Genetic Disruption of Circadian Rhythms Impairs Hippocampus-Dependent Memory

Sarah M. Wardlaw

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Reading Committee:

Daniel Storm, Chair

Horacio de la Iglesia

Jeansok Kim

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Abstract

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Chair of Supervisory Committee:

Daniel Storm, Professor of Pharmacology

Pharmacology

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 for memory. *Bmal1*$^{-/-}$ mice, which are arrhythmic under constant conditions, were examined for hippocampus-dependent memory, LTP at the Schaffer collateral/commisurral-CA1 synapse, and signal transduction activity in the hippocampus. *Bmal1*$^{-/-}$ mice exhibited impaired contextual fear and spatial memory. Furthermore, LTP in hippocampal slices from *Bmal1*$^{-/-}$ mice was also significantly decreased relative to wildtype 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 were also lost in *Bmal1*$^{-/-}$ mice, suggesting that the memory defects were due to loss of the memory consolidation pathway in the hippocampus. We conclude that critical signaling events in the hippocampus required for

memory depend on BMAL1, however BMAL1 is not required in area CA1 of the hippocampus.

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CHAPTER 1: INTRODUCTION

The desire to understand memory reflects a desire to understand ourselves. We know who we are in remembering what we have experienced. Given our personal investment in the question of memory, science has clarified and systematized how we think about memory. Memory can be divided into declarative, episodic or knowing what memory versus nondeclarative, procedural or knowing how memory (reviewed in Milner et al., 1998). Alternatively, memory can be categorized as associative or nonassociative (reviewed in Fanselow & Poulos, 2005). Memories develop through distinct stages. Information must first be learned or acquired. New memories are stabilized hours to days after acquiring new information through the process of consolidation. When we access or retrieve a memory, we may update and reconsolidate it. Time is an important theme in memory, as memory is the longevity of information over time. We can further classify memory as short-term, long-term or long-lasting (reviewed in McGaugh, 2000). This dissertation will consider the interaction of the circadian system, the biological timekeeping mechanism, and memory.

The Hippocampus

While there are many memory systems in the brain, the hippocampus is the most studied. H.M., the most famous patient in the study of memory (Scoville & Milner, 1957), launched the focus on the hippocampus and its role in memory. H.M. had a substantial amount of both hemispheres of his temporal lobe surgically removed, including his hippocampus, to relieve severe, debilitating seizures. While H.M. had no effect on his intelligence, or learning

procedural, motor tasks, he had difficulty forming new, episodic memories. He could attend to new information in the short-term, but as soon as his attention shifted, the information would be lost. Intriguingly, his memories pre-surgery remained intact. Patients like H.M. raised the possibility of the existence of a neuroanatomical correlate for specific types of memory.

Lesion studies in both humans and animals (Mishkin, 1978; Becker et al., 1980; Eichenbaum et al., 1986; Sutherland & McDonald, 1990; Squire & Zola-Morgan, 1991) have revealed that declarative memory requires the hippocampus formation, which includes the hippocampus, dentate gyrus, subicular cortex, entorhinal cortex, as well as the surrounding perirhinal and parahippocampal cortices. The more of the hippocampal area is compromised, the more severe the memory deficits (Zola & Squire, 2001). Investigations of the hippocampus in rodents revealed that the hippocampus is required for spatial memories. The hippocampus contains place cells that fire when a rat is in a particular location, implicating the hippocampus in encoding a spatial map (O'Keefe and Dostrovsky, 1971). Lesioning the hippocampus disrupts rats' ability to remember the location of a hidden platform in the Morris water maze (Morris et al., 1982). Furthermore, contextual fear conditioning, the associative pairing of a novel place with an emotionally significant and aversive foot shock, requires the hippocampus (Kim & Fanselow, 1992; Frankland et al., 1998).

Canonically, based on hippocampal lesion studies, the hippocampus is regarded as a temporary site for memory storage while the neocortex is regarded as the storage site for long-lasting or remote memories (Kim & Fanselow, 1992; Teng & Squire, 1999; Bontempi et al., 1999). The limited, time-dependent role of the hippocampus in accessing remote memories has also been questioned. Reversibly compromising hippocampal function with lidocaine infusion 30 days after Morris water maze training still impaired performance in rats (Broadbent et al., 2006).

Inhibiting protein synthesis locally in the hippocampus during memory retrieval suggests that the hippocampus may play an ongoing role in the reconsolidation of long-lasting memories (Debiec et al., 2002; Morris et al., 2006). A recent optogenetic study finds that the real-time inhibition of hippocampus activity interferes with remote contextual fear memory (Goshen et al., 2011). The authors suggest that the fast inhibition of neuronal activity does not give neural processes enough time to compensate for the impaired hippocampus.

The distinct laminar organization of the hippocampus has aided its extensive study (see Figure 1). Generally, information enters the dentate gyrus from cortical and subcortical structures, namely the entorhinal cortex, via the perforant pathway. Information then travels to area CA3 via mossy fibers, and finally to area CA1 via the Schaffer collateral pathway.

The laminar structure of the hippocampus facilitated the discovery of long-term potentiation (LTP) in the rabbit hippocampus (Bliss and Lomo, 1973). Bliss and Lomo's discovery changed how we think about memory. It provided evidence for Donald Hebb's speculations on synaptic plasticity in his 1949 publication *The Organization of Behavior*. Hebb proposed that memory could be explained on a cellular level by considering the strength of connections in networks of distributed cells. Bliss and Lomo demonstrated that cells in the hippocampus could hold information regarding recent excitation events for hours. Many features of LTP parallel memory, including the existence of short-term and long-term phases and signal transduction mechanisms. While treating LTP as the reduced correlate of learning and memory is controversial (reviewed in Martin et al., 2000; Lynch, 2004), LTP is a useful model of the sort of synaptic changes required for memory. Furthermore, LTP is not unique to the hippocampus; the hippocampus just happens to be where it was initially discovered.

The hippocampus is also unique in that it harbors one of the adult neurogenic niches. Both the subventricular zone (SVZ) in the lateral ventricles and the subgranular zone (SGZ) of the hippocampus maintain a population of stem cells to regenerate neurons and glia in the adult animals. The discovery of adult neurogenesis in mammals overturned a long-standing assumption that new neurons aren't added to the adult brain (reviewed in Gross, 2000; Zhao et al., 2008). Though the SVZ and SGZ are the canonical neurogenic niches in the adult brain, some researchers contend that there may be more (Gould et al., 1999a). Stress and aging decrease adult neurogenesis (Kuhn et al., 1996; Gould et al., 1998; reviewed in Gould & Tanapat 1999b). Exercise and novelty increase neurogenesis (Kempermann et al., 1998; Nilsson et al., 1999; van Praag et al., 1999). Though most of the newly generated cells don't survive in the hippocampus, there is indication that they are important for memory. In particular, animals deficient in adult neurogenesis show subtle memory deficits, including pattern separation, learning a new platform location in the Morris water maze, associating a weak shock paradigm with a fearful context, and maintaining object memory (Pan et al., 2012). A learning event appears to increase the likelihood that a neuron will survive (Leuner et al., 2004). New neurons will even be pulled into a memory trace as they're maturing (Deng et al., 2009). There is also evidence that more recent neurons are more likely to be recruited into a new memory trace (Kee et al., 2007).

Signal Transduction Involved in Memory and Long-term Potentiation

The key to making memories and LTP long-term is protein synthesis (reviewed in Davis & Squire, 1984). There's a long history of using proteins synthesis inhibitors to probe the role of de novo proteins in memory consolidation. Initial acquisition and short term retention of new

information is resistant to protein synthesis inhibitors. Signaling events translate neuronal activity at the membrane into the de novo protein transcription and translation (see Figure 2).

Hippocampus-dependent learning and memory is initiated by an increase in intracellular calcium through NMDA-receptors (Morris et al., 1986; Tsien et al., 1996a), L-type voltage gated calcium channels (Moosmang et al., 2005), and/or release from intracellular stores (reviewed in Ross et al., 2005*).* Similarly, LTP of the Schaffer collateral pathway is mediated through NMDA-receptors (Collingridge et al., 1983; Coan & Collingridge, 1987). This increase in calcium activates many second messenger signaling pathways, including the cAMP, Ca^{2+}/calmodulin kinases (CAMK) and ERK MAPK signaling pathways (reviewed in Adams & Sweatt, 2002; Wang & Storm, 2003; Wayman et al., 2008). The activation of second messenger signaling cascades culminates in CRE (cAMP responsive element)-mediated transcription (Impey at al., 1998a; Athos et al., 2002; Sindreu et al., 2007).

The influx of intracellular calcium activates adenylyl cyclases and increases the production of cAMP. cAMP activates PKA. Calcium also activates the G-coupled protein Ras through RasGEFS (guanine exchange factors), which phosphorylates Raf (reviewed in Ye and Carew, 2010). Raf activates MEK. MEK phosphorylates ERK MAPK. The ERK MAPK pathway can also be activated by cAMP through the action of the Epac protein Rap1 (De Rooij et al., 1998). The crosstalk of both of these pathways leads to the translocation of activated MAPK to the nucleus and the phosphorylation of CREB (cAMP response element binding protein) at Ser-133 (Impey et al., 1998b). After contextual fear conditioning, coactivation of PKA, ERK MAPK and MSK1 leads to the phosphorylation of CREB in the hippocampus and is dependent on the calcium-activated adenylyl cyclases, AC1 and AC8 (Sindreu et al., 2007). CREB is part of a family of transcriptional activators that bind CRE (reviewed in Silva et al., 1998). This family

also includes CREM (cAMP response element modulator) and ATF (activating transcription factor). CREB has binding sites for PKA and nuclear CaMKIV, in addition to other secondary messengers, making CREB well positioned to converge several signaling inputs. CREB is necessary but not sufficient for the activation of CRE-mediated transcription. CRE-mediated transcription also requires the co-activator CBP (CREB binding protein; Chrivia et al., 1993; Kwok et al., 1994). Genes immediately activated with CRE include c-fos, zif268, C/EBPβ and BDNF.

Perturbing any of these signaling events disrupts the consolidation of hippocampus-dependent memory (Bourtchuladze et al., 1994; Mayford et al., 1996; Atkins et al., 1998; Wong et al., 1999; Kang et al., 2001; Athos et al., 2002; Pittenger, 2002). Interfering with these signaling events also disrupts LTP in the Schaffer collateral pathway (Impey et al., 1996; English & Sweatt, 1996; Mayford et al., 1996; Kang et al., 2001).

Many of these same signal transduction pathways also contribute to neurogenesis. CREB, as a convergent transcription factor, plays an important role in neurogenesis (reviewed in Merz et al., 2011). Integration of newly born neurons into the dentate gyrus circuitry requires NMDA-receptors. cAMP/CREB signaling and activation of MAPKs fosters neuronal survival in the adult animal (Nakagawa et al., 2002; Watt et al., 2004; Miwa & Storm, 2005; Pan et al., 2012). Specifically, Pan et al. (2012) found that while ERK5, another member of the MAPK family, is essential for neuronal development, its levels decline as the brain matures until eventually its expression is specific to the SVZ and SGZ. By conditionally knocking out ERK5 in the adult animal, Pan et al. (2012) were able to generate a neurogenesis-specific knockout mouse.

Signaling events culminating in protein synthesis contribute to neuroplasticity by changing several features of the neuron, including receptor expression, neurotransmitter availability, and

dendritic spine density. However, these proteins degrade. How do memories persist in the dynamic environment of protein turnover? Reoccurring rounds of transcription and protein synthesis may contribute to the persistence of memories (Bekinsteschtein et al., 2007; Eckel-Mahan, 2008). Specifically, Eckel-Mahan et al. (2008) showed that the cAMP/MAPK/CREB transcriptional pathway undergoes a circadian oscillation in area CA1 of the hippocampus that when blocked, inhibits the maintenance of contextual fear memory in mice.

The Circadian System

Time-of-day may set a consistent cue to maintain existing memories. In fact, evidence suggests that time of day can influence hippocampus function and memory. Synaptic excitability in the dentate gyrus undergoes a circadian rhythm in monkeys and rats, peaking in the dark phase (Barnes et al., 1977). LTP of the Schaffer collaterals is stronger in the night than during the day in mice (Chaudhury et al., 2005). BDNF, contributing to synaptic growth following CRE-mediated transcription, mRNA, protein and trkB receptor undergo a circadian oscillation in the rat hippocampus (Bova et al., 1998; Berchtold et al., 1999; Pollock et al., 2001; Dolci et al., 2003). Even adult neurogenesis may regulated in a time-dependent manner (Kochman et al., 2006; Guzman-Marin et al., 2007; Bouchard-Cannon et al., 2013), though one report calls into question if this time-of-day dependence is caused by sleep (Mueller et al., 2011). Mice trained during the day for fear memory exhibit stronger memory than mice trained at night (Chaudhury et al., 2002; Eckel-Mahan et al., 2008). Rats demonstrate improved performance in the Morris water maze when they are tested during their active phase (Valentinuzzi et al., 2004). A time-of-day dependence of memory and long-term potentiation suggests involvement of the circadian system.

Many biological processes, including memory, exhibit a 24-hour period, including body temperature, metabolism, and the sleep-wake cycle. Endogenous circadian rhythms will persist or free-run in constant conditions, without any input from the environment. In mammals, these circadian rhythms are controlled by the suprachiasmatic nucleus (SCN), the master circadian regulator (see Figure 3). Ablating the SCN results in behavioral arrhythmicity (Moore & Eichler, 1972; Rusak and Zucker, 1979; Schwartz & Zimmerman, 1990; Mistlberger et al., 1996), which can be restored with transplantation (Ralph et al., 1990). The SCN operates as an integrated output of autonomous, single celled oscillators (Welsh et al., 1995). The SCN receives environmental input about lighting conditions in the external environment via glutamatergic innervation from melanopsin positive retinal ganglion cells (Berson et al., 2002). Rods and cones can compensate in the absence of malanopsin retinal ganglion cells (Drouyer et al., 2007).

