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Circadian Regulation of Hippocampal Long Term Potentiation.

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Abstract:

The goal of this study is to investigate the possible circadian regulation of hippocampal excitability and long term potentiation (LTP) measured by stimulating the Schaffer collaterals (SC) and recording the field excitatory post-synaptic potential (fEPSP) from the CA1 dentritic layer or the population spike (PS) from the soma in brain slices from C3H and C57 mice. These two strains of mice were of interest because the C3H mice secrete melatonin rhythmically while the C57 mice do not. We found that the magnitude of the enhancement of the PS was significantly greater in LTP recorded from night slices compared to day slices of both C3H and C57 mice. We also found significant diurnal variation in the decay of LTP measured with fEPSPs with the decay slower during the night in both strains of mice. There was evidence for a diurnal rhythm in the input/output function of pyramidal neurons measured at the soma in C57 but not C3H mice. Furthermore, LTP in the PS measured in slices prepared during the day, but recorded during the night, had a profile remarkably similar to the night group. Finally, PS recordings were carried out in slices from C3H mice maintained in constant darkness prior to experimentation. Again, we found that the magnitude of the enhancement of the PS was significantly greater in LTP recorded from subjective night slices compared to subjective day slices. These results provide the first evidence that an endogenous circadian oscillator modulates synaptic plasticity in the hippocampus.

Key words: circadian rhythm, hippocampus, LTP, melatonin, synaptic plasticity, mice

<u>Introduction:</u>

    Daily rhythms in behavior and physiology have been shown to occur in most organisms,

including humans. The physiological system responsible for these rhythms is known as the

circadian system; in mammals, the core of this rhythm generating system is localized to a site in the

hypothalamus known as the suprachiasmatic nucleus (SCN: Reppert and Weaver, 2002; Buijis et

al., 2003). The endogenous rhythms generated by the SCN have a period close to, but not quite, 24-

hours. The function of the circadian system is thought to allow the temporal coordination of various

physiological processes within an organism, as well as enabling an organism to temporally

coordinate with the external world. In order to fulfill these functions, these rhythms must be

synchronized to the exact 24-hr cycle of the physical world. The dominant cue used by most

organisms that enables their circadian system to synchronize to the environment is the daily cycle of

light and dark. Within an organism, the biological clock regulates many physiological processes and

behaviors. Recent data suggests that the SCN functions as a master clock that coordinates the

activity of multiple, damped circadian oscillators outside the SCN (Yamazaki et al., 2000).

    It is well established that the hippocampus is critically involved in memory formation and that

synaptic connections within the hippocampus are well known to undergo activity-dependent

changes in synaptic strength referred to as long-term potentiation (LTP). LTP in the CA1

pyramidal cell layer has been particularly well studied and can be measured by stimulating the

Schaffer collaterals and recording the field excitatory post-synaptic potential (fEPSP) from the CA1

dentritic layer or the population spike (PS) from the soma. Changes in the strength of the SC/CA1

synaptic connection are commonly viewed as a model for understanding activity-dependent changes

in synaptic strength that may ultimately be linked to learning and memory (Martin et al., 2000;

Sweatt, 2001). Previous work makes it clear that the strength of LTP at the SC/CA1 synapse can be

regulated by a number of signaling pathways including those activated by modulatory neurotransmitters and hormones (Lisman et al., 2003; Silva, 2003). If LTP is indeed a neural correlate of learning and memory formation, then it seems logical that the many factors, including circadian rhythms, which modulate learning and memory will also influence LTP. Indeed, *in vivo* electrophysiological studies have described a diurnal rhythm of synaptic excitability in the dentate gyrus (DG) of monkeys and rats (Barnes et al., 1977; West and Deadwyler, 1980; Cauller et al., 1985). In addition, *in vivo* recordings from anesthetized rats have also revealed a diurnal rhythm in LTP from hippocampal granule cells (Dana and Martinez, 1984). Finally, *in vitro* studies have reported diurnal variations in the incidence and magnitude of LTP in the CA1 region of the rat and hamster hippocampus with the largest responses occurring in the day (Harris and Teyler, 1983; Ragavan et al., 1999).

In the current study, we first examined the possibility of a diurnal variation in excitability and LTP in hippocampal brain slices from C3H and C57 BL/6 mice. While this type of work has been done in rats and hamsters, extending this work to mice with their well-characterized genetics allows a range of experiments not possible with other mammals. In addition, the daily rise and fall of melatonin (MEL) are under the control of the circadian system and previous studies suggest that this hormone can regulate synaptic plasticity (e.g. Collins and Davies, 1997; El-Sherif et al., 2003). Therefore, in the current study, we compared LTP in fEPSP and PS in a strain of mice that secretes MEL rhythmically (C3H) to another strain (C57 BL/6) that carries a mutation that does not allow the production of MEL (Ebihara et al., 1986). Finally, we determined whether the day/night differences seen in a light-dark (LD) cycle continues when the animals were maintained in constant darkness (DD). This experiment provides a critical test of the hypothesis that an endogenous circadian oscillator drives the observed diurnal variations.

