one peak that does cross the line of significance, $\tau = 1130$ min, or 18.8 h.

Bmal1$^{-/-}$ mice have impaired LTP

The Schaffer collateral efferent fibers in the stratum radiatum of hippocampal area CA1 were stimulated in the acute brain slice and the resulting fEPSPs recorded. Before inducing LTP, the input/output (I/O) curve and paired-pulse facilitation were characterized. There was no significant difference between the *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ I/O values at each stimulus (Tukey's multiple comparison test) (Fig. 3A). There was also no significant difference between the normalized slopes of the second fEPSP at each interpulse interval for paired-pulse facilitation (Tukey's multiple comparison test) (Fig. 3B). To induce LTP, three tetanic stimuli separated by 5 min were delivered to the acute slice and fEPSPs monitored for 2 h (Fig. 3C). For the first 30 min following LTP induction, the *Bmal1*$^{+/+}$ hippocampus mean fEPSP slope response was $251.50 \pm 3.43\%$ of baseline, while the *Bmal1*$^{-/-}$ hippocampus demonstrated significantly reduced LTP with a mean fEPSP slope response of $152.20 \pm 2.51\%$ of baseline ($U = 0.0$, $P < 0.0001$, $r = 1.21$, Mann–Whitney). The *Bmal1*$^{-/-}$ hippocampus continued to demonstrate a significantly reduced response ($228.7 \pm 1.2\%$ of baseline vs.

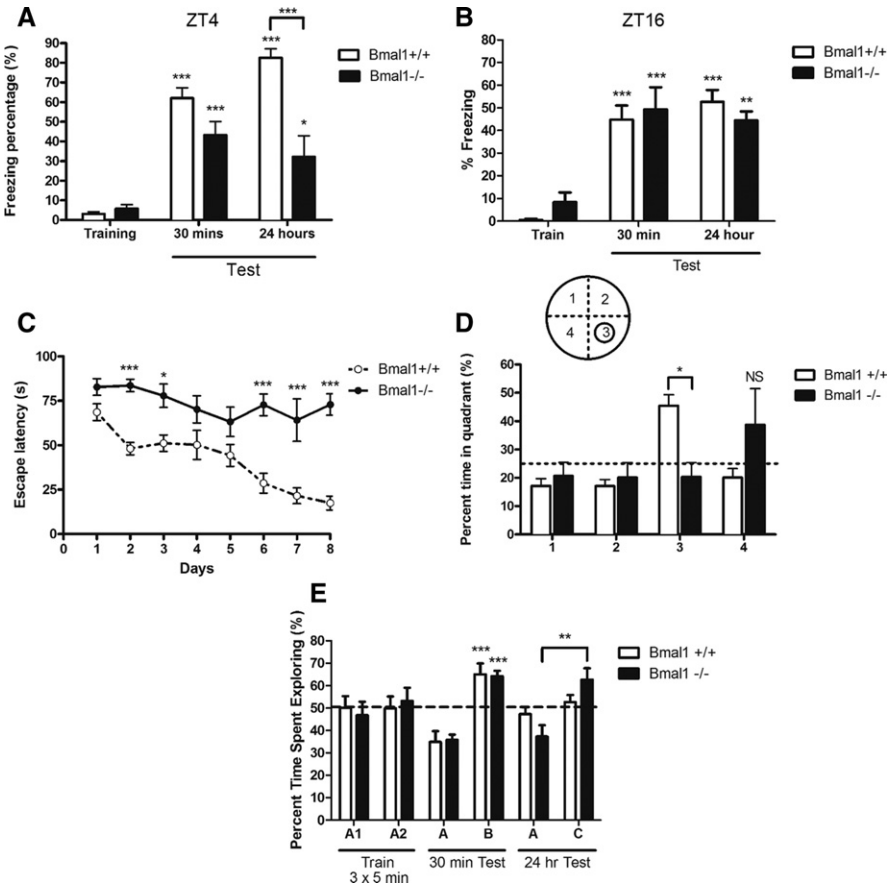

Figure 2. *Bmal1*$^{-/-}$ mice demonstrate impaired learning and memory in hippocampus-dependent tasks. (*A*) Contextual fear freezing behavior at ZT4. *Bmal1*$^{-/-}$ mice freeze significantly less 24 h ($F_{(5,45)} = 31.44$, $P < 0.0001$) after contextual fear conditioning training with a 0.7-mA foot shock. One-way ANOVA with Tukey post-hoc test. (*B*) Contextual fear freezing behavior at ZT16. Both groups demonstrate significant freezing behavior 30 min and 24 h after training ($F_{(5,41)} = 17.49$, $P < 0.0001$, one-way ANOVA, Tukey post-hoc test). (C) Morris water maze training curve. *Bmal1*$^{-/-}$ consistently took longer to find the platform for four trials of training per day over 8 d. There is a significant effect of genotype ($F_{(1,98)} = 34.25$, $P < 0.001$) and day of training ($F_{(7,98)} = 9.043$, $P < 0.001$). Two-way ANOVA with Bonferroni post-hoc test. (*D*) Morris water maze probe trial. *Bmal1*$^{+/+}$ mice spend significantly more time in quadrant 3, where the platform used to be, compared to other quadrants and *Bmal1*$^{-/-}$ mice ($F_{(3,52)} = 4.080$, $P < 0.01$). Two-way ANOVA with Bonferroni post-hoc test. (*E*) Novel object recognition. Thirty minutes after training to object A, both *Bmal1*$^{-/-}$ and *Bmal1*$^{+/+}$ mice spend significantly more time exploring novel object B ($F_{(3,22)} = 18.12$, $P < 0.001$). *Bmal1*$^{-/-}$ mice spent more time interacting with novel object C 24 h after exposure to object A ($P < 0.001$). One-way ANOVA, Tukey post-hoc test. All values represent mean ± SEM, $n = 6$–10.