Light affects activity in the SCN through similar signaling mechanisms required for memory: through CRE-mediated transcription, speaking to the ubiquity of signaling mechanisms in biology. With nighttime light stimulation, phosphorylated ERK MAPK activates CREB and leads to CRE-mediated transcription (Obrietan et al., 1998; Obrietan et al., 1999). Even the pacemaker functionality of cells in the SCN depend on cAMP signaling (O'Neill et al., 2008). Similar to hippocampus signaling, signaling in the SCN is activated by an increase in intracellular calcium, whether through NMDA-receptors, voltage-gated calcium channels or a release from calcium stores (reviewed in Golombek & Rosentein, 2010).

The SCN updates or entrains peripheral (also known as slave) oscillators in the rest of the brain and periphery about the time of day. A variety of signals other than light, including exercise, melatonin, feeding and temperature changes, can also entrain circadian rhythms. These slave oscillators are capable of autonomous oscillations, though they depend on the SCN to

remain synchronized to each other and the external environment. Cultured brain regions are capable of oscillations of *mPer1,* though these oscillations are dampened (Abe et al., 2002). Peripheral organs will even maintain some rhythmicity in PER2:luciferase in vivo after SCN lesion (Tahara et al., 2012). However, these rhythms are decreased in amplitude. Also, while the peak phase of oscillations in each organ was similar in SCN intact and SCN lesioned mice, the peripheral PER2:luciferase rhythms of the SCN lesioned group had a greater SEM.

Some peripheral oscillators and entraining cues are stronger than others. For example, feeding behavior can strongly affect peripheral oscillators like the liver. During restricted feeding, rats or mice are fed only for a narrow window during the light period. Though normally nocturnal, they will shift their activity to be more active during the day in response to food availability. This schedule will uncouple oscillation of circadian gene expression in the SCN from the liver (Damiola et al., 2000).

The SCN synchronizes peripheral oscillations through its direct GABAergic output (reviewed in Bartness et al., 2001) and the secretion of circadian-regulated diffusible factors (Silver et al., 1996; reviewed in LeSauter & Silver, 1998). Disrupting circadian system's functionality can compromise memory. Phase shifting rats, a rapid change in the light:dark cycle akin to jet lag, compromises their memory of the shock chamber after passive avoidance training (Tapp & Holloway, 1981). Phase shifting mice compromises their memory of contextual fear conditioning (Loh et al., 2010). Housing rats in a 22-hour light:dark cycle disassociates their circadian locomotor rhythms and compromises passive avoidance memory (Neto et al., 2008). Arrhythmic hamsters exhibit deficits in distinguishing novel and familiar objects throughout the light dark cycle (Ruby et al., 2008). Lesioning the SCN compromises maintenance of contextual fear memory, Morris water maze performance, and the diurnal change of hippocampal signaling

activity (Phan et al., 2011). Disrupting the circadian system also appears to affect neurogenesis in the SGZ. Exposing mice to constant light conditions will decrease cell proliferation (Fujioka et al., 2011) or phase shifting hamsters will decrease cell proliferation and neuronal survival (Gibson et al., 2010).

A functioning circadian system thus seems crucial to many functions of the hippocampus. The following section will discuss what drives oscillatory activity in the single cell.

The Molecular Clock

Endogenous circadian rhythms are set in motion by a molecular clock that consists of positively and negatively regulated transcriptional and translational loops (reviewed in Ko & Takahashi, 2006). In mammals, BMAL1 and CLOCK dimerize and bind to E-box elements to regulate the expression of many circadian-regulated genes, including their negative regulators *Period* and *Cryptochrome* (see Figure 4). PERIOD (PER) and CRYPTOCHROME (CRY) dimerize and translocate back to the nucleus to inhibit the activity of the BMAL1:CLOCK heterodimer. BMAL1 protein levels are regulated by another loop involving the competitive binding of REV-ERBα and RORα at RORE sites in the *Bmal1* promoter. The molecular clock is found in cells and capable of oscillating throughout the body, not just in the SCN.

Due to redundant isoforms of circadian clock proteins, finding a completely arrhythmic transgenic animal by knockout out a single circadian clock protein proves difficult. Mutant $Clock^{\Delta 19/\Delta 19}$ mice are arrhythmic when exposed to prolonged dark conditions (Vitaterna et al., 1994), but $Clock^{-/-}$ mice can sustain circadian rhythms (DeBruyne et al., 2006). The functional isoform NPAS2 is sufficient to compensate for the absence of CLOCK (Reick et al., 2001). While BMAL1 preferentially binds CLOCK, it will bind NPAS2 in CLOCK's absence.

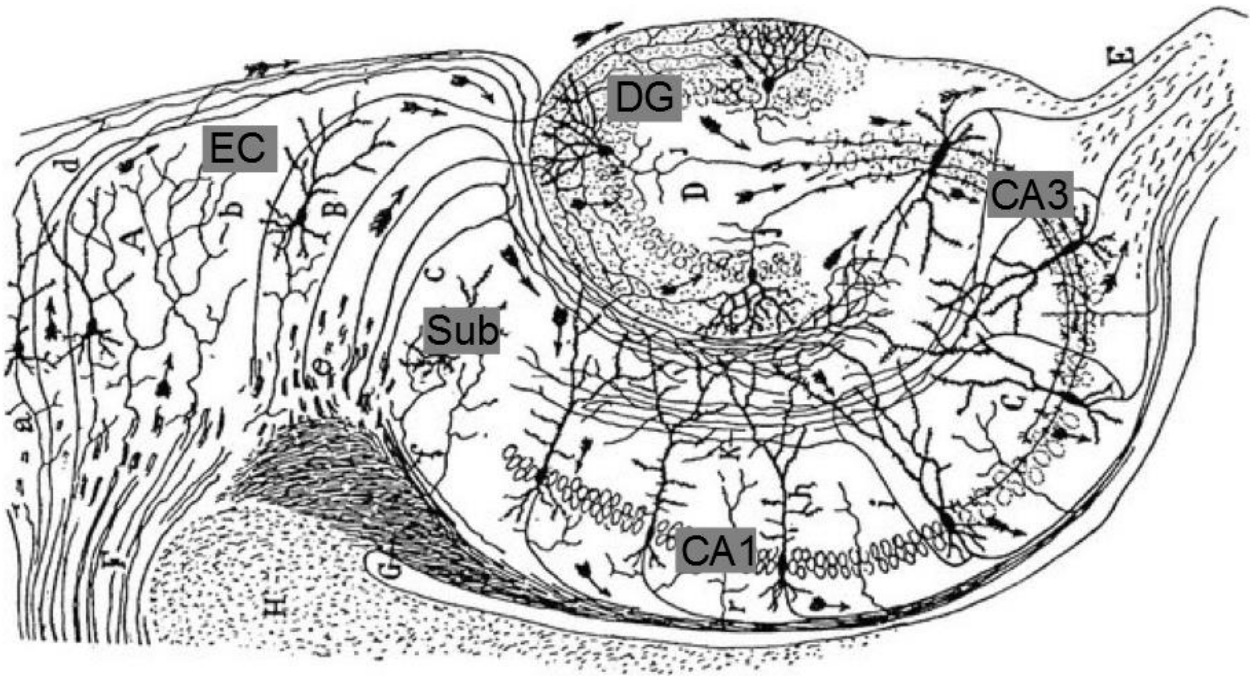
Knocking out both PER1 and PER2 or CRY1 and CRY2 will result in an arrhythmic animal, but deficits in each single knockout are more subtle. Mutant *Per1*^{Brdm1} and *Per2*^{Brdm1} mice exhibit shorter free-running periods (Zheng et al., 1999, 2001). *Cry1*$^{-/-}$ mice exhibit a shorter free running period while *Cry2*$^{-/-}$ mice exhibit a longer free running period. *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice are behavioral arrhythmic but only in constant dark conditions (van der Horst et al., 1999). *Bmal1*$^{-/-}$ mice are severely arrhythmic in constant dark conditions and exhibit difficulties entraining to a light dark cycle (Bunger et al., 2000). Knocking out BMAL1 also functionally knocks out its redundant isoform BMAL2, as the *Bmal2* promoter is regulated by the BMAL1:CLOCK heterodimer (Shi et al., 2010)

As a completely arrhythmic animal consisting of the knockout of a single circadian protein, the *Bmal1*$^{-/-}$ mouse provides an interesting opportunity to understand the interaction between the molecular clock and memory. Other transgenic, circadian clock knockout models present subtle and varied memory deficits. *NPAS2*$^{-/-}$ mice exhibit deficits 24 hours after cued and contextual fear conditioning, but not Morris water maze or passive avoidance memory paradigms (Garcia et al., 2000). Mutant *Per1*^{Brdm1} and *Per2*^{Brdm1} demonstrate no deficits in the Morris water maze or contextual fear conditioning (Zueger et al., 2006). Mutant *Per2*^{Brdm1} mice demonstrate deficits in recall of contextual fear conditioning when tested 24 hours to 7 days after training but showed no impairment in cued fear conditioning (Wang et al., 2009). Furthermore, these mutant *Per2*^{Brdm1} exhibit impaired LTP and a decrease in phosphorylated CREB protein expression in the hippocampus. Mutant *Per2*^{Brdm1} mice also show an increase in cell proliferation in the SVZ with no effect on neuronal maturation and survival (Borgs et al., 2009). *Per1*$^{-/-}$ mice exhibit deficits in the radial arm maze (Jilg et al., 2010). *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice are impaired in time-place

learning, but not in a Y maze reference task or contextual fear conditioning (Van der Zee, 2008). How does the memory of *Bmal1*$^{-/-}$ mice, such a compromised arrhythmic strain, compare?

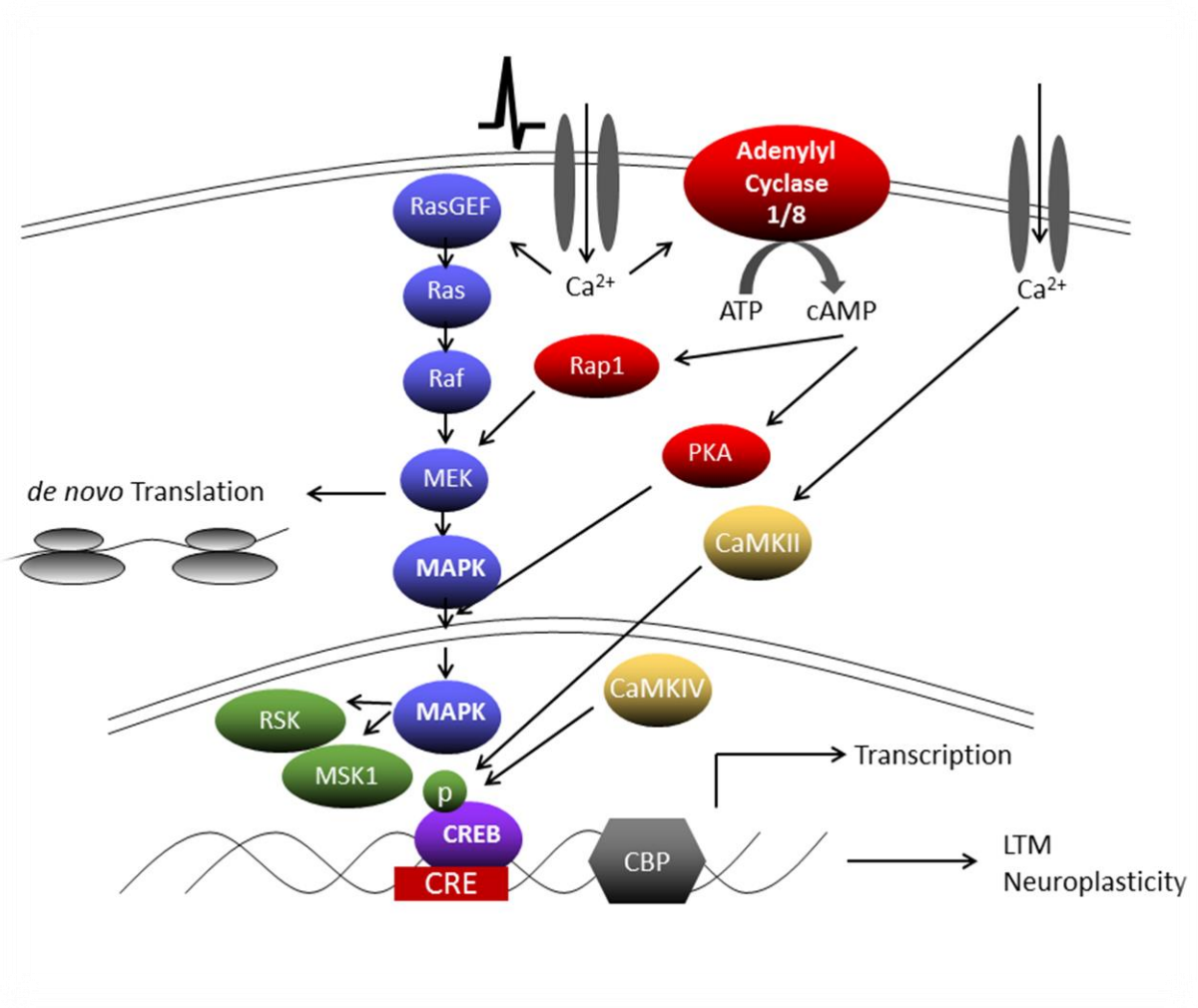
We hypothesize that the molecular clock is required for the diurnal oscillation of signaling activity in the hippocampus and hippocampus-dependent memory. Here, we report that *Bmal1*$^{-/-}$ mice have impaired contextual fear memory, defects in learning and spatial memory, impaired long-lasting LTP at the Schaffer-collateral synapse as well as no diurnal change in cAMP and MAPK activity. However, a local *Bmal1* knockdown in area CA1 in the hippocampus has no effect on contextual fear conditioning, Morris water maze performance, or the diurnal change in MAPK activity.