Materials and Methods

Animals and Lighting Conditions: Two to four month old male mice (C-57 BL/6 or C3H) were purchased from Charles River Laboratories. The University of California, Los Angeles Animal Research Committee approved the experimental protocols used in this study. Three types of experiments were performed. First, to investigate possible diurnal variations in any of the measured values, animals were maintained on a daily LD cycle consisting of 12 hrs of light followed by 12 hrs of dark. It is already well established that cells in the SCN continue to show circadian oscillations when isolated from the animal in a hypothalamic brain slice preparation (e.g., Green and Gillette, 1982) and we sought to determine if diurnal differences in LTP could also be found in the hippocampus. For the day group, brain slices were prepared at zeitgeber time (ZT) 3 and recordings made between ZT 4 and 12. For the night group, brain slices were prepared at ZT 15 and recordings made between ZT 16 and 24. By convention, ZT 12 is the time that the lights go off for organisms held in an LD cycle. Second, some mice were kept in an LD cycle and killed in the day (ZT 11) and evoked responses were recorded at night (from ZT 13-18). With these experiments, we sought to determine if the hippocampal slices were able to transition from the day to the night state *in vitro*. Finally, experiments were conducted to determine if the rhythm continues in DD and could be considered a circadian oscillation. For these experiments, mice were housed individually and their wheel-running activity was recorded as revolutions per 3 min intervals and the time of activity onset determined. The running wheels and data acquisition system were obtained from Mini-Mitter Company (Sunriver, OR). After entrainment to an LD cycle, the mice were placed into DD for a week to assess their free-running activity pattern. For the day group, brain slices were prepared at circadian time (CT) 3 and recordings made between CT 4 and 12. For the night group, brain slices were prepared at CT 15 and recordings made between CT 16 and 24.

By convention, CT 12 is the time of activity onset for nocturnal organisms held in DD. All

handling of animals was carried out either in the light portion of the LD cycle or in the dark with the

aid of an infrared viewer (FJW Optical Systems, Palatine, IL).

Slice Preparation: Brain slices were prepared using standard techniques from mice between 2-4

months of age. Mice were anaesthetized with halothane and decapitated either in the day or the

night as described above. The brains were dissected and placed in cold oxygenated artificial

cerebral spinal fluid (ACSF) containing (in mM) NaCl 130, $NaHCO_3$ 26, KCl 3, $MgCl_2$ 5,

NaH_2PO4 1.25, $CaCl_2$ 1, glucose 10 (pH 7.2-7.4; osmolality 290-300 mOsm). Coronal

hippocampal slices (400μM thick) were prepared using a microslicer (DSK Microslicer; Ted Pella,

Redding, CA). Slices were then transferred into an ACSF in which the $CaCl_2$ was increased to 2

mM, $MgCl_2$ was decreased to 2 mM. Slices were allowed to recover for at least 1 hour prior to

starting electrophysiology recordings. Slices were constantly oxygenated with $95\%O_2$-5% CO_2.

Besides the hippocampus, the slice contained some elements of the entorhinal cortex,

parahippocampal gyrus, and subcortical white matter.

Electrophysiology Recording: Briefly, slices were placed in an interface chamber (Fine Science

Tools, Foster City, CA) and continuously superfused with oxygenated ACSF (30^oC) at 2-3 ml/min.

A bipolar stimulating electrode constructed from nichrome wire (0.0015" diameter, A-M Systems,

Carlborg, WA). The electrode was placed in the stratum radiatum in the CA1 region of the

hippocampus to stimulate presynaptic fibers arising from the CA3 pyramidal cells. The slices were

stimulated with negative current pulses with an A-M Systems stimulator at 0.02 Hz (100 μs

duration). The recording electrodes were pulled on a multistage puller (Sutter P-97, Novato, CA)

and filled with ACSF (5-10 MΩ filled with ACSF). The field potentials were typically 1-3 mV in

amplitude and were amplified 100X with Axon Instruments 2A amplifier (Axon Instruments, Foster City, CA) and an external amplifier. Responses were filtered at 5 kHz and digitized (10 kHz) using data acquisition and analysis programs (pClamp9, Axon Instruments).