133.3 ± 1.1% of baseline) 60–90 min following induction ($U = 0.0$, $P < 0.0001$, $r = 1.22$, Mann–Whitney). These results are consistent with behavioral data, suggesting a reduction in long-term synaptic plasticity.

The hippocampus of *Bmal1*$^{-/-}$ mice does not demonstrate diurnal oscillation of MAPK activity or cAMP

Previous studies have shown that wild-type mice exhibit a diurnal oscillation in MAPK activity and cAMP that is required for long-term hippocampus-dependent memory (Eckel-Mahan et al. 2008). Therefore, *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ hippocampi were collected at ZT4 and ZT16, the peak and trough of the MAPK oscillation, and analyzed for MAPK activity. Two-way ANOVA revealed significant effects of genotype ($F_{(1,26)} = 6.085$, $P < 0.05$) and time of day ($F_{(1,26)} = 6.875$) on diurnal phospho-MAPK levels. *Bmal1*$^{+/+}$ hippocampi demonstrated increased levels of phospho-MAPK at ZT4 compared to ZT16 ($P < 0.05$, Bonferroni post-hoc test) (Fig. 4B). *Bmal1*$^{-/-}$ hippocampi showed no diurnal change in phospho-MAPK at ZT4 and exhibited lower levels of phospho-MAPK at ZT4 compared to *Bmal1*$^{+/+}$ hippocampi ($P < 0.05$, Bonferroni post-hoc test) (Fig. 4B). Similarly, two-way ANOVA revealed a significant effect of genotype ($F_{(1,54)} = 21.56$, $P < 0.0001$) and time of day ($F_{(1,54)} = 9.101$, $P = 0.0039$) on diurnal cAMP levels. *Bmal1*$^{+/+}$ hippocampi demonstrated increased levels of cAMP at ZT4 compared to ZT16 ($P < 0.05$, Bonferroni post-hoc test) (Fig. 4C). *Bmal1*$^{-/-}$ hippocampi showed no diurnal change in cAMP at ZT4 and exhibited lower levels of cAMP at ZT4 compared to *Bmal1*$^{+/+}$ hippocampi ($P < 0.001$, Bonferroni post-hoc test) (Fig. 4C).

The *Bmal1*$^{-/-}$ hippocampus shows no training-induced activation of MAPK

When wild-type mice are trained for contextual fear memory, there is a measurable increase in MAPK activity in area CA1 of the hippocampus (Sindreu et al. 2007). Therefore, *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice were exposed to a novel context (ZT4 to ZT6) and either immediately received a 0.7-mA foot shock or were first allowed to explore for 2 min. *Bmal1*$^{+/+}$ mice that experienced the paired association between the novel context and foot shock showed an increase in phospho-MAPK in the hippocampus 30 min after training compared to unpaired controls which were shocked immediately (*Bmal1*$^{+/+}$ hippocampi [$t_{(16)} = 2$, $P = 0.0121$, two-tailed unpaired *t*-test]) (Fig. 5). There was no difference between *Bmal1*$^{-/-}$ paired and unpaired hippocampi ($t_{(15)} = 1.585$, $P = 0.1339$, two-tailed unpaired *t*-test) (Fig. 5), indicating that MAPK activity did not increase when *Bmal1*$^{-/-}$ mice were trained for contextual fear memory.

Discussion

Bmal1$^{-/-}$ mice offer the opportunity to investigate the relationship between a functional molecular clock and memory. We discovered that *Bmal1*$^{-/-}$ mice are impaired in hippocampus-dependent memory tasks. At 24 h after training for contextual fear conditioning, *Bmal1*$^{-/-}$ mice freeze less than wild-type littermates when trained and tested during the day. While *Bmal1*$^{-/-}$ mice do learn and exhibit fear memory for the foot shock, their memory for contextual fear is similar to that of wild-type littermates during the night. *Bmal1*$^{-/-}$ mice are also impaired in spatial learning and memory, as measured by the Morris water maze.

Consistent with these memory deficits, LTP at the Schaffer-collateral pathway is reduced in the *Bmal1*$^{-/-}$ hippocampus. The *Bmal1*$^{-/-}$ hippocampus also does not demonstrate