FIGURE 1: HIPPOCAMPUS CIRCUITRY



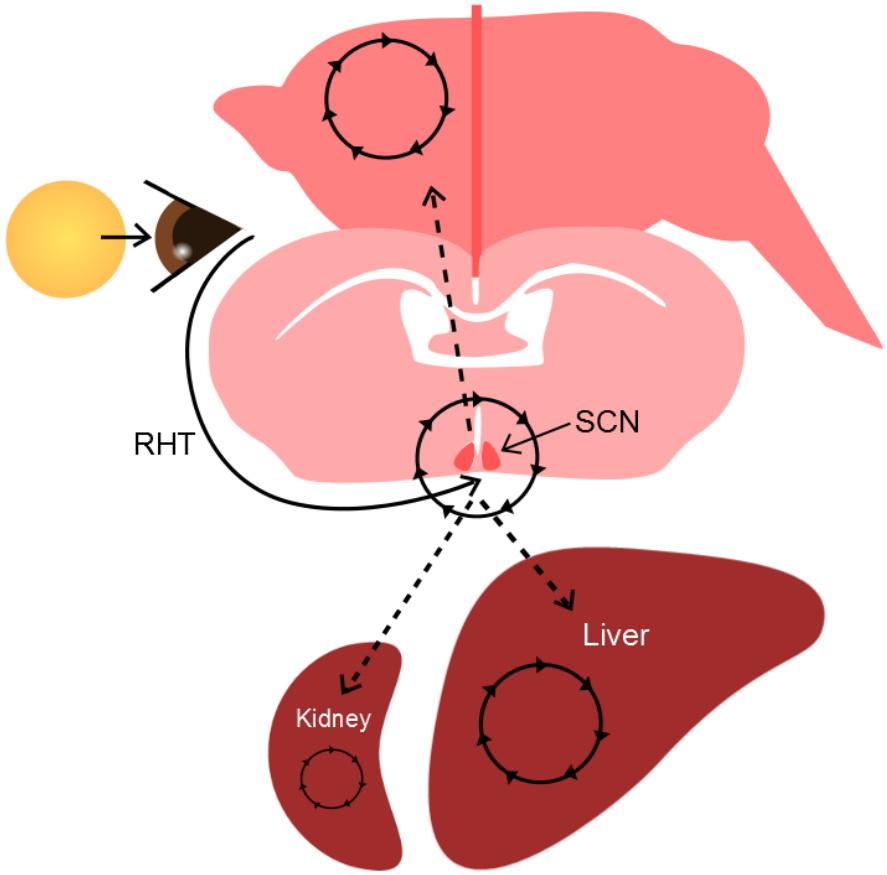
The circuitry of the hippocampus, as revealed by Golgi stain and illustrated by Ramon y Cajal. The Golgi stain readily illuminates the laminar structure of the hippocampus. Even though Ramon y Cajal did not have the means to electrically record from the hippocampus, he surmised the directive flow of information through this curled brain structure. Information largely enters the dentate gyrus (DG) from the entorhinal cortex (EC) via the perforant pathway. Mossy fibers transmit information from the DG to CA3. The Schaffer collateral pathway carries information from CA3 to CA1. Information exits CA1 to the subiculum (Sub) and entorhinal cortex. Of course, this is a largely simplified outline of the circuit, as feedback exists within the circuit and the EC has direct projects to areas CA3 and CA1 as well. (Annotated image from Ramon y Cajal)

FIGURE 2: THE cAMP/MAPK/CREB TRANSCRIPTIONAL PATHWAY



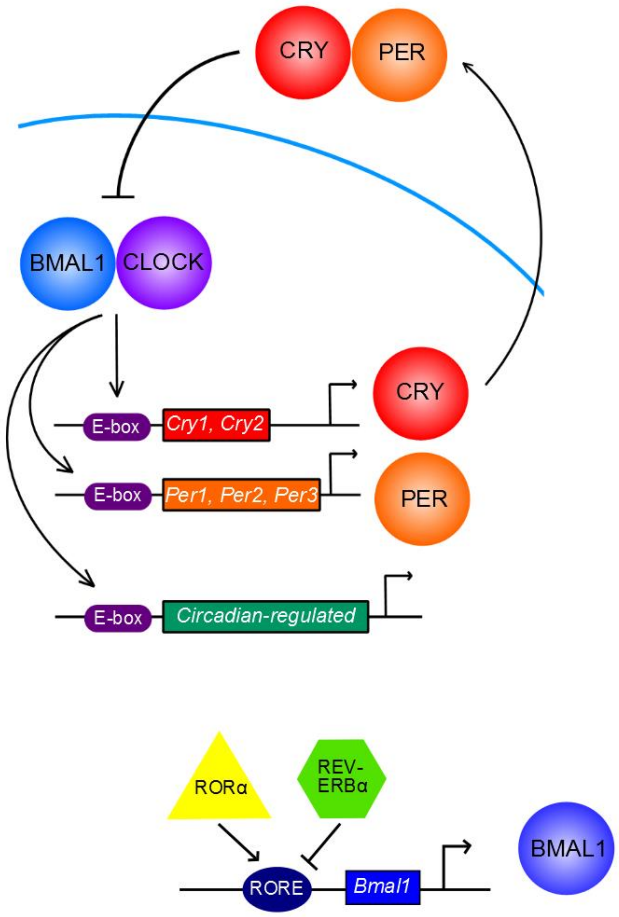
Many signaling pathways are involved in the activation of CREB, including cAMP, ERK-MAPK and CaMK signaling. An activity-induced increase in intracellular calcium activates ERK-MAPK through two pathways: one directly through RasGEF/Ras and one indirectly through stimulation of adenylyl cyclases. The cross talk between both pathways results in the translocation of MAPK to the nucleus, which leads to phosphorylation of CREB through MSK1 and RSK. CaMKII, CaMKIV, and PKA can all directly phosophorylate CREB as well. Binding of phosphorylated CREB and CBP induce gene transcription and de novo translation. (Modified image from Eckel-Mahan, 2008)

FIGURE 3: THE SUPRACHIASMATIC NUCLEUS



The suprachiasmatic nucleus (SCN), located in the hypothalamus, is the master circadian pacemaker in mammals. The SCN receives information about light from the eyes via the retinohypothalamic tract. This light information modifies oscillations in the SCN. The SCN then conveys this information to oscillators throughout the rest of the brain and periphery (such as the cortex, kidney, and liver) via synaptic connections and diffuse hormonal signals to synchronize oscillations throughout the animal.

FIGURE 4: THE MOLECULAR CLOCK



Within a single cell, circadian oscillations are driven by positively and negatively regulated transcriptional and translational loops. In mammals, BMAL1 and CLOCK heterodimerize and bind to E-box elements to enhance transcription of many circadian-regulated genes, including their negative regulators *Period* and *Cryptochrome*. PER and CRY then provide negative feedback by interacting with CLOCK and BMAL1 to inhibit transcription. As PER and CRY levels fall, the BMAL1:CLOCK heterodimer can again induce transcription. This cycle occurs in about 24 hours. A separate transcriptional loop involving the binding of RORα and REV-ERBα to RORE sites in the *Bmal1* promoter also regulates BMAL1 levels. RORα positively regulates *Bmal1* transcription whereas REV-ERBα inhibits *Bmal1* transcription.

CHAPTER 2: BMAL1 AND HIPPOCAMPUS-DEPENDENT FUNCTION

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 tau close to 24 hours (Figure 5C, D). *Bmal1*$^{-/-}$ mice also exhibit difficulties entraining to a 12:12 hour light:dark cycle (Figure 5A, B), thus all experiments were performed under diurnal conditions.

Bmal1$^{+/+}$ and *Bmal1*$^{-/-}$ mice were trained and tested for contextual fear conditioning from ZT3 (four hours after light onset) to ZT5, the peak of MAPK activity (Eckel-Mahan et al., 2008), and under red light from ZT15 to ZT17, the trough of MAPK activity. Mice explored a novel context for 2 minutes before receiving a 0.7 mA foot shock. *Bmal1*$^{-/-}$ mice froze significantly less than their wildtype littermates when they were tested 24 hours after training (Figure 6A, $F(5,45)=31.44$, $p<0.0001$, one-way ANOVA, Tukey post-hoc test). While *Bmal1*$^{-/-}$ mice did learn to associate the novel context with a foot shock (Figure 6A, $p<0.0001$ and $p<0.05$, Tukey post-hoc test), their long-term memory for contextual fear conditioning was not as strong as *Bmal1*$^{+/+}$ controls. *Bmal1*$^{-/-}$ mice contextual fear memory was comparable to *Bmal1*$^{+/+}$ controls when trained and tested during the night (Figure 6B). Both groups freeze more 30 minutes and 24 hours 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 MAPK activity.

In the Morris water maze, *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice were trained to navigate to a

hidden platform for eight days from ZT4 to ZT8. Throughout this training, *Bmal1*$^{-/-}$ mice consistently took longer to find the hidden platform compared to their wildtype littermates (Figure 6C, Bonferroni post-hoc test). 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 ± 3.9% versus 20.3 ± 5.0% of probe trial (Figure 2D, ($F(3,52)=4.080$, $p<0.01$, two-way ANOVA with Bonferroni post-hoc test). Furthermore, *Bmal1*$^{+/+}$ mice crossed the platform's previous location more frequently than *Bmal1*$^{-/-}$ mice, 3.9 ± 0.8 versus 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 minutes, three times. Both *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice spent more time exploring novel object B 30 minutes after training (Figure 6E, $F(3,22)=18.12$, $p<0.001$, one-way ANOVA, Tukey post-hoc test). In fact, *Bmal1*$^{-/-}$ mice even spent more time interacting with a novel object 24 hours after exploring the original object (Figure 6E, $p<0.001$).

Bmal1$^{+/+}$ and *Bmal1*$^{-/-}$ mice do not significantly differ in their levels of anxiety, as measured by the elevated plus maze and open field activity. In the open field, both *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ spend more time in the margins as opposed to the center (Figure 7A, $F(1,20)=241.8$, $p<0.0001$, two-way ANOVA, Bonferroni post-hoc test). Though there is a trend of *Bmal1*$^{-/-}$ mice spending more time in the open arms, *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice percentage of time spent in the open arms is not significantly different, (Figure 7B, $t(14)=1.073$, $p=0.3013$, two-tailed unpaired t-test).

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 (Figure 8A, Tukey's multiple comparison test). There was also no significant difference between the normalized slopes of the second fEPSP at each interpulse interval for paired-pulse facilitation (Figure 8B, Tukey's multiple comparison test). To induce LTP, three tetanic stimuli separated by 5 minutes were delivered to the acute slice and fEPSPs monitored for 2 hours (Figure 8C). For the first 30 minutes following LTP induction, the *Bmal1*$^{+/+}$ hippocampus mean fEPSP slope response was 251.50 ± 3.43 % of baseline, while the *Bmal1*$^{-/-}$ hippocampus demonstrated significantly reduced LTP with a mean fEPSP slope response of 152.20 ± 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 ± 1.2% of baseline versus 133.3 ± 1.1% of baseline) 60 to 90 minutes 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 do 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 the persistence of 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 level of phospho-MAPK at ZT4 compared to ZT16 (Figure 9B, $p<0.05$, Bonferroni post-hoc test). *Bmal1*$^{-/-}$ hippocampi showed no diurnal change in phospho-MAPK at ZT4 and exhibit lower levels of phospho-MAPK at ZT4 compared to *Bmal1*$^{+/+}$ hippocampi (Figure 9B, $p<0.05$, Bonferroni post-hoc test). 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 level of cAMP at ZT4 compared to ZT16 (Figure 4C, $p<0.05$, Bonferroni post-hoc test). *Bmal1*$^{-/-}$ hippocampi showed no diurnal change in cAMP at ZT4 and exhibited lower levels of cAMP at ZT4 compared to *Bmal1*$^{+/+}$ hippocampi (Figure 9C, $p<0.001$, Bonferroni post-hoc test).

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

When wildtype 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 minutes. *Bmal1*$^{+/+}$ mice that experienced the paired association between the novel context and foot shock showed an increase in phospho-MAPK in the hippocampus 30 minutes after training compared to unpaired controls which were shocked immediately (*Bmal1*$^{+/+}$ hippocampi (Figure 10, $t(16)=2$, $p=0.0121$, two-tailed unpaired t-test). There was no difference between *Bmal1*$^{-/-}$ paired and unpaired hippocampi (Figure 5, $t(15)=1.585$, $p=0.1339$, two-tailed unpaired t-test) indicating that

MAPK activity did not increase when *Bmal1*$^{-/-}$ mice were trained for contextual fear memory.

The *Bmal1*$^{-/-}$ hippocampus demonstrates decreased cell proliferation in the SGZ

When mice are injected with BrdU at ZT4 and transcardianly perfused two hours later, the *Bmal1*$^{-/-}$ SGZ exhibits fewer BrdU positive cells compared to the *Bmal1*$^{+/+}$ SGZ (Figure 11C, t(15)=4.169, p=0.0008, two-tailed unpaired t-test). Ki67 cell counts corroborate the BrdU findings (Figure 11D, t(18)=4.895, p=0.0001, two-tailed unpaired t-test). While BrdU only marks dividing cells that have progressed to the S-phase of the cell cycle, Ki67 is expressed throughout all phases of the cell cycle. When mice are injected with BrdU at ZT4 for five consecutive days and then allowed to recover for one month, the *Bmal1*$^{-/-}$ SGZ exhibits fewer surviving BrdU+ cells compared to the *Bmal1*$^{+/+}$ SGZ (Figure 12C, t(13)=2.122, p=0.0536, two-tailed unpaired t-test).