Typically, stable baseline measurements were obtained within an hour after placing hippocampal slices in the interface chamber (0.02 Hz stimulation). At this point, post-synaptic responses were recorded for at least 10 minutes prior to the induction of LTP. During this time, input–output (I–O) relations were generated by varying the stimulation intensity from 0 to 100 μA in steps of 10 μA. Information was also gathered about the peak amplitude, slope, and duration of evoked responses at half maximal amplitude. These evoked responses were stable and changed less than 10% over the course of 60 min of recording ($5 \pm 8\%$, N=5). For LTP experiments, the baseline presynaptic stimulation was delivered at 0.02Hz (100 μs duration) using a stimulation intensity that evoked approximately 50% of maximal post-synaptic response. After tetanus, stimulation was again delivered at 0.02Hz (100 μs duration). In most cases, LTP was evoked by a single tetanizing stimulus (1 x 100 Hz, 1sec duration). In some cases, a more robust form of LTP was evoked by repeated tetanizing stimuli (3 x 100 Hz, 1sec duration, inter-trial interval 15 sec). Different groups of animals were used for the PS and fEPSP recordings.

Analyses: For LTP experiments, post-tetanic responses were normalized to baseline, as is standard in the field. We used 3 separate statistical analyses to assess possible between-group differences in LTP. First, possible differences in average LTP were assessed using Friedman Repeated Measures Analysis of Variance (RM ANOVA) on Ranks followed by post-hoc pair-wise comparison (Dunnett's Method). The alpha level for all analyses was set at 0.05. Next, the post-tetanus data was grouped into 10 min bins (10, 20, 30, 40, 50, 60 min) and pair-wise comparisons made using Tukey's t-test or Mann-Whitney Rank Sum Test. Finally, the post-tetanus data from each slice was

fitted with a nonlinear regression. The best fit (R>0.9) was obtained with an equation that had an exponential decay and linear component ($y = y_0 + ae^{-bx} + cx$). This equation was used to determine the slope of the linear component of the curve ("c" in the equation) that we took for a measure of the rate of decay of LTP.   The slope of the exponential decay was determined in large part by the first couple of measurements after tetanus. This exponential decay did not significantly vary between groups. The best-fit values were then compared using Tukey's t-test. In the text, the sample size (N) refers to the number of slices in each group. In all cases, the slices in an experimental group come from at least 5 mice. Some control groups came from just 3 mice. For example, the control slices prepared in the day and recorded in the late day were made from just 3 mice. Values were considered significantly different if $P < 0.05$. All tests were performed using SigmaStat (SPSS, Chicago, IL). In the text, values are shown as mean ± SEM.

Results:

LTP in C3H mice

A diagram of the hippocampus showing placement of the stimulating and recording electrode is shown in Fig. 1A. The first set of experiments was designed to determine if LTP recorded from the CA1 region varies as a function of time of day in C3H mice. For these experiments, we recorded fEPSP or PS from the CA1 region of hippocampal slices evoked by stimulation of the SC pathway before and after application of a high frequency stimulus train (1x100 Hz, 1 sec duration, Fig. 1B). To determine if the magnitude of LTP varies within the day or the night, we compared data collected during ZT 4-6 (early day) with ZT 7-12 (late day) as well as data collected between ZT 16-18 (early night) with ZT 19-24 (late night). There were no significant differences between the magnitude of LTP recorded during the early and late segments of the day or night. Accordingly for these studies, the data collected between ZT 4-12 and ZT 16-24 were pooled to form the day and night groups, respectively (Fig. 1C). Overall, we found that the magnitude of the enhancement of the PS was greater in LTP recorded from night slices compared to day slices of C3H mice (Fig. 2A, RM ANOVA, Chi-square = 146, $P < 0.001$). The average PS slope was $334 \pm 12\%$ (N=7) of baseline during the night was larger and $177 \pm 15\%$ (N=9) of baseline during the day. The amplitude of the PS increased more (Mann-Whitney Rank Sum Test, T = 120.5, P = 0.014) during the night (Pre-tetanus: -1.98 ± 0.2 mV; Post-tetanus: -5.0 ± 0.5 mV) than during the day (Pre-tetanus: -1.28 ± 0.2 mV; Post-tetanus: -2.25 ± 0.4 mV). PS slopes were significantly larger in the night at all time points (Mann-Whitney Rank sum test, T = 82-87, P = 0.02-0.004). There were no significant differences in the latency-to-peak induction (Day: 1.6 ± 0.2 min; Night: 2.4 ± 0.5 min). Finally, in order to measure the rate of decay of the potentiated PS, the post-tetanus data collected from each slice were fitted to a non-linear regression. The rate of decay was measured as the slope

of the fitted equation. By this measure, the decay of LTP was faster in the day (slope = -0.48, R=0.95) than in the night (slope = 1.6, R=0.92) but this difference was not significant.