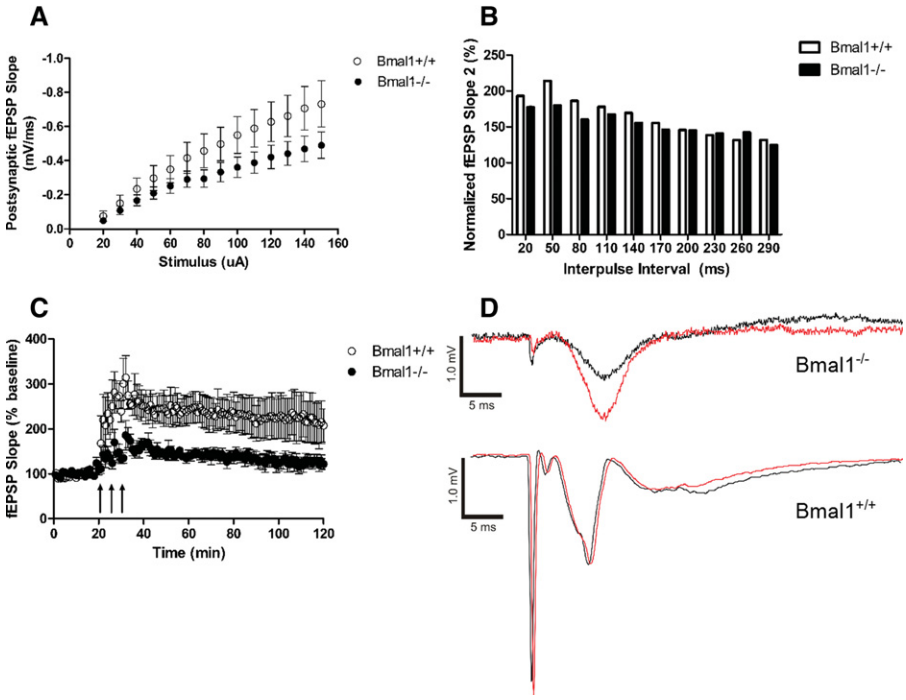

Figure 3. LTP at the Schaffer-collateral synapse is impaired in the *Bmal1*$^{-/-}$ hippocampus. (*A*) Input/output (I/O) relationship between stimulus (μA) and the slope of the postsynaptic evoked fEPSP (mV/ms). Stimulus ranged from 20 to 150 μA. There is no difference between the *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ I/O values at each stimulus. Tukey's multiple comparison test. $n = 10–12$. (*B*) Paired-pulse facilitation with interpulse interval ranging from 20 to 290 msec. There was no difference between the normalized slopes of the second fEPSP at each interpulse interval. Tukey's multiple comparison test. $n = 7–9$. (*C*) Averaged LTP response of the Schaffer-collateral fibers, represented by fEPSP slope and normalized to pretetanic baseline. To induce LTP, three tetanic stimuli (1-sec, 100-Hz train with 0.1-msec pulse length) separated by 5 min were delivered. *Bmal1*$^{-/-}$ hippocampus demonstrated significantly reduced LTP with a mean fEPSP slope response within first 30 min following induction ($U = 0.0$, $P < 0.0001$, $r = 1.21$, Mann–Whitney). The *Bmal1*$^{-/-}$ hippocampus continued to demonstrate a significantly reduced response 60–90 min following induction ($U = 0.0$, $P < 0.0001$, $r = 1.22$, Mann–Whitney). (*D*) Representative LTP traces from individual slices. Black is 5 min after tetanic stimulations. Red is 60 min after tetanic stimulations. All values represent mean ± SEM.

diurnal changes in cAMP or MAPK activity in the hippocampus, which Eckel-Mahan et al. (2008) demonstrated are necessary for the maintenance of contextual fear learning. Furthermore, the *Bmal1*$^{-/-}$ hippocampus exhibits impaired training-induced activation of MAPK, which is normally seen when wild-type mice are trained for contextual fear conditioning (Sindreu et al. 2007). The hippocampal memory deficits exhibited by *Bmal1*$^{-/-}$ mice are most likely due to deficiencies in key signaling events required for formation and maintenance of hippocampus-dependent memory, including activation of MAPK and diurnal oscillation of cAMP and MAPK activity. The dampened level of MAPK activity in the *Bmal1*$^{-/-}$ hippocampus may still be sufficient to permit learning and memory of contextual fear conditioning. Alternatively, other signaling pathways leading to the phosphorylation of CREB and CRE-mediated transcription could compensate for the reduction of MAPK activity (Johannessen et al. 2004).

We were surprised to find that *Bmal1*$^{-/-}$ mice demonstrate no deficits in novel object recognition, and even enhancement 24 h later compared to wild-type littermates. Though it is clear that the hippocampus is required for spatial memory, there exists debate as to the extent of the hippocampus' involvement for recognition memory (Clark et al. 2000; Broadbent et al. 2004; Winters et al. 2004; Good et al. 2007). Phan et al. (2011) similarly find that SCN lesioned mice demonstrate no deficit in novel object recognition, despite the impairment to both contextual fear and Morris water maze memory. Kondratova et al. (2010) report that *Bmal1*$^{-/-}$ mice exhibit altered exploratory activity, which may explain their behavior in the novel object recognition task. Specifically, *Bmal1*$^{-/-}$ mice are hyperactive when exposed to novelty, as assessed by open field activity.

Given that sleep is also strongly associated with hippocampus-dependent memory (Diekelmann and Born 2010) and *Bmal1*$^{-/-}$ mice have an impaired sleep architecture and diurnal distribution, even in LD conditions (Laposky et al. 2005), sleep disruption could contribute to impaired hippocampus-dependent function in *Bmal1*$^{-/-}$ mice. *Bmal1*$^{-/-}$ mice spend more time in REM and NREM sleep, but this increase in sleep is not concentrated during the day as it is with wildtype mice. Eckel-Mahan et al. (2008) find that the peak in MAPK signaling activity in the hippocampus occurs during the day in wild-type mice, when mice spend the most time sleeping. Luo et al. (2013) further demonstrate that the cAMP/MAPK/CREB transcriptional pathway is higher specifically during REM sleep compared to NREM and wake stages when evaluated at ZT4–ZT8 under 12-h LD conditions. Perturbing sleep can disrupt hippocampus-dependent memory, synaptic plasticity, and molecular signaling. In rats, sleep deprivation negatively effects consolidation of contextual fear conditioning, but not cued fear conditioning (Graves et al. 2003), and decreases LTP, synaptic transmission, and MAPK activation in the hippocampus (Ravassard et al. 2009). Thus, alterations in sleep may contribute to the reduction of diurnal, hippocampal cAMP/MAPK signaling activity and impairments in hippocampus-dependent memory in *Bmal1*$^{-/-}$ mice.