Disruption of *Bmal1* expression in area CA1 of the hippocampus does not affect hippocampus-dependent memory

When mice are trained for contextual fear memory, the major increases in cAMP, MAPK and CRE-mediated transcription are in area CA1 of the hippocampus (Impey et al., 1998; Athos et al., 2002; Sindreu et al., 2007). Since BMAL1 is also expressed in area CA1 of the hippocampus (Wyse and Coogan, 2010), our reported deficits in *Bmal1*$^{-/-}$ mice could reflect an important role of hippocampal BMAL1 in learning and memory. To test this possibility, *Bmal1*$^{loxP/loxP}$ mice were crossed with *CaMKIIa*:*Cre recombinase* mice, a strain that gives CRE expression specifically in area CA1 (Tsien et al., 1996b), for two generations to generate mice without BMAL1 in area CA1. *CaMKIIa:Cre*$^{+}$*Bmal1*$^{loxP/loxP}$ mice showed no expression of

BMAL1 in area CA1 of the hippocampus and maintained normal expression in the SCN (Figure 13). *CaMKIIa:Cre*$^{+}$*Bmal1*$^{loxP/loxP}$ mice exhibited no difference in freezing behavior from *CaMKIIa:Cre*$^{+}$*Bmal1*$^{+/+}$ mice at 1 hour and 24 hours after associating a novel context with a 0.7 mA foot shock (Figure 14A, $F(5,36)=12.50$, $p<0.0001$, one-way ANOVA with Tukey post-hoc test). When the paradigm was made more challenging by exposing mice three times to a 0.3 mA foot shock, *CaMKIIa:Cre*$^{+}$*Bmal1*$^{loxP/loxP}$ mice still exhibited no difference in freezing behavior from *CaMKIIa:Cre*$^{+}$*Bmal1*$^{+/+}$ controls 24 hours after training (Figure 14B, $F(3,20)=151.7$, $p<0.0001$, one-way ANOVA with Tukey post-hoc test).

CaMKIIa:Cre^{+}*Bmal1*$^{loxP/loxP}$ mice also demonstrated no difference from controls in the Morris water maze. There was no difference in the training curve between *CaMKIIa:Cre*$^{+}$*Bmal1*$^{loxP/loxP}$ mice and *CaMKIIa:Cre*$^{+}$*Bmal1*$^{+/+}$ controls (Figure 14C). Two-way ANOVA revealed an effect of day of training ($F(7,84)=16.67$, $p<0.0001$) but no effect of genotype ($F(1,84)=0.3506$, $p=0.5648$). During the probe test, both groups spent significantly more time in the quadrant where the platform used to be (Figure 14D, $F(7,52)=22.86$, $p<0.001$, One-way ANOVA, Tukey post-hoc test). These data support that the memory defects observed with the global knockout of BMAL1 are not due to loss of the protein in area CA1 of the hippocampus.

Disruption of *Bmal1* expression in area CA1 of the hippocampus does not affect diurnal oscillation of MAPK activity in the hippocampus.

MAPK activity exhibits a diurnal oscillation in area CA1 of the hippocampus that is required for the persistence of contextual fear memory (Eckel-Mahan et al., 2008). Both *CaMKIIa:Cre*$^{+}$*Bmal1*$^{+/+}$ and *CaMKIIa:Cre*$^{+}$*Bmal1*$^{loxP/loxP}$ hippocampi show a significant

difference in MAPK activity between ZT4 and ZT16 (Figure 15B). Two-way ANOVA reveals a significant effect of time ($F(1,21)=30.74$, $p<0.0001$) but not genotype ($F(1,21)=0.7724$, $p=0.3894$). Thus, the *CaMKIIa:Cre*$^{+}$*Bmal1*$^{loxP/loxP}$ hippocampus is still capable of oscillations of signaling activity despite the local knockdown of the molecular clock.

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. *Bmal1*$^{loxP/loxP}$ (B6.129S4(Cg)-Arnt1^{tm1Weit}/J, stock number 007668) and *CaMKIIa:Cre recombinase* (B6.Cg-Tg(Camk2a-cre)T29-1Stl/J, stock number 005359) mice were obtained from Jackson Labs (Bar Harbor, Maine). *Bmal1*$^{loxP/loxP}$ mice were crossed with *CaMKIIa*:*Cre recombinase* mice to yield heterozygous mice. The heterozygous mice were then bred to generate mice without BMAL1 in area CA1 of the hippocampus. The genotype of all mice was confirmed using PCR. *CaMKIIa:Cre recombinase*$^{+}$*Bmal1*$^{+/+}$ littermates were used as controls. All experiments were performed on adult, male mice aged 3-5 months. Food and water were provided *ab libitum* and animals were maintained on a 12:12 hour light:dark cycle, with light onset ZT0 at 8 AM, unless otherwise specified. Experiments were in accordance of 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 VitalView Data Acquisition System (Mini Mitter, version 4.1). Actograms and periodograms were generated with the ImageJ plugin ActogramJ (University of Wuerzburg). Mice were monitored for 7 days in 12:12 hour LD conditions and 14 days in DD conditions. Only the last 7 days in DD conditions were included in

depicted actograms and periodogram analysis.

Contextual Fear Conditioning

Mice were handled for at least 5 days before all behavioral testing. For contextual fear training, mice were exposed to a 10 (W) x 10 (D) x 16 (H) inches plexiglass 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 two minutes before receiving a 0.7 mA foot shock. They were returned to their home cage after a 1 minute 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 ZT3-5, during the light phase, or under red light from ZT15-17, during the night phase.

Morris Water Maze.

Mice were placed in a 122 cm diameter pool filled with room temperature water (22ºC) made opaque with nontoxic, white, tempora paint. They were trained for 8 days, 4 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 seconds to find the platform and guided to the platform when they did not find it. They sat on the platform for 30 seconds before removal. Their memory was tested using a probe trial, where the platform is removed, lasting 90 seconds. The probe trial was video recorded and their swim behavior was analyzed using Ethovision (Noldus). Mice were trained and tested from ZT3-ZT8, during the light phase.

Novel Object Recognition.

Mice were habituated to a 17(W) x 8.5(D) x 6(H) cage overnight. After habituation, they explored two identical plastic objects (A) for 5 minutes, 3 times, in the habituated cage. 30 minutes after training, they were presented with object A and a new object B for 5 minutes. 24 hours after training, they were presented with object A and a new object C for 5 minutes. 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 towards 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-ZT6, during the light phase.

Open Field.

Mice explored a 10 (W) x 10 (D) x 16 (H) inches plexiglass chamber for 10 minutes for three consecutive days. For analysis, the activity in these three exposure trials were averaged. Between each subject, the chamber was wiped with diluted acetic acid.

Elevated Plus Maze.

Mice were placed in the center of 67.3 cm long, 15 cm wide elevated plus maze that was elevated 40 cm off of the ground. Mice explored the maze for 10 minutes while observers monitored open versus closed arm entries and time spent in each arm.

Electrophysiology.

Hippocampal LTP was compared between *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ male mice at ages 2-3 months. 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 two hours 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 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-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 Axopatch 200B (Axon Instruments) and digitized by 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 ms to 310 ms in steps of 30 ms. To induce LTP, three tetanic stimuli separated by five minutes were delivered. Each stimulus consisted of a 1 second, 100 Hz train with a pulse length of 0.1 ms. fEPSPs were monitored for two hours 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 x g for 10 minutes. 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 seconds. 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. 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 1,000 g at 4ºC. Resulting supernatants were evaporated in a heat block at

55ºC 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.

BrdU Injection.

To mark and visualize proliferating cells, 4-month-old *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ were i.p. injected with 5'-bromo-2-deoxyuridine (BrdU; Sigma Aldrich) at ZT2-ZT4 at the concentration 100 mg/kg of body weight. Mice were sacrificed 2 hours later. To assess the number of surviving cells, 4-month-old *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ were i.p. injected with 100 mg/kg BrdU at ZT4 for 5 consecutive days. The mice were sacrificed 4 weeks after the last injection for analysis.

Immunohistochemistry.

Mice were anesthetized with ketamine/xylazine and transcardially perfused at ZT4-ZT6 with 4% formaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were cryoprotected in 30% sucrose, frozen in crushed dry ice, and cut into serial 30 µm coronal sections using a cryostat. Free-floating sections were washed in 0.1 M PB, permeabilized with 0.1 M PB with saline and 0.2% Triton X-100 (PBST), and blocked for 2 hours with 0.1 M PBS, 0.2% Triton X-100, 2% BSA, 10% goat serum, 50 mM glycine. Sections were incubated in rabbit anti-BMAL1 overnight (1:200, Novus) and goat anti-rabbit Alexa Fluor 488 (1:500) for 2 hours, with PBST washings in between. Hoescht (5 µM) was used as a nuclear counterstain.

To stain BrdU-injected 30 µm coronal brain slices, free-floating sections were incubated in 2 N HCl for 30 minutes at 37°C and neutralized in a 0.1 M sodium borate buffer solution (pH

8.5). After rinses and permeabilization (see above), slices were incubated in rat anti-BrdU overnight (1:500) and goat anti-rat Alexa Fluor 488 (1:500, AbD serotec) for 2 hours. To stain for Ki-67, slices were incubated overnight in rabbit anti-Ki67 (1:1000, Vector) and goat anti-rabbit Alexa Fluor 594 (1:500) for 2 hours.

Statistical Analysis.

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

CHAPTER THREE: DISCUSSION AND FUTURE DIRECTIONS

On Memory

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 hours after training for contextual fear conditioning, *Bmal1*$^{-/-}$ mice freeze less than wildtype 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 wildtype 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 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 wildtype 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 that are required for 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).

Impaired LTP in the *Bmal1*$^{-/-}$ hippocampus, even immediately within 30 minutes following induction, suggests a role of BMAL1 in synaptic plasticity. As transcription factors, BMAL1 and CLOCK promote the transcription of many genes and even play a role in epigenetic regulation (Doi et al., 2006; Menet et al., 2014). Neuronal structure and plasticity vary across the circadian cycle (Mehnert et al., 2007; Fernández et al., 2008), but much there is research on synaptic scaling as it relates to sleep.

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 L:D conditions (Lapolsky 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 wildtype 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 12:12 hour light:dark 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.

We found it surprising that as impaired as *Bmal1*$^{-/-}$ mice are in many memory paradigms, they show no deficits in novel object recognition. These results led us to question how important the hippocampus is for performance in the novel object recognition task. 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.

Hippocampal lesions do impair novel object recognition across longer delays (10 min, 1 hour, 24 hours) of object presentation (Clark et al., 2000). The extent of hippocampal lesion affects novel object recognition (Broadbent et al., 2004). Only rats with 75-100% of the hippocampus destroyed demonstrated impaired recognition memory. Winters et al. (2004) argue that the perirhinal cortex, but not the hippocampus, is required for recognition memory. Good et al. (2007) demonstrate that recognition memory requires the hippocampus when the task involves contextual or spatial associations. Phan et al. (2011) similarly find that SCN lesioned mice demonstrate no deficit in novel object recognition, despite the impairment to contextual fear and Morris water maze memory. These findings do not completely address how important the hippocampus is to performance in the novel object recognition task. What else would recognition memory involve if not the hippocampus?

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. BMAL1, and other molecular clock proteins, have also been implicated in the regulation of dopamine and mood (Yujnovsky et al., 2006; Roybal et al., 2007; Jiang et al., 2013). However, we do not find any significant differences in the level of exploration and anxiety of *Bmal1*$^{-/-}$ mice, as measured by open field and elevated plus maze activity.

On Neurogenesis

We find that cell proliferation and survival is reduced in the *Bmal1*$^{-/-}$ SGZ, with no effect on cell proliferation in the SVZ. We have not determined the number of surviving neurons versus surviving glia using colocalization of the neuronal marker NeuN or the glial marker GFAP. Such an investigation would further clarify if there are any true differences in cell survival based on cell type in the *Bmal1*$^{-/-}$ SGZ.

Previous reports have implicated a role of the molecular clock in neurogenesis in the SGZ is altered in *Bmal1*$^{-/-}$ and mutant *Per2*^{Brdm1} mice (Borgs et al., 2012; Bouchard-Cannon, 2013). However, these reports find that molecular clock dysfunction results in an increase in cell proliferation with no effect on neuronal maturation and survival, completely opposite to our findings. Borgs et al. and Bouchard-Cannon et al. conclude that PER2 and BMAL1 play a direct role in cell cycle entry, controlling the number of dividing neural progenitor cells. Thus, in the absence of functional PER2 and BMAL1, more cells divide. This conclusion is supported by Matsuo et al.'s (2003) report that the circadian clock regulates cell cycle entry in the mouse liver. Specifically, the circadian clock controls expression of cyclin B1-Cdc2 kinase, an important regulator of mitosis. However, neither report addresses whether PER2 or BMAL1 protein is specifically required in the SGZ to regulate cell cycle entry. Furthermore, they do not consider proliferation in the SVZ. Not much is known in the differences in regulation of neurogenesis in different neurogenic niches. Our findings that the absence of BMAL1 does not appear to affect proliferation in the SVZ is certainly intriguing.

The biggest difference between our investigation and the report by Bouchard-Cannon et al. (2013) is the age of the mice. Bouchard-Cannon et al. used 6-week-old mice while we used 4-

month-old mice. Given that *Bmal1*$^{-/-}$ mice have an accelerated aging phenotype (Kondratov et al., 2006) and neurogenesis is known to decrease with age (Kuhn et al., 1996), our observations could be more related to effects of age rather than the absence of BMAL1.