Our data are consistent with the observation that lesioning the SCN impairs memory persistence and inhibits the diurnal oscillation of MAPK activity and cAMP in the hippocampus (Phan et al. 2011). There is no direct synaptic link between the SCN and the hippocampus (Watts et al. 1987; Morin et al. 1994) thereby implicating indirect synaptic input and/or diffusible factors in entraining hippocampal rhythms. The SCN indirectly projects to the limbic system (including the amygdala and hippocampus) through the paraventricular nucleus, the lateral septum, and the bed nucleus of the stria terminalis (Morin et al. 1994). Potential diffusible candidates include glucocorticoids (Girotti et al. 2009; Dickmeis et al. 2013) and melatonin (Stehle et al. 2013) as they are susceptible to circadian influence from the SCN and also act on the hippocampus (Reul and de Kloet 1985; Musshoff et al. 2002). For example, melatonin is a night cue for mammals which inhibits the activity of type 1 adenylyl cyclase and compromises LTP (Wang et al. 2005). However, C57BL/6J mice, the most background in transgenic mouse strains, including the global *Bmal1*$^{-/-}$ mouse, have a natural melatonin knockdown due to a mutation in arylalkylamine-*N*-acetyltransferase (AANAT) (Roseboom et al. 1998). As C57BL/6J mice maintain circadian oscillations outside of the SCN (Stehle et al. 2002) despite dramatically

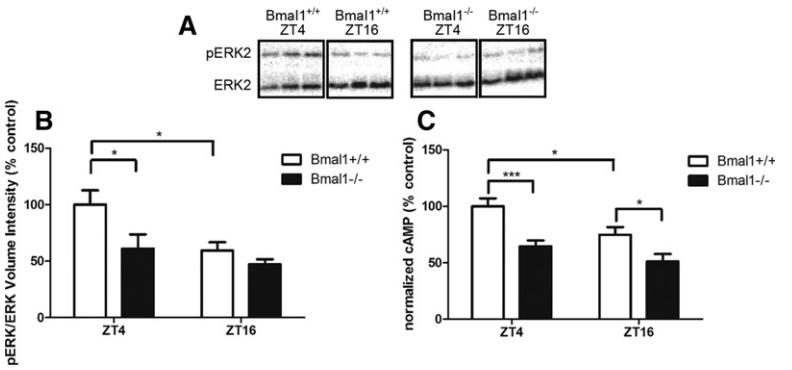

Figure 4. There is no diurnal oscillation of ERK activity or cAMP in *Bmal1*$^{-/-}$ hippocampi. (*A*) Western blot image depicts representative phosphorylated ERK and ERK intensities from isolated hippocampi of individual mice at ZT4 or ZT16. (*B*) Ratio of pERK to ERK volume intensities normalized to control *Bmal1*$^{+/+}$ ZT4 levels. There is a significant effect of genotype ($F_{(1,26)} = 6.085$, $P < 0.05$) and time of day ($F_{(1,26)} = 6.875$) on diurnal phospho-MAPK levels. For *Bmal1*$^{+/+}$ hippocampi, phospho-MAPK is higher at ZT4 compared to ZT16 ($P < 0.05$). *Bmal1*$^{-/-}$ hippocampi showed no change in phospho-MAPK activity between ZT4 and ZT16 and exhibited lower levels of phospho-MAPK at ZT4 compared to *Bmal1*$^{+/+}$ hippocampi ($P < 0.05$). Two-way ANOVA with Bonferroni post-hoc test. All values represent mean ± SEM, $n = 6–9$. (C) Ratio of cAMP concentration (pmol) to protein (mg) normalized to *Bmal1*$^{+/+}$ ZT4 group. There are significant effects of genotype ($F_{(1,54)} = 21.56$, $P < 0.0001$) and time of day ($F_{(1,54)} = 9.101$, $P = 0.0039$) on diurnal cAMP levels. *Bmal1*$^{+/+}$ hippocampi demonstrated increased levels of cAMP at ZT4 compared to ZT16 ($P < 0.05$). *Bmal1*$^{-/-}$ hippocampi showed no diurnal change in cAMP at ZT4 and exhibited lower levels of cAMP at ZT4 compared to *Bmal1*$^{+/+}$ hippocampi ($P < 0.001$). Two-way ANOVAs with Bonferroni post-hoc test. All values represent mean ± SEM, $n = 12–16$.

reduced melatonin signaling, melatonin is not essential. Similar to melatonin, glucocorticoids are not essential in entraining peripheral circadian rhythms. In adrenalectomized Per1:luciferase rats, only some peripheral tissues exhibited a phase shift of activity (Pezuk et al. 2012). The SCN likely contributes to the molecular oscillations of signaling activity in the hippocampus through redundant inputs rather than relying on a single mechanism. The connection between the SCN and signaling oscillations in the hippocampus warrants further investigation.

We find that genetically abolishing the molecular clock compromises hippocampus-dependent learning and memory tasks. This report extends our understanding of the relationship between diurnal rhythms and the hippocampus and is consistent with substantial literature implicating the circadian system in memory (Gerstner and Yin 2010).

Materials and Methods

Mice

Bmal1$^{+/-}$ breeders in the C57/Bl6 background were generously provided by Dr. Christopher Bradfield from the University of Wisconsin Medical School, Madison, WI. Mice were genotyped with PCR as previously described (Bunger et al. 2000). *Bmal1*$^{+/+}$ littermates were used as controls. All experiments were performed on adult, male mice aged 3–5 mo. Food and water were provided ab libitum and animals were maintained on a 12-h light:dark (LD) cycle, with light onset ZT0 at 8 a.m., unless otherwise specified. Experiments were in accordance with the Institutional Animal Care and Use Committee's recommendations at the University of Washington.

Activity monitoring

Mice were individually housed and their voluntary activity data were acquired using QA-4 activity input modules coupled to infrared motion detectors and the VitalView Data Acquisition System (Mini Mitter, version 4.1). Actograms and periodograms were generated with the ImageJ plug-in ActogramJ (University of Wuerzburg). Mice were monitored for 7 d in 12-h LD conditions and 14 d in DD conditions. Only the last 7 d in DD conditions were included in depicted actograms and periodogram analysis.

Contextual fear conditioning

Mice were handled for at least 5 d before all behavioral testing. For contextual fear training, mice were exposed to a 10 (W) × 10 (D) × 16 (H)-inch Plexiglas chamber with a metal shock grid (Colbourn Instruments). Between each subject, the chamber was wiped with diluted acetic acid. Mice were allowed to explore the novel context for 2 min before receiving a 0.7-mA foot shock. They were returned to their home cage after a 1-min recovery. Behavior during testing was video recorded and the percentage of freezing activity was scored by a blinded investigator. Freezing was scored as all four paws on the ground and no movement other than respiration. Mice were trained and tested from ZT3 to ZT5, during the light phase, or under red light from ZT15 to ZT17, during the night phase.

Morris water maze

Mice were placed in a 122-cm-diameter pool filled with room temperature water (22°C) made opaque with nontoxic, white, tempera paint. They were trained for 8 d, four times a day, to locate the hidden and fixed platform (circular, 5-cm diameter) using visual cues to navigate. Mice were placed in randomized locations of the pool for each trial. During training, the mice were given 90 sec to find the platform and guided to the platform when they did not find it. They sat on the platform for 30 sec before removal. Their memory was tested using a probe trial, where the platform is removed, lasting 90 sec. The probe trial was video recorded and their swim behavior was analyzed using Ethovision (Noldus). Mice were trained and tested from ZT3 to ZT8, during the light phase.

Novel object recognition

Mice were habituated to a 17 (W) × 8.5 (D) × 6 (H) cage overnight. After habituation, they explored two identical plastic objects (A) for 5 min, three times, in the habituated cage. Thirty minutes after training, they were presented with object A and a new object B for 5 min. Twenty-four hours after training, they were presented with object A and a new object C for 5 min. Objects were sprayed with ethanol in between animal subjects. Investigators video recorded each trial and later scored exploration time with each object. Exploration was defined as anytime a mouse's head was oriented toward the object and their nose was within 1 cm of the object. Time spent climbing on objects was eliminated from exploration time. Mice were trained and tested from ZT3 to ZT6, during the light phase.

Electrophysiology

Hippocampal LTP was compared between *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ male mice at ages 2–3 mo. After dissection at ZT2, brains were put in ice-cold cutting aCSF (12.5 mM NaCl, 0.25 mM KCl, 0.6 mM $MgCl_2$, 0.05 mM $CaCl_2$, 0.125 mM NaH_2PO_4, 10 mM glucose, 25 mM $NaHCO_3$) containing APV (40 μM, obtained from Tocris) and then cut into 400-μm coronal sections using a vibratome (Leica VT1000S, Leica Microsystems). Slices recovered for 2 h in recording aCSF (12.5 mM NaCl, 0.25 mM KCl, 0.1 mM $MgCl_2$, 0.25 mM $CaCl_2$, 0.125 mM NaH_2PO_4, 10 mM glucose, 25 mM $NaHCO_3$). Slices were continuously