On BMAL1 in Area CA1 of the Hippocampus

Since BMAL1 is present in the hippocampus, we tested if BMAL1 in the hippocampus plays a role in learning and memory. We crossed *Bmal1*$^{loxP/loxP}$ mice with a *CaMKIIa:Cre recombinase* line that is specific to the CA1 region of the hippocampus (Tsien et al., 1996b). This yielded mice devoid of BMAL1 expression in area CA1 of the hippocampus. We targeted CA1 since this area demonstrates activation of MAPK and CRE-mediated transcription 30 minutes after contextual fear conditioning (Sindreu et al., 2007) and a robust diurnal oscillation in MAPK activity (Eckel-Mahan et al., 2008).

It was especially important to consider a regional *Bmal1* knockdown because of the profound deficits of the *Bmal1*$^{-/-}$ mice. *Bmal1*$^{-/-}$ mice die young due to systemic organ failure (Sun et al., 2006). They demonstrate accelerated aging phenotypes, including increased reactive oxygen species (Kondratov et al., 2006). The increase in reactive oxygen species also lead a recent report to consider the role of BMAL1 in neurodegeneration (Musiek et al., 2013). Furthermore, *Bmal1*$^{-/-}$ mice exhibit a perturbed sleep architecture (Laposky et al., 2005). Though they do not consolidate sleep in the light, inactive phase, they spend more time in both REM and NREM sleep compared to wildtype mice. McDearmon et al. (2006) demonstrate that BMAL1 has tissue-specific roles. When they restore BMAL1 in the brain, they find that circadian rhythmicity is restored but mice remain small, move less and die young. When they restore BMAL1 in muscle, size and activity is restored but the mice remain arrhythmic.

In contrast to *Bmal1*$^{-/-}$ mice, the CA1-specific *Bmal1*$^{-/-}$ mice exhibited no deficits in contextual fear conditioning or spatial memory measured in the Morris water maze. The CA1-specific *Bmal1*$^{-/-}$ mice exhibited a diurnal change in MAPK activity, suggesting that a local molecular clock is not required to sustain the diurnal change of signaling activity in area CA1. This result is surprising, as the literature suggests that a local, molecular clock could have a direct role in hippocampus function. *Per2* mRNA and protein is rhythmically expressed in the hippocampus and persists in vitro (Wang et al., 2009). Expression of the other molecular clock genes (*Per1*, *Cry1*, *Cry2*, *Bmal1*, *Clock*) and their protein products is also rhythmic in the hippocampus (Jilg et al., 2010).

On the SCN and Slave Oscillators

We hypothesize that the SCN is the most important in establishing circadian oscillations in the hippocampus. This hypothesis is supported by Phan et al. (2011) finding that lesioning the SCN compromises the maintenance of hippocampus-dependent memory and the diurnal oscillation of signaling activity. These reported results help us understand the relationship between the SCN and one of its slave oscillators, the hippocampus.

Understanding how the central, master circadian oscillator in the SCN synchronizes the rest of the oscillators throughout the brain and periphery is still an active research question. This question carries substantial weight when considering human health. The adverse effects of shift work and jet lag in part are a consequence of the internal desynchronization of oscillators. Peripheral circadian rhythms become out of phase from environment signals. While the SCN is updated immediately to a change in the light:dark cycle, other oscillations, like those in the digestive system or regulating sleep, take longer to catch up (reviewed in Albrecht, 2012).

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 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 synchronizing candidates include glucocorticoids (Girotti et al., 2009; reviewed in Dickmeis et al., 2013) and melatonin (reviewed in Stehle et al., 2003) as they are susceptible to influence from the SCN and also act on the hippocampus.

Melatonin is a conserved time signal indicating night in vertebrates (reviewed in von Gall et al., 2002). Depending on the animal, it has varied effects on behavior. As diurnal animals, melatonin in humans induces sleep behavior. However, as nocturnal animals, melatonin in rats and mice fosters activity. Melatonin is converted from serotonin in the pineal gland (Ganguly et al., 2002). AANAT (arylaklylamine-N-acetyltransferase) converts serotonin to N-acetylserotonin and hydroxyindole-O-methyltransferase converts N-acetylserotonin to melatonin. Because melatonin is released into circulation as soon as it is synthesized, its production is tightly regulated. With nighttime exposure to light, N-acetylserotonin and melatonin levels rapidly fall. This time-of-day and light sensitive regulation of melatonin production is due to the SCN's input to the pineal gland. SCN lesion will disrupt melatonin rhythms (reviewed in Moore et al., 1996). The SCN projects to the pineal gland through the paraventricular nucleus (PVN), intremediolateral cell column of the spinal chord and superior cervical ganglia. The release of norepinephrine to the pineal gland stimulates β_1-adrenergic receptors, adenylyl cyclase activity and the production of cAMP. PKA binds and phosphorylates AANAT to mark for degradation.

Melatonin is a timing cue that can affect the hippocampus and memory. Melatonin receptors are G protein coupled and inhibit the activity of type I adenylyl cyclases (Chan et al., 2001). Melatonin can entrain circadian rhythms. It can entrain free-running rhythms in blind humans (Sack et al., 2000; Lockley et al., 2000). Melatonin inhibits long-term potentiation of Schaffer-collateral pathway (Wang et al., 2005). Melatonin also inhibits formation of memory at night in zebrafish (Rawashdeh et al., 2007).

C57BL/6J mice, the majority of the background in most transgenic mouse strains including the global *Bmal1*$^{-/-}$ mouse, have a natural melatonin knockdown due to a mutation in AANAT (Roseboom et al., 1998). However, cultured pineal glands from C57BL/6J mice will synthesize melatonin in response to NE stimulation (von Gall et al., 2000). AANAT is still present in C57BL/6J mice, though dramatically reduced. Moving mice to a short photoperiod and extending activation of AANAT at the beginning of the night will actually make melatonin synthesis detectable in vivo, though detected levels are still only 10% of the levels detected in CH3 mice. As C57BL/6J mice maintain circadian oscillations outside of the SCN (reviewed in Stehle et al., 2002) despite dramatically reduced melatonin signaling, melatonin is not essential.

Glucocorticoids are steroid hormones secreted by the adrenal glands and regulated by the hypothalamus-pituitary-adrenal (HPA) axis in response to acute stress (reviewed in Groeneweg et al., 2011). There are two types of glucocorticoid receptors, the type I mineralcorticoid receptor (MR) and the type II glucocorticoid receptor (GR). MRs have a higher affinity for glucocorticoids, thus there are more MRs bound during basal, circulating levels of glucocorticoids. Glucocorticoid receptors expressed throughout the brain, except for the SCN (Rosenfield et al., 1988). Thus, glucocorticoids influence a variety of physiological systems, including memory (reviewed in Sandi, 1998) and neurogenesis (reviewed in Dickmeis et al.,

2011), which makes adaptive sense for an organism's ability to respond to stress (reviewed in Trollope et al., 2012).

Glucocorticoids exhibit a daily peak occurring right before an animal's activity phase, in addition to ultradian variations throughout the day (reviewed in Dickmeis et al., 2013). SCN lesion disrupts ACTH and glucocorticoid rhythms (Moore and Eichler, 1972; Szafarczyk et al., 1983; Meyer-Bernstein et al., 1999). The SCN can influence changes in the HPA axis, including the release of ACTH and CRH from the PVN. The SCN can also influence the sensitivity of the adrenal glands to ACTH through the autonomic nervous system.

Glucocorticoids can similarly affect peripheral circadian rhythms. Glucocorticoids can change phase of *Per1* oscillations in the liver (Balsalobre et al., 2000). Glucocorticoids can modulate adaptation of locomotor activity to light environment (Sage et al., 2004). The release of glucocorticoids by the adrenal clock is involved in reentrainment after jet lag in mice (Kiessling et al., 2010). Glucorticoids slow the rate of adaptation of peripheral oscillations to daytime feeding in mice (Le Minh et al., 2001). Adrenalectomy will increase cell proliferation in the SGZ of the hippocampus (Gould et al., 1992; Cameron & Gould, 1994), similar to the result of compromising PER2 or BMAL1 (Borgs et al., 2012; Bouchard-Cannon et al., 2013). High levels of corticosterone with decrease cell proliferation in the SGZ of rats (Brummelte & Galea, 2010). However, 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).

When glucocorticoids bind to receptors in the plasma membrane, the receptor undergoes a conformational change, internalizes, and translocates to the nucleus. The homodimeric receptor can bind glucocorticoid response elements (GREs) to positively or negatively regulate gene

transcription. Given the time frame of an animal's response to stress, glucocorticoids can also have rapid, nongenomic effects. Many physiological responses to stress are too fast to be attributable to genomic action alone. Within 5 minutes of corticosterone application in the acute hippocampal slice frequency of mEPSPs in area CA1 is enhanced (Karst et al., 2005; Olijslagers et al., 2008). There is evidence of cross talk of activated GRs with pERK1/2-MSK1-Elk1 signaling (Gutierrez-Mecinas et al., 2011), which may be relevant to signaling activity required for memory maintenance.

This thesis extends our understanding of the relationship between the molecular clock and hippocampus functionality and is consistent with a substantial literature implicating the circadian system in memory (reviewed in Gerstner and Yin, 2010). Future investigations should confirm if BMAL1 in the SCN specifically is required for the circadian oscillation of signaling activity in the hippocampus and hippocampus-dependent memory. The SCN may be the most important in driving the oscillation of signaling activity in the hippocampus that is crucial for memory maintenance. Targeting the SCN specifically, either by local molecular clock knockout or lesion, would also address whether hippocampus-specific molecular clock proteins play a direct role in cell cycle entry in the SGZ, or if signals emerging from the SCN are sufficient. The SCN likely contributes to the molecular oscillations of signaling activity in the hippocampus through redundant inputs rather than relying on a single mechanism. Potential connections between the SCN and signaling oscillations in the hippocampus warrants further investigation.

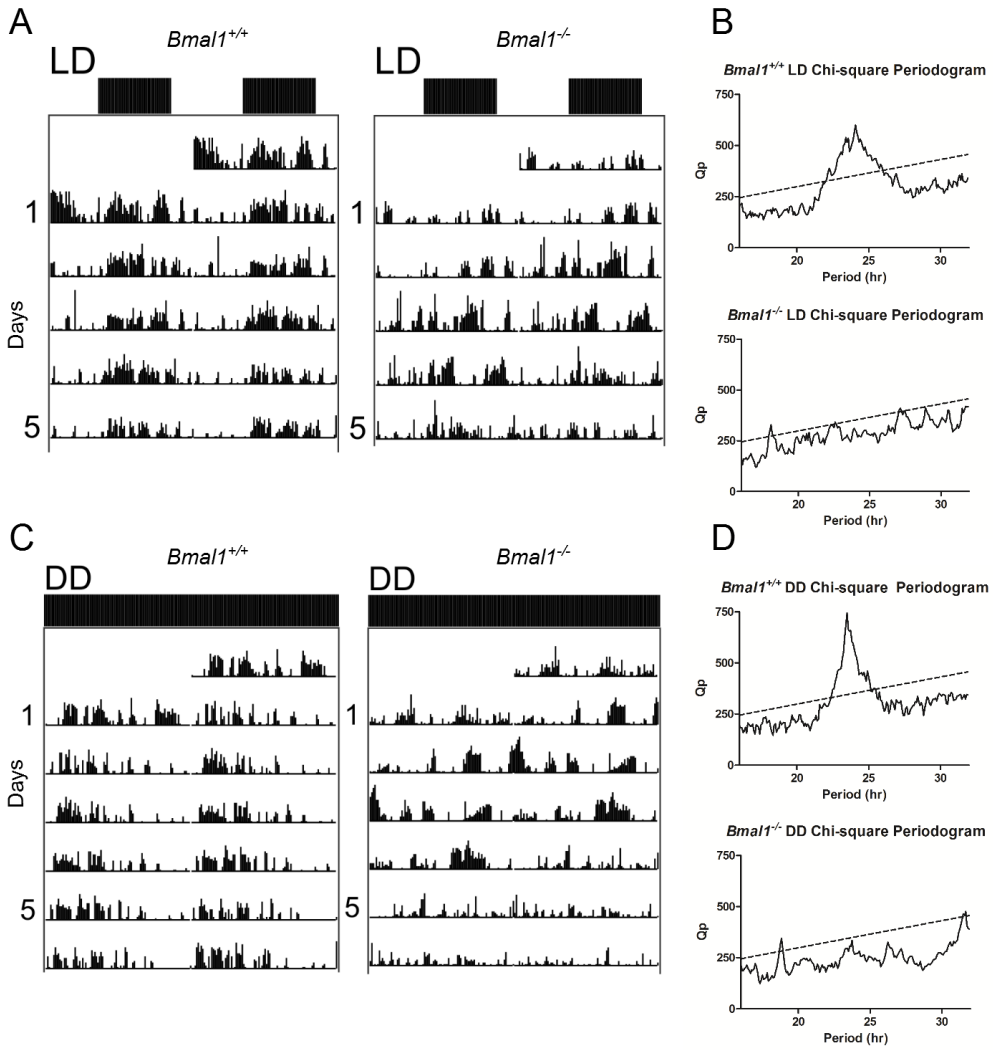


Figure 5. *Bmal1*$^{-/-}$ mice locomotor activity. Mice were monitored in 12:12 hour L:D conditions for one week before exposed to DD conditions for two weeks. Analysis of DD conditions is restricted to the second week of monitoring. A. Representative *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ double-plotted actograms in 12:12 hour L:D conditions. Each line represents 48 hours. B. *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ chi-square periodograms. *Bmal1*$^{+/+}$ periodogram reveals τ = 1445 minutes, or 24.1 hours. *Bmal1*$^{-/-}$ periodogram does not reveal a period of 24 hours. For the one peak that does cross the line of significance, τ = 1085 minutes, or 18.1 hours. C. Representative *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ double-plotted actograms in DD conditions. D. *Bmal1*$^{+/+}$ periodogram reveals τ = 1410 minutes, or 23.5 hours. *Bmal1*$^{-/-}$ periodogram does not reveal a period of 24 hours. For the one peak that does cross the line of significance, τ = 1130 minutes, or 18.8 hours.