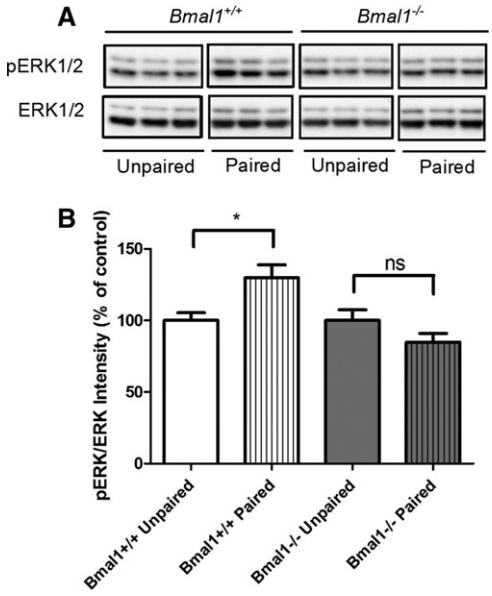

Figure 5. There is no increase in phosphorylated ERK activity in *Bmal1*$^{-/-}$ hippocampi 30 min after contextual fear conditioning. (*A*) Western blot image depicts phosphorylated ERK and ERK intensities from isolated hippocampi of individual mice 30 min after contextual fear conditioning. Unpaired animals immediately received a 0.7-mA foot shock after placement in a novel cage. Paired animals explored the novel context for 2 min before receiving a foot shock. (*B*) Ratio of pERK to ERK intensities normalized to unpaired group for each genotype. pERK is higher for *Bmal1*$^{+/+}$ paired hippocampi compared to *Bmal1*$^{+/+}$ unpaired hippocampi ($t_{(16)} = 2$, $P = 0.0121$). There is no change in pERK between the unpaired and paired *Bmal1*$^{-/-}$ hippocampi ($t_{(15)} = 1.585$, $P = 0.1339$). Two-tailed unpaired *t*-tests. All values represent mean ± SEM, $n = 8–9$.

oxygenated with a biological mix (95% oxygen, 5% carbon dioxide). After recovery, the Schaffer collateral efferent fibers in the stratum radiatum of hippocampal area CA1 were stimulated and the resulting fEPSPs recorded from ZT4 to ZT10. The slice was stimulated with the S88 Stimulator (Astro-Med Inc., Grass Instrument Division) using a concentric bipolar electrode (Frederick Haer & Co). Electric responses were amplified by the Axopatch 200B (Axon Instruments) and digitized by the Digidata 1440A (Axon CNS). Before inducing LTP, the basal field responsiveness was characterized by generating an input/output (I/O) curve. The stimulus was varied from 10 to 150 μA in 10-μA increments. Paired pulse facilitation was also measured while varying the interpulse interval from 20 msec to 310 msec in steps of 30 msec. To induce LTP, three tetanic stimuli separated by 5 min were delivered. Each stimulus consisted of a 1-sec, 100-Hz train with a pulse length of 0.1 msec. fEPSPs were monitored for 2 h following LTP induction.

Western analysis

Mice were cervically dislocated under dim, red light at ZT4 and ZT16. Both lobes of the hippocampus were dissected out and immediately frozen on liquid nitrogen. The hippocampi were homogenized in buffer containing 10 mM Tris base, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 10% glycerol, 1% NP-40, 100 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, phosphatase inhibitor I and III diluted 1:100 (Sigma), and Complete Mini protease inhibitor tablet (Roche). Homogenates were centrifuged at 14,000*g* for 10 min. The supernatant was isolated and an equal volume of Laemmli Sample Buffer (BioRad) with β-mercaptoethanol (1:20) was added. The samples were sonicated for 10 sec. Samples were boiled and run on a 12.5% Tris-HCl polyacrylamide gel (BioRad). Proteins were transferred to a PVDF membrane (Millipore) and blocked with 5% milk in a phosphate-buffered saline (PBS) solution with 0.05% Tween. Proteins were detected with rabbit polyclonal antibody to phospho-p44/42 MAPK (Thr202/Tyr204, 1:1000, Cell Signaling, RRID: AB_331775), mouse polyclonal antibody to pan-ERK (1:1000, BD Transduction Laboratories, RRID: AB_397447), and mouse antibody to actin (1:2000, Millipore, RRID:AB_94235). Blots were probed with alkaline phosphatase-conjugated goat to rabbit IgG (Sigma) and alkaline phosphatase-conjugated goat to mouse IgG (Sigma). Immunoreactivity was developed with the CDP-star western alkaline phosphatase detection system (Tropix). Blots were imaged with ChemiDoc XRS+ and analyzed with Image Lab (BioRad). pERK intensity was normalized to ERK intensity, which served as a loading control.

cAMP ELISA

The ELISA-based cAMP Biotrak Enzyme immunoassay System protocol (Amersham Biosciences) was used with some minor deviations. Hippocampal tissue was collected and homogenized as described in the Western methods but with the addition of 1 mM of the PDE inhibitor IBMX. Ice-cold ethanol (100%) was added to the homogenate. Homogenates were spun for 2 min at a speed of 1000*g* at 4°C. Resulting supernatants were evaporated in a heat block at 55°C and precipitates were resuspended in 100 μL assay buffer. Competition binding was carried out according to the Enzyme Immunoassay System instructions. Extracts were diluted 1:30 to achieve concentrations in the linear range of the assay. cAMP levels were normalized to protein concentrations.

Statistical analysis

Statistical tests were performed in GraphPad Prism. α value was set at 0.05. The specific tests used are reported with results.

Acknowledgments

This work was supported by National Institutes of Health Grants NS 20498 and MH 073601.

References

Adams JP, Sweatt JD. 2002. Molecular psychology: roles for the ERK MAP kinase cascade in memory. *Annu Rev Pharmacol Toxicol* **42:** 135–163.

Antle MC, Silver R. 2005. Orchestrating time: arrangements of the brain circadian clock. *Trends Neurosci* **28:** 145–151.

Athos J, Impey S, Pineda VV, Chen X, Storm DR. 2002. Hippocampal CRE-mediated gene expression is required for contextual memory formation. *Nat Neurosci* **5:** 1119–1120.

Atkins CM, Selcher JC, Petraits JJ, Trzaskos JM, Sweatt JD. 1998. The MAPK cascade is required for mammalian associative learning. *Nat Neurosci* **1:** 602–609.