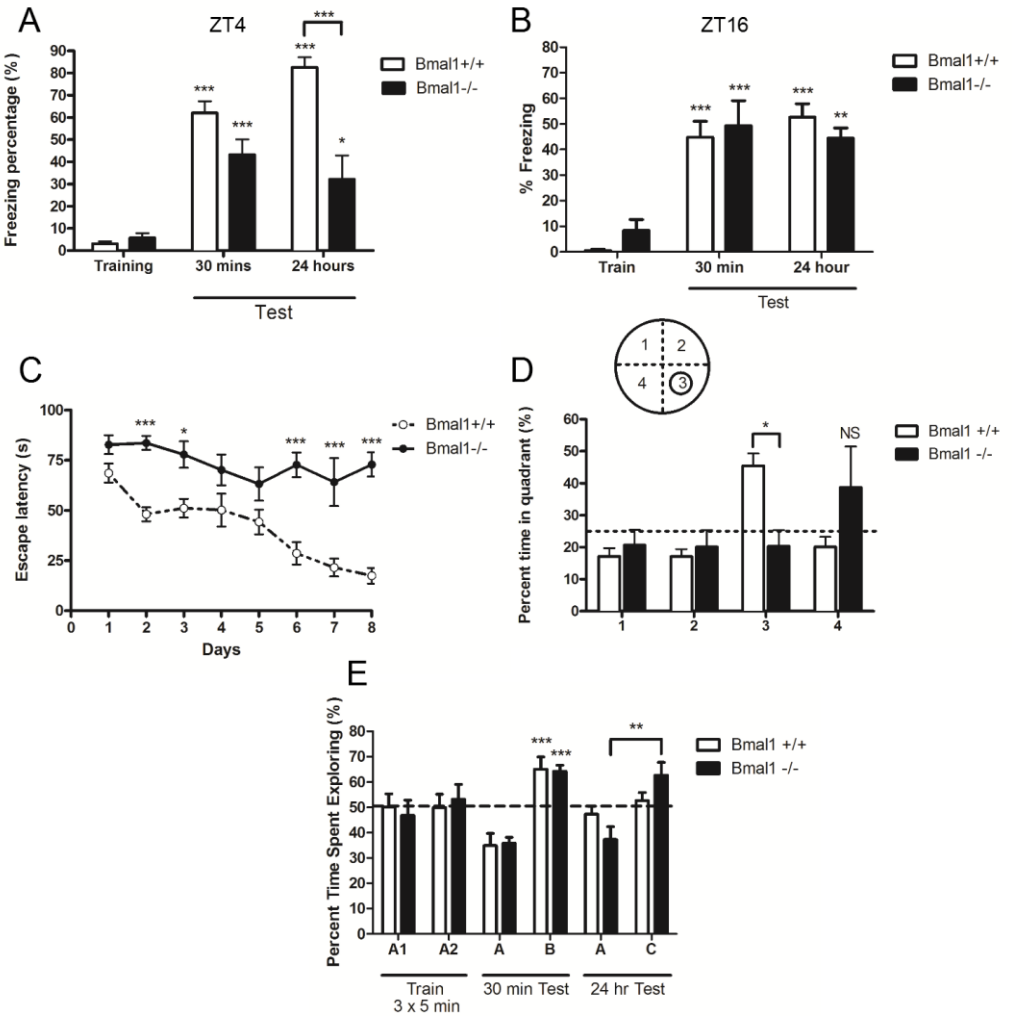


Figure 6: *Bmal1*$^{-/-}$ mice demonstrate impaired learning and memory in hippocampus-dependent tasks. A. Contextual fear freezing behavior at ZT4. *Bmal1*$^{-/-}$ mice freeze significantly less 24 hours ($F(5,45)=31.44$, $p<0.0001$) after contextual fear conditioning training with a 0.7 mA foot shock. One-way ANOVA with Tukey post-hoc test. B. Contextual fear freezing behavior at ZT16. Both groups demonstrate significant freezing behavior 30 minutes and 24 hours 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 4 trials of training per day over 8 days. 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 significantly more time in quadrant 3, where the platform used to be, compared to other quadrants and *Bmal1*$^{-/-}$ mice ($F(7,52)=5.039$, $p<0.01$). One-way ANOVA with Tukey post-hoc test. D. Novel object recognition. 30 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 hours after exposure to object A ($p<0.001$). One-way ANOVA, Tukey post-hoc test. All values represent mean ± SEM, n=6-10.

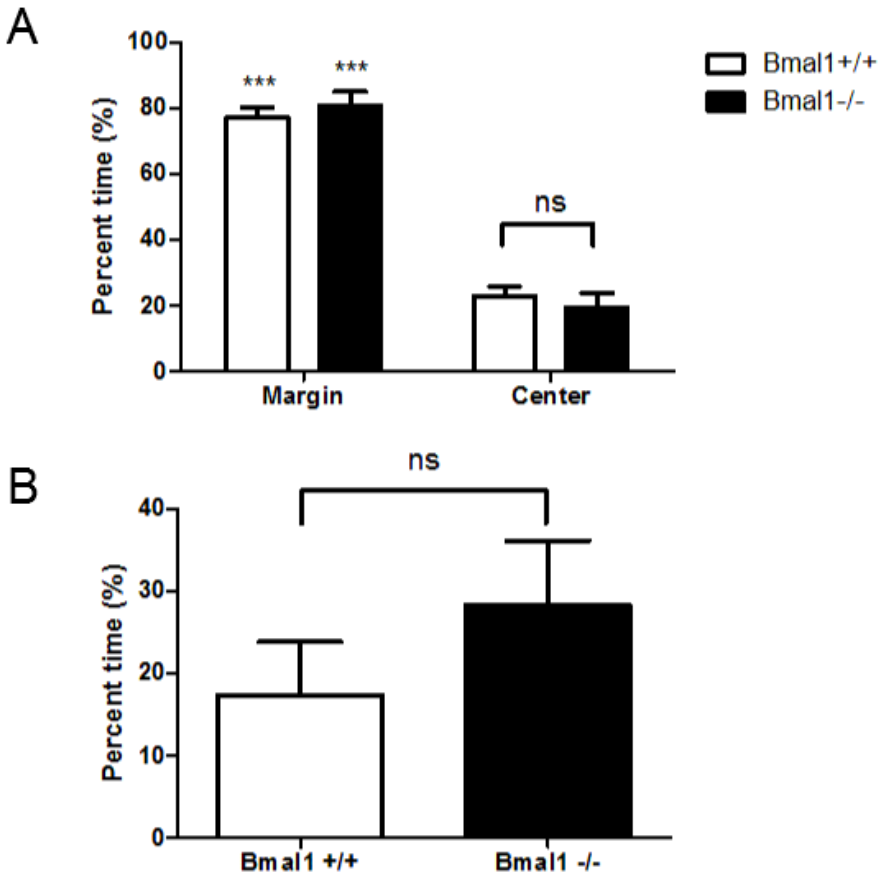


Figure 7: *Bmal1*$^{-/-}$ mice do not significantly differ in anxiety levels as measured by elevated plus maze and open field activity. A. Open field margin versus center time. Both *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ spend more time in the margins as opposed to the center ($F(1,20)=241.8$, $p<0.0001$) Two-way ANOVA with Bonferroni post-hoc test. B. Elevated plus maze open arm time. Though there is a trend of *Bmal1*$^{-/-}$ mice spending more time in the open arms, *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice percentage of time spent in the open arms is not significantly different, (Figure 7B, $t(14)=1.073$, $p=0.3013$, two-tailed unpaired t-test).

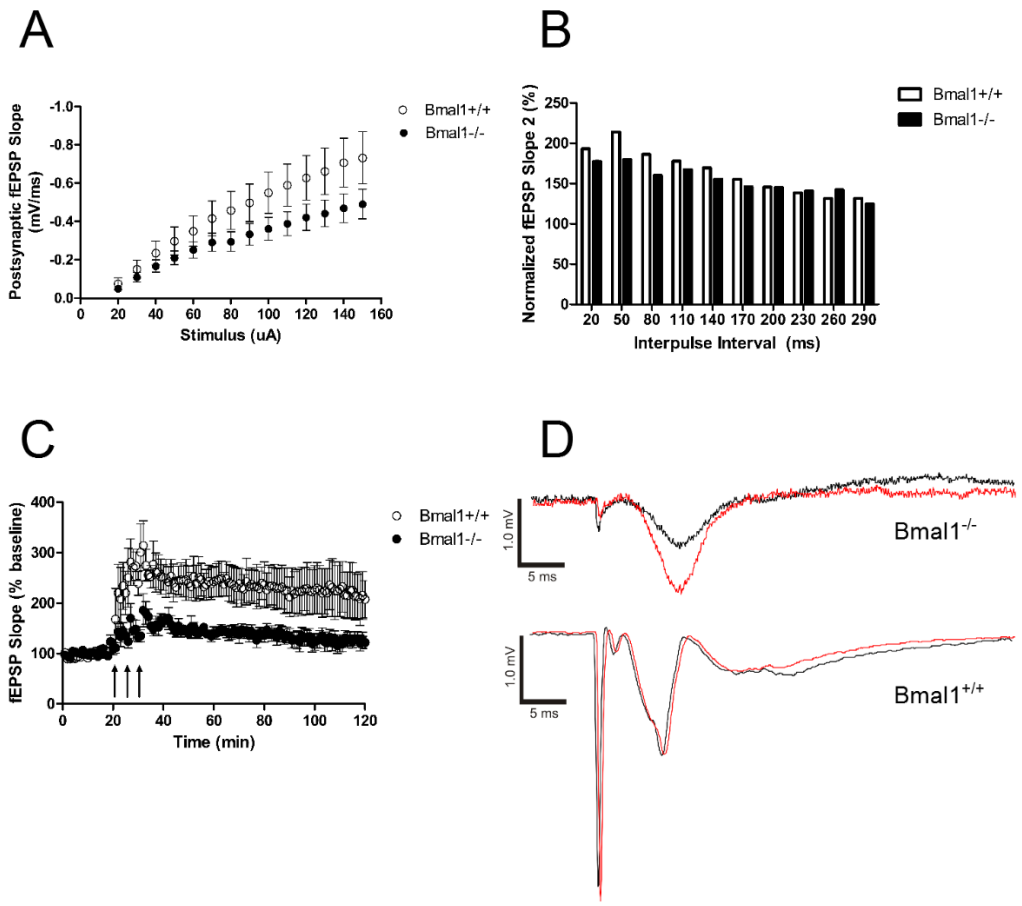


Figure 8: LTP at the Schaffer-collateral synapse is impaired in the *Bmal1*$^{-/-}$ hippocampus. A. Input/output (I/O) relationship between stimulus (µA) and the slope of the postsynaptic evoked fEPSP (mV/ms). Stimulus ranged from 20 to 150 µA. There is no difference between the *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ 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 ms. 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 pre-tetanic baseline. To induce LTP, three tetanic stimuli (1 s, 100 Hz train with 0.1 ms pulse length) separated by five minutes were delivered. *Bmal1*$^{-/-}$ hippocampus demonstrated significantly reduced LTP with a mean fEPSP slope response within first 30 minutes following induction ($U=0.0$, $p<0.0001$, $r = 1.21$, Mann-Whitney). The *Bmal1*$^{-/-}$ hippocampus continued to demonstrate a significantly reduced response 60 to 90 minutes following induction ($U=0.0$, $p<0.0001$, $r = 1.22$, Mann-Whitney) D. Representative LTP traces from individual slices. Black is 5 minutes after tetantic stimulations. Red is 60 minutes after tetanic stimulations. All values represent mean ± SEM.