Bekinschtein P, Cammarota M, Igaz LM, Bevilaqua LR, Izquierdo I, Medina JH. 2007. Persistence of long-term memory storage requires a late protein synthesis- and BDNF-dependent phase in the hippocampus. *Neuron* **53:** 261–277.

Bourtchuladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G, Silva AJ. 1994. Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell* **79:** 59–68.

Broadbent N, Squire L, Clark R. 2004. Spatial memory, recognition memory, and the hippocampus. *Proc Natl Acad Sci* **40:** 14515–14520.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA. 2000. Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* **103:** 1009–1017.

Chaudhury D, Colwell CS. 2002. Circadian modulation of learning and memory in fear-conditioned mice. *Behav Brain Res* **133:** 95–108.

Chaudhury D, Wang LM, Colwell CS. 2005. Circadian regulation of hippocampal long-term potentiation. *J Biol Rhythms* **20:** 225–236.

Clark R, Zola S, Squire L. 2000. Impaired recognition memory in rats after damage to the hippocampus. *J Neurosci* **20:** 8853–8860.

Debruyne JP, Noton E, Lambert CM, Maywood ES, Weaver DR, Reppert SM. 2006. A clock shock: Mouse CLOCK is not required for circadian oscillator function. *Neuron* **50:** 465–477.

Devan BD, Goad EH, Petri HL, Antoniadis EA, Hong NS, Ko CH, Leblanc L, Lebovic SS, Lo Q, Ralph MR, et al. 2001. Circadian phase-shifted rats