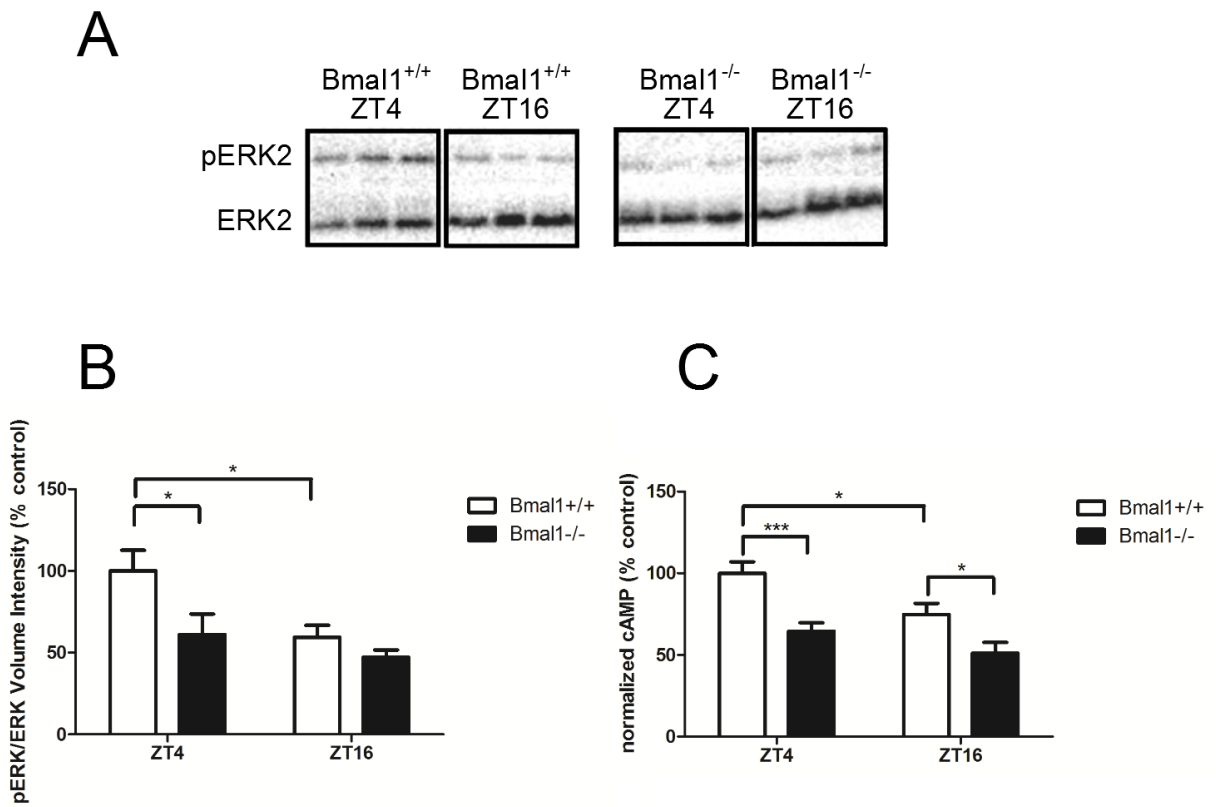


Figure 9: There is no diurnal oscillation of ERK activity or cAMP in *Bmal1*$^{-/-}$ hippocampi. A. Western blot image depicts representative phosphorylated ERK and ERK intensities from isolated hippocampi of individual mice at ZT4 or ZT16. B. Ratio of pERK to ERK volume intensities normalized to control *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 exhibit 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 level of cAMP at ZT4 compared to ZT16 ($p<0.05$). *Bmal1*$^{-/-}$ hippocampi showed no diurnal change in cAMP at ZT4 and exhibit 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.

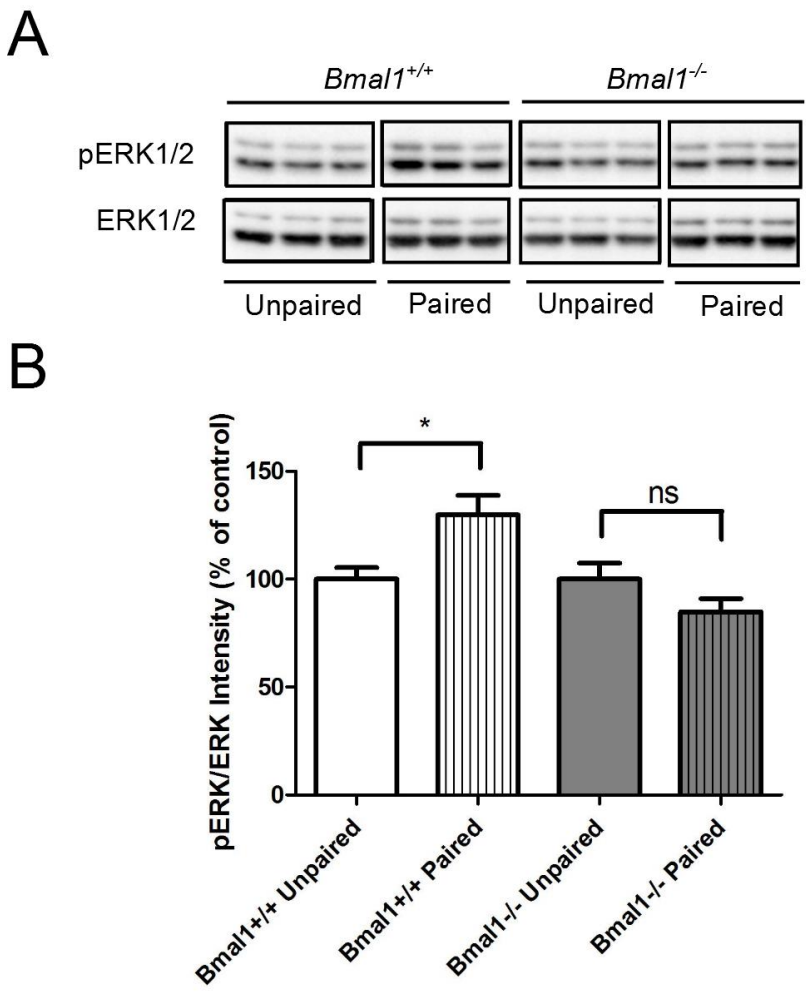


Figure 10: There is no increase in phosphorylated ERK activity in *Bmal1*$^{-/-}$ hippocampi 30 minutes after contextual fear conditioning. A. Western blot image depicts phosphorylated ERK and ERK intensities from isolated hippocampi of individual mice 30 minutes after contextual fear conditioning. Unpaired animals immediately received a 0.7 mA foot shock after placement in a novel cage. Paired animals explored the novel context for 2 minutes 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.

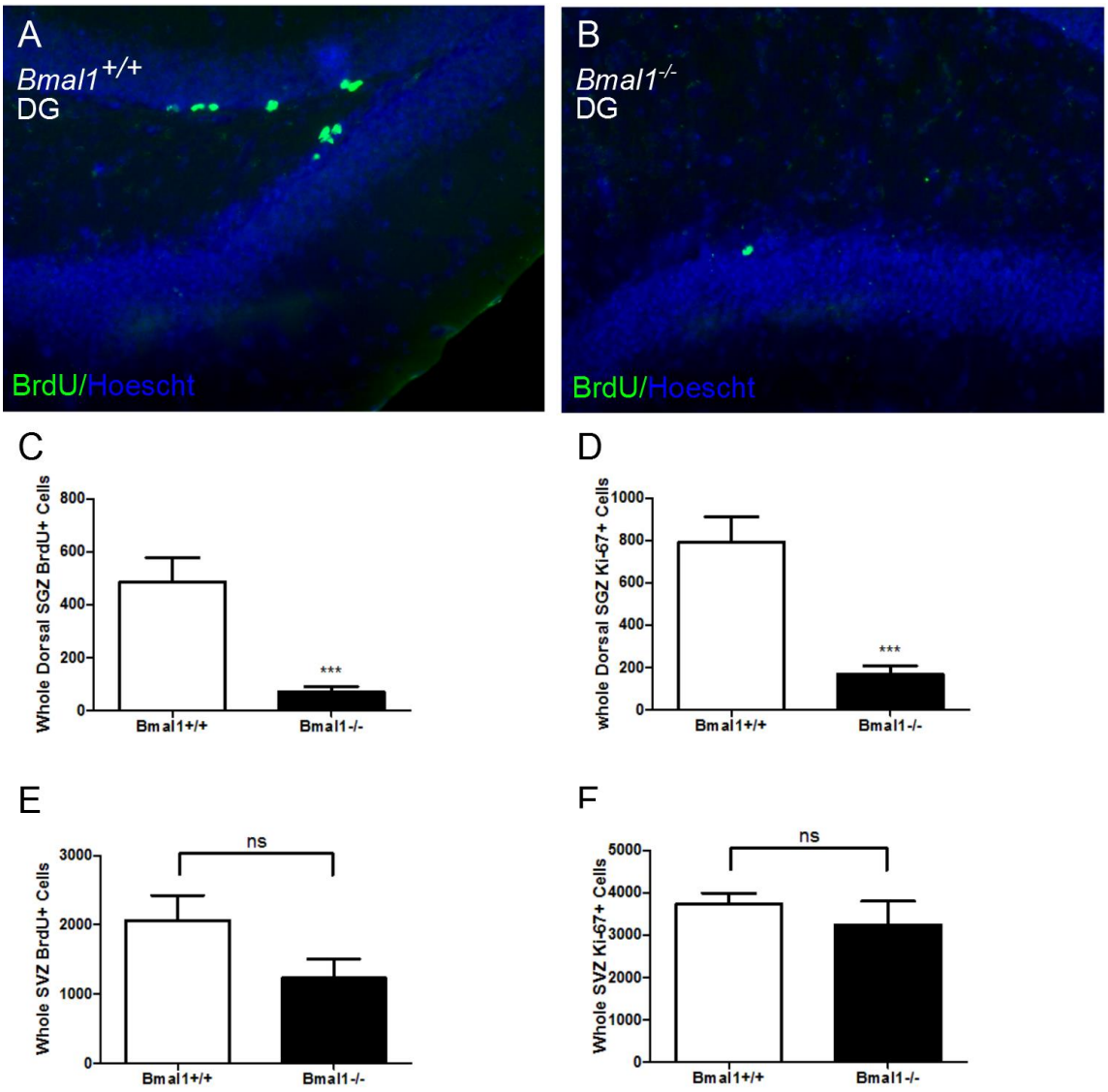


Figure 11: *Bmal1*$^{-/-}$ SGZ, but not the SVZ, demonstrates a decrease in cell proliferation. A,B. *Bmal1*$^{+/+}$ (A) and *Bmal1*$^{-/-}$ (B) dentate gyrus stained for BrdU after a 2-hour pulse and nuclear counterstained with Hoescht. C. The *Bmal1*$^{-/-}$ SGZ has significantly less BrdU positive cells in the SGZ ($t(15)=4.169$, $p=0.0008$). D. The *Bmal1*$^{-/-}$ SGZ has significantly less Ki-67 positive cells in the SGZ ($t(18)=4.895$, $p=0.0001$). E. There is no significant difference in BrdU positive cells between the *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ SVZ ($t(15)=1.797$, $p=0.0924$). F. There is no significant difference in Ki-67 positive cells between the *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ SVZ ($t(18)=0.7895$). Two tailed unpaired t-tests. All values represent mean ± SEM, n=8-9.

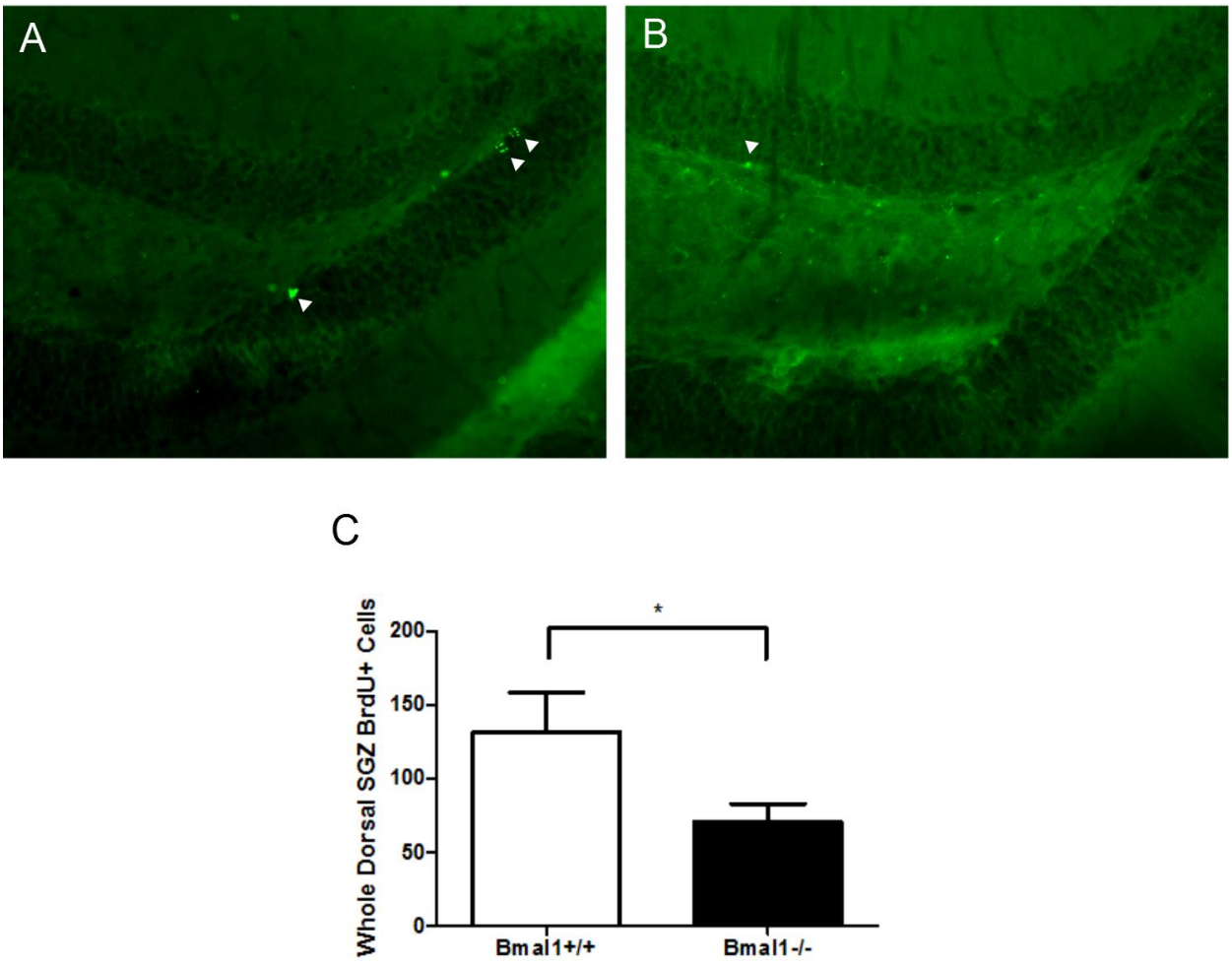


Figure 12: Cell survival is reduced in the *Bmal1*$^{-/-}$ SGZ. A,B. *Bmal1*$^{+/+}$ (A) and *Bmal1*$^{-/-}$ (B) dentate gyrus stained for BrdU after 5 days of consecutive BrdU-pulses and a month recovery. White arrows indicate BrdU+ cells. C. There are less BrdU positive cells in the *Bmal1*$^{-/-}$ SGZ ($t(13)=2.122$, $p=0.0536$). Unpaired t-test. All values represent mean ± SEM, n=7-8.

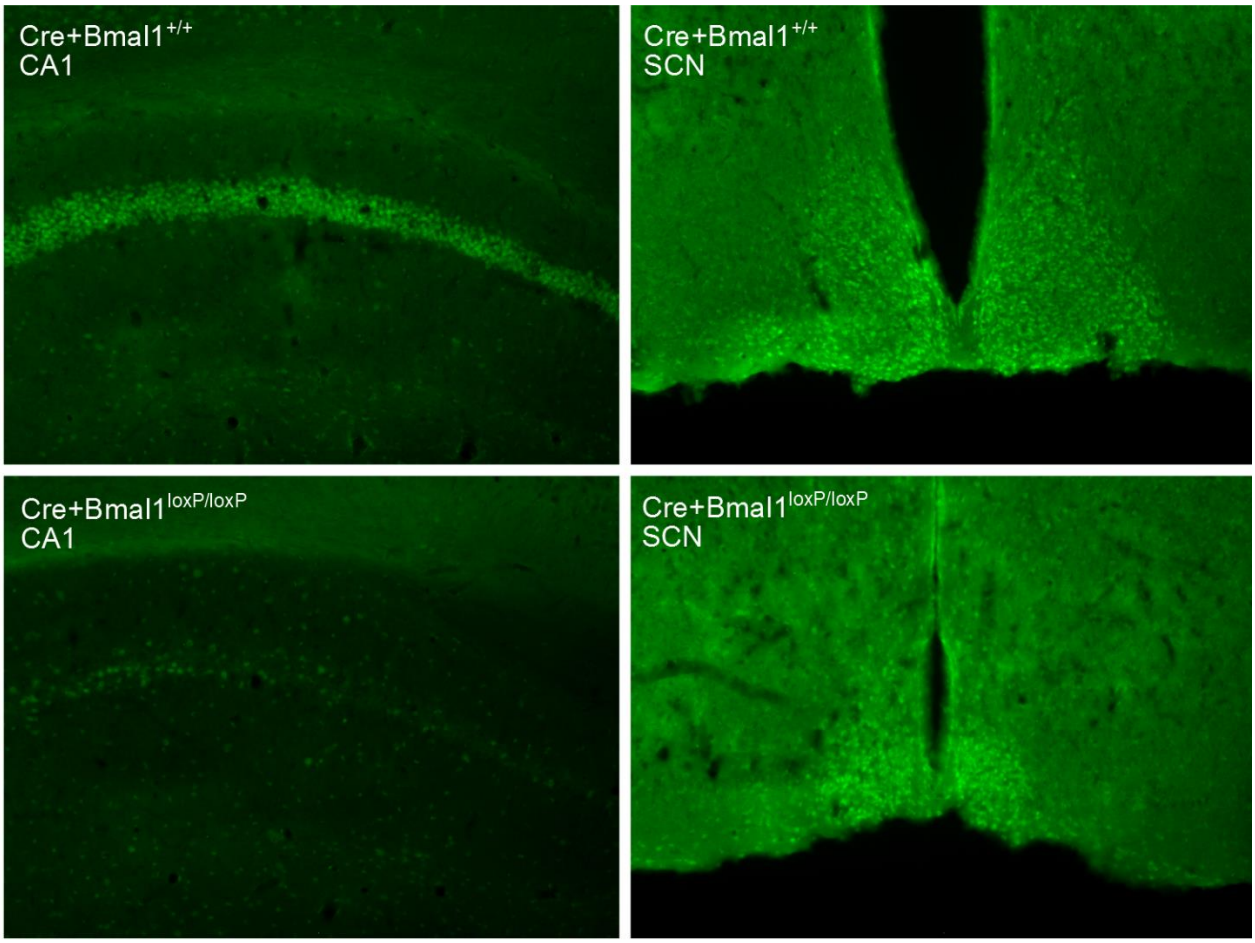


Figure 13: **Immunohistochemical staining demonstrates that *CaMKIIa:Cre^{+}Bmal1$^{loxP/loxP}$* mice lack BMAL1 in area CA1 of the hippocampus compared to *CaMKIIa:Cre^{+}Bmal1$^{+/+}$* control mice**. Both groups are positive for BMAL1 in the SCN. 30 µm coronal slice.

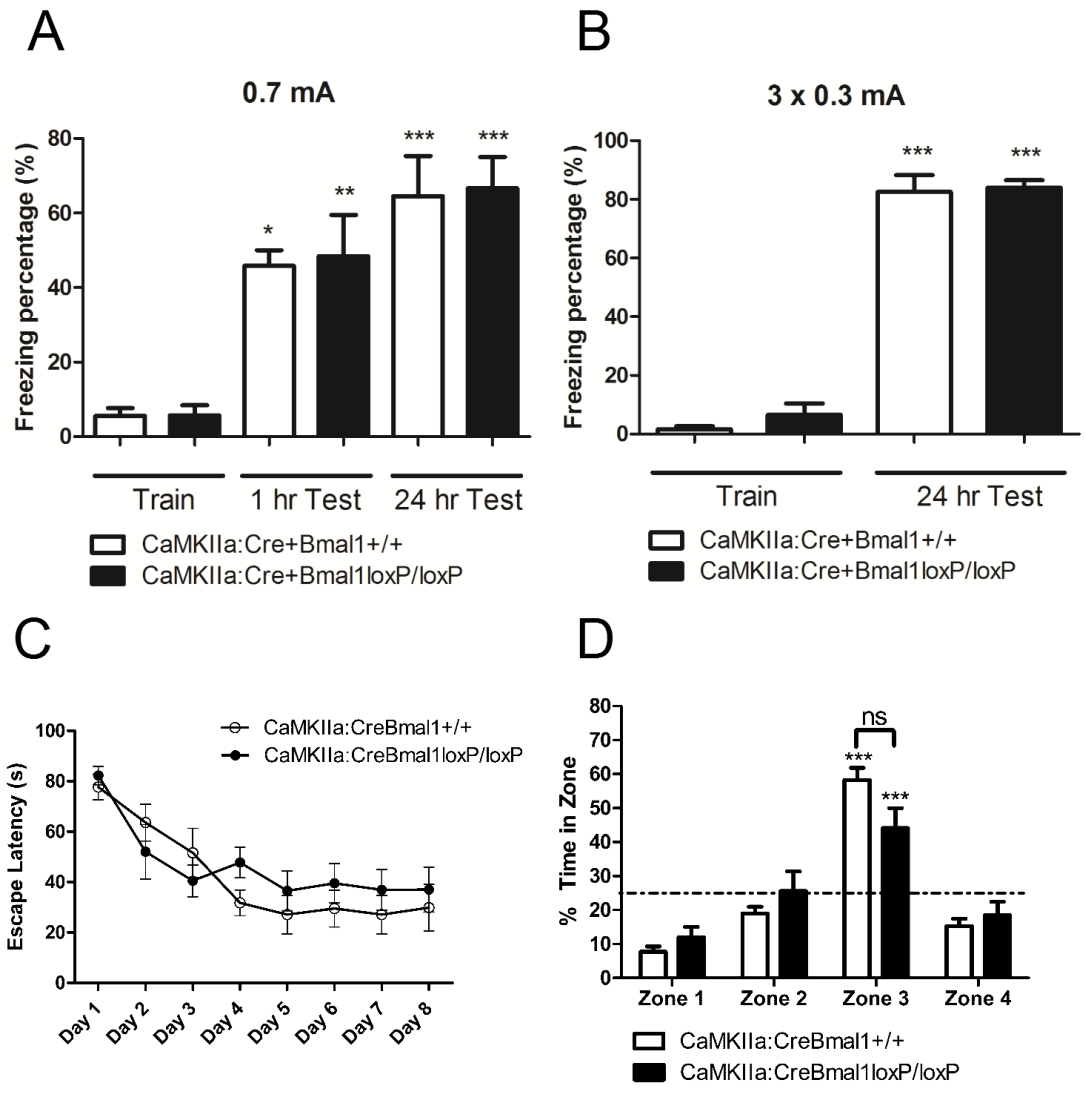


Figure 14: *CaMKIIa:Cre*$^{+}$*Bmal1*$^{loxP/loxP}$ mice show no deficit in contextual fear conditioning or Morris water maze. A. 0.7 mA contextual fear freezing behavior. Both *CaMKIIa:Cre*$^{+}$*Bmal1*$^{loxP/loxP}$ and *CaMKIIa:Cre*$^{+}$*Bmal1*$^{+/+}$ mice exhibit increased freezing at both 1 hour and 24 hours after training ($F(5,36)=12.50$, $p<0.0001$). One-way ANOVA with Tukey post-hoc test. B. 3 x 0.3 mA contextual fear freezing behavior. When using a more challenging paradigm, Both *CaMKIIa:Cre*$^{+}$*Bmal1*$^{loxP/loxP}$ and *CaMKIIa:Cre*$^{+}$*Bmal1*$^{+/+}$ mice exhibit increased freezing behavior 24 hours after training ($F(3,20)=151.7$, $p<0.0001$). One-way ANOVA with Tukey post-hoc test. C. Morris water maze training curve. There is an effect of day of training ($F(7,84)=16.67$, $p<0.0001$) but no effect of genotype ($F(1,84)=0.3506$, $p=0.5648$). Two-way ANOVA with Bonferroni post-hoc test. D. Morris water maze probe trial. Both *CaMKIIa:Cre*$^{+}$*Bmal1*$^{+/+}$ and *CaMKIIa:Cre*$^{+}$*Bmal1*$^{loxP/loxP}$ groups spend more time in quadrant 3, where the platform used to be ($F(3,52)=48.22$, $p<0.0001$), but there is no difference between the groups ($F(1,52)=0.00$, $p=0.997$). Two-way ANOVA with Bonferroni post-hoc test. All values represent mean ± SEM, n=5-8.

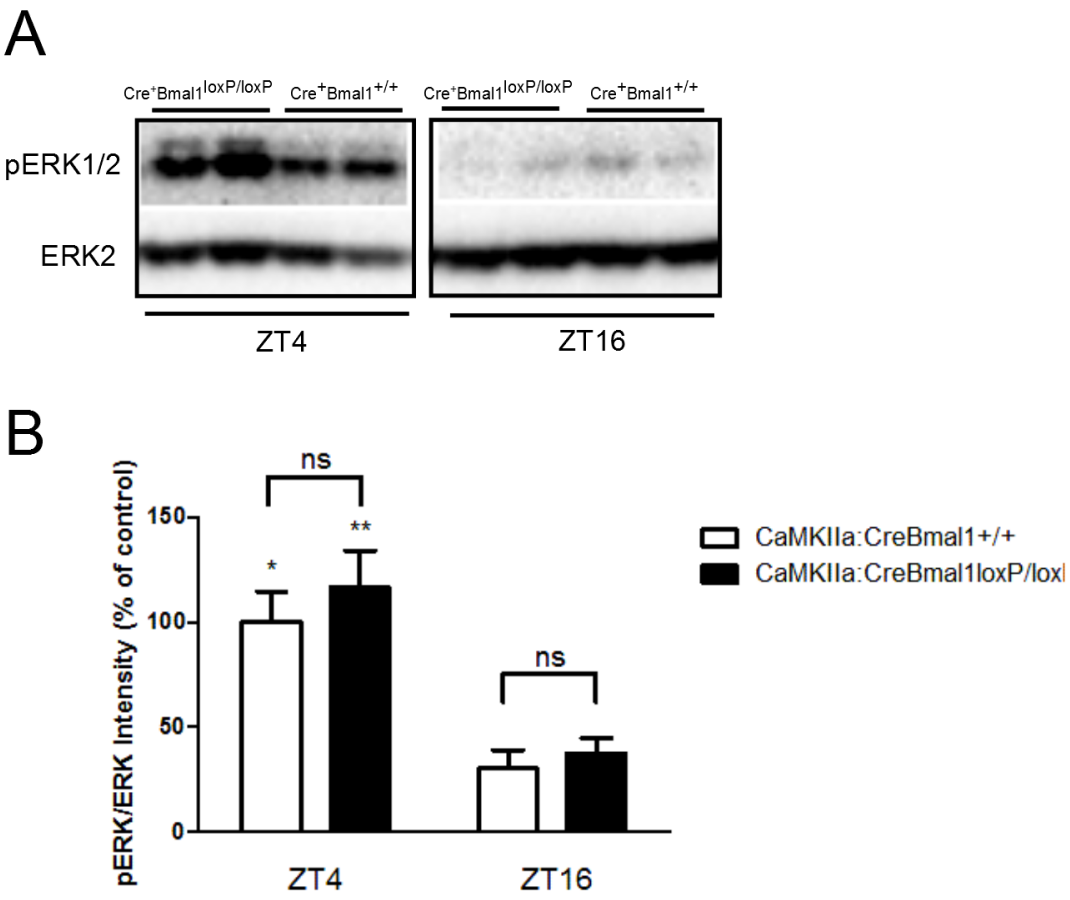


Figure 15: *CaMKIIa:Cre*$^{+}$*Bmal1*$^{loxP/loxP}$ mice show diurnal oscillation in phospho-ERK activity in the hippocampus. A. Western blot image depicts representative phosphorylated ERK and ERK intensities from isolated hippocampi of individual mice at ZT4 or ZT16. B. Ratio of pERK to ERK volume intensities normalized to *CaMKIIa:Cre*$^{+}$*Bmal1*$^{+/+}$ ZT4 group. There is a significant effect of ZT ($F(1,21)=30.74$, $p<0.0001$) but not genotype ($F(1,21)=0.7724$, $p=0.3894$). Two-way ANOVA with Bonferroni post-hoc test. All values represent mean ± SEM, n=5-7.

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