show normal acquisition but impaired long-term retention of place information in the water task. *Neurobiol Learn Mem* **75:** 51–62.
Dickmeis T, Weger BD, Weger M. 2013. The circadian clock and glucocorticoids—interactions across many time scales. *Mol Cell Endocrinol* **380:** 2–15.
Diekelmann S, Born J. 2010. The memory function of sleep. *Nat Rev Neurosci* **11:** 114–126.
Eckel-Mahan KL, Phan T, Han S, Wang H, Chan GC, Scheiner ZS, Storm DR. 2008. Circadian oscillation of hippocampal MAPK activity and cAmp: implications for memory persistence. *Nat Neurosci* **11:** 1074–1082.
Garcia JA, Zhang D, Estill SJ, Michnoff C, Rutter J, Reick M, Scott K, Diaz-Arrastia R, McKnight SL. 2000. Impaired cued and contextual memory in NPAS2-deficient mice. *Science* **288:** 2226–2230.
Gerstner JR, Yin JC. 2010. Circadian rhythms and memory formation. *Nat Rev Neurosci* **11:** 577–588.
Girotti M, Weinberg MS, Spencer RL. 2009. Diurnal expression of functional and clock-related genes throughout the rat HPA axis: system-wide shifts in response to a restricted feeding schedule. *Am J Physiol Endocrinol Metab* **296:** E888–E897.
Good MA, Barnes P, Staal V, McGregor A, Honey RC. 2007. Context- but not familiarity-dependent forms of object recognition are impaired following excitotoxic hippocampal lesions in rats. *Behav Neurosci* **121:** 218–223.
Graves LA, Heller EA, Pack AI, Abel T. 2003. Sleep deprivation selectively impairs memory consolidation for contextual fear conditioning. *Learn Mem* **10:** 168–176.
Impey S, Smith DM, Obrietan K, Donahue R, Wade C, Storm DR. 1998. Stimulation of cAMP response element (CRE)-mediated transcription during contextual learning. *Nat Neurosci* **1:** 595–601.
Johannessen M, Delghandi MP, Moens U. 2004. What turns CREB on? *Cell Signal* **16:** 1211–1217.
Ko CH, Takahashi JS. 2006. Molecular components of the mammalian circadian clock. *Hum Mol Genet* **15:** 271–277.
Kondratova AA, Dubrovsky YV, Antoch MP, Kondratov RV. 2010. Circadian clock proteins control adaptation to novel environment and memory formation. *Aging* **2:** 285–297.
Laposky A, Easton A, Dugovic C, Walisser J, Bradfield C, Turek F. 2005. Deletion of the mammalian circadian clock gene BMAL1/Mop3 alters baseline sleep architecture and the response to sleep deprivation. *Sleep* **28:** 395–409.
Loh DH, Navarro J, Hagopian A, Wang LM, Deboer T, Colwell CS. 2010. Rapid changes in the light/dark cycle disrupt memory of conditioned fear in mice. *PLoS One* **5:** e12546.
Luo J, Phan T, Yang Y, Garelick MG, Storm DR. 2013. Increases in cAMP, MAPK activity, and CREB phosphorylation during REM sleep: implications for REM sleep and memory consolidation. *J Neurosci* **33:** 6460–6468.
Lyons LC, Sol Collado M, Khabour O, Green CL, Eskin A. 2006. The circadian clock modulates core steps in long-term memory formation in *Aplysia*. *J Neurosci* **26:** 8662–8671.
Morin LP, Goodless-Sanchez N, Smale L, Moore RY. 1994. Projections of the suprachiasmatic nuclei, subparaventricular zone and retrochiasmatic area in the golden hamster. *Neuroscience* **61:** 391–410.
Morris RG, Anderson E, Lynch GS. 1986. Selective impairment of learning and blockade of long-term potentiation by an *N*-methyl-D-aspartate receptor antagonist, AP5. *Nature* **319:** 774–776.
Musshoff U, Riewenherm D, Berger E, Fauteck JD, Speckmann EJ. 2002. Melatonin receptors in rat hippocampus: molecular and functional investigations. *Hippocampus* **12:** 165–173.
Pezuk P, Mohawk JA, Wang LA, Menaker M. 2012. Glucocorticoids as entraining signals for peripheral circadian oscillators. *Endocrinology* **153:** 4775–4483.
Phan TX, Chan GC, Sindreu CB, Eckel-Mahan KL, Storm DR. 2011. The diurnal oscillation of MAP (mitogen-activated protein) kinase and adenylyl cyclase activities in the hippocampus depends on the suprachiasmatic nucleus. *J Neurosci* **31:** 10640–10647.
Pittenger C, Huang YY, Paletzki RF, Bourtchouladze R, Scanlin H, Vronskaya S, Kandel ER. 2002. Reversible inhibition of CREB/ATF transcription factors in region CA1 of the dorsal hippocampus disrupts hippocampus-dependent spatial memory. *Neuron* **34:** 447–462.
Ravassard P, Pachoud B, Comte J, Mejia-Perez C, Scoté-Blachon C, Gay N, Claustrat B, Touret M, Luppi P, Salin PA. 2009. Paradoxical (REM) sleep deprivation causes a large and rapidly reversible decrease in long-term potentation, synaptic transmission, glutamate receptor protein levels, and ERK/MAPK activation in dorsal hippocampus. *Sleep* **32:** 227–240.
Reul JM, de Kloet ER. 1985. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology* **117:** 2505–2511.
Roseboom PH, Aryan Namboodiri MA, Zimonjic DB, Popescu NC, Rodriguez IR, Gastel JA, Klein DC. 1998. Natural melatonin 'knockdown' in C57BL/6J mice: rare mechanism truncates serotonin *N*-acetyltransferase. *Mol Brain Res* **63:** 189–197.
Ruby NF, Hwang CE, Wessells C, Fernandez F, Zhang P, Sapolsky R, Heller HC. 2008. Hippocampal-dependent learning requires a functional circadian system. *Proc Natl Acad Sci* **105:** 15593–15598.
Sindreu CB, Scheiner ZS, Storm DR. 2007. Ca^{2+}-stimulated adenylyl cyclases regulate ERK-dependent activation of MSK1 during fear conditioning. *Neuron* **53:** 79–89.
Stehle JH, von Gall C, Korf HW. 2002. Organisation of the circadian system in melatonin-proficient CH3 and melatonin-deficient C57BL mice: a comparative investigation. *Cell Tissue Res* **309:** 173–182.
Stehle JH, von Gall C, Korf HW. 2013. Melatonin: a clock-output, a clock-input. *J Endocrinol* **15:** 383–389.
Tapp WN, Holloway FA. 1981. Phase shifting circadian rhythms produces retrograde amnesia. *Science* **211:** 1056–1058.
Tsien JZ, Huerta PT, Tonegawa S. 1996. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell* **87:** 1327–1338.
Van der Zee EA, Havekes R, Barf RP, Hut RA, Nijholt IM, Jacobs EH, Gerkema MP. 2008. Circadian time-place learning in mice depends on Cry genes. *Curr Biol* **18:** 844–848.
Wang H, Storm DR. 2003. Calmodulin-regulated adenylyl cyclases: cross-talk and plasticity in the central nervous system. *Mol Pharmacol* **63:** 463–468.
Wang LM, Suthana NA, Chaudhury D, Weaver DR, Colwell CS. 2005. Melatonin inhibits hippocampal long-term potentiation. *Eur J Neurosci* **22:** 2231–2237.
Watts AG, Swanson LW, Sanchez-Watts G. 1987. Efferent projections of the suprachiasmatic nucleus: I. Studies using anterograde transport of *Phaseolus vulgaris* leucoagglutinin in the rat. *J Comp Neurol* **258:** 204–229.
Winters BD, Forwood SE, Cowell RA, Saksida LM, Bussey TJ. 2004. Double disassociation between the effects of peri-postrhinal cortex and hippocampal lesions on tests of object recognition and spatial memory: heterogeneity of function in the temporal lobe. *J Neurosci* **24:** 5901–5908.
Wong ST, Athos J, Figueroa XA, Pineda VV, Schaefer ML, Chavkin CC, Muglia LJ, Storm DR. 1999. Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. *Neuron* **23:** 787–798.
Zueger M, Urani A, Chourbaji S, Zacher C, Lipp HP, Albrecht U, Spanagel R, Wolfer DP, Gass P. 2006. mPer1 and mPer2 mutant mice show regular spatial and contextual learning in standardized tests for hippocampus-dependent learning. *J Neural Transm* **113:** 347–356.

Received April 14, 2014; accepted in revised form June 11, 2014.

Erratum

Learning & Memory 21: 417–423 (2014)

Genetic disruption of the core circadian clock impairs hippocampus-dependent memory
Sarah M. Wardlaw, Trongha X. Phan, Amit Saraf, Xuanmao Chen, and Daniel R. Storm

In the aforementioned article, the second author's name has been corrected to Trongha X. Phan, as noted above. The PDF and full-text versions online have been updated to reflect this change as well.

Genetic disruption of the core circadian clock impairs hippocampus-dependent memory

Sarah M. Wardlaw, Trongha X. Phan, Amit Saraf, et al.

Learn. Mem. 2014, **21:**
Access the most recent version at doi:10.1101/lm.035451.114