<mark>Figure 2, 1 column, 6 inches</mark>

A day/night difference was also found in LTP recorded from the fEPSPs in C3H mice. The average fEPSP slope was $165 \pm 1.2\%$ (N = 21) of baseline during the night and $152 \pm 1.9\%$ (N = 21) of baseline during the day (RM ANOVA, Chi-square = 69, P < 0.001). A comparison of the fEPSP slopes indicated that slopes were significantly larger in the night only at the 50 min (t-test, t = -2.532, df = 40, P = 0.015) and 60 min (t-test, t = -2.95, df = 40, P = 0.005) time points. While there were no significant day/night differences in the latency-to-peak induction (day: 1.7 ± 0.2 min; night: 1.7 ± 0.1 min), the rate of decay of LTP was significantly faster in the day (slope = -0.66; R = 0.97) than the night (slope = -0.19; R = 0.93) groups (t-test, t = -2.08, df =38, P =0.04). We did not see a diurnal difference in LTP in response to a stronger tetanizing protocol (3x100 Hz, 1sec, inter-trial interval 15 sec; data not shown). The average fEPSP slope was $229 \pm 2\%$ of baseline during the night (N = 12) and $221 \pm 5\%$ of baseline during the day (N = 12). No day/night differences were found in the fEPSP slopes averaged into 10 min bins using a stronger tetanus (data not shown).

LTP in C57 BL/6 mice

The next set of experiments was designed to determine if LTP recorded from the CA1 region also varies as a function of time of day in C57 BL/6 mice. Again, we compared data collected during early (ZT 4-6) and late (ZT 7-12) day as well as data collected between early (ZT 16-18) and late (ZT 19-24) night. There were no significant differences between the magnitude of LTP recorded during the early and late portions of the day or night. Accordingly for these studies, the data collected between ZT 4-12 and ZT 16-24 were pooled to form the day and night groups,

respectively. We found that the magnitude of the enhancement of the PS was greater in LTP recorded from night slices compared to day slices of C57 mice (Fig. 2B; RM ANOVA, Chi-square = 74, P < 0.001). This diurnal difference was significant but more modest than that observed with the C3H mice. The average PS slope was 168 ± 7% of baseline during the night (N=12) and 140 ± 5% of baseline during the day (N = 12). The amplitude of the PS increased more (t-test, t = -2.508, p = 0.020) during the night (Pre-tetanus: -1.5 ± 0.2 mV; Post-tetanus: -2.6 ± 0.4 mV) than during the day (Pre-tetanus: -1.4 ± 0.1 mV; Post-tetanus: -1.8 ± 0.4 mV). Although the PS slopes were always larger during the night, these differences were only significant at the 50 min (t-test, t = -2.656, df = 22, P = 0.014) and 60 min (t-test, t = -2.231, df = 22, P = 0.036) time points. There were no significant differences in the latency-to-peak induction (Day: 1.4 ± 0.1 sec; Night: 1.7 ± 0.3 sec) or in the rate of the decay of LTP between the day (slope = -0.28; R = 0.92) and night (slope = -0.16; R = 0.97) groups.

Some evidence for a diurnal difference was also found in LTP recorded from the fEPSPs in C57 mice. The average fEPSP slope was 151 ± 5% (N = 21) of baseline during the night and 133 ± 41% (N = 28) of baseline during the day (RM ANOVA, Chi-square = 27, P < 0.001). The fEPSP slopes were significantly larger in the night at the 50 min (t-test, t = -2.446, df = 47, P = 0.018) and 60 min (t-test, t = -2.656, df = 47, P = 0.011) time points. While there were no significant day/night differences in the latency-to-peak induction (Day: 1.4 ± 0.1 sec; Night: 1.4 ± 0.1 sec), the rate of decay of the LTP was faster in the day (slope = -0.21; R=0.98) than the night (slope = 0.23; R = 0.94) groups. With the stronger protocol, no diurnal differences were observed (data not shown). The average fEPSP slope was 192 ± 1.7% of baseline during the night (N=13) and 190 ± 1.6% of baseline during the day (N=14). No day/night differences were found in the fEPSP slopes averaged into 10 min bins using the stronger tetanus (data not shown).

Current-response relationships in C3H and C57 mice

We also carried out experiments designed to determine whether there was a diurnal difference in evoked fEPSP and PS in hippocampal slices prepared from C3H or C57 mice measure in day (ZT 4-12) or night (ZT 16-24). For these experiments, we varied the stimulus intensity and recorded the resulting slope to characterize the input/output (IO) relationship for these evoked responses (Fig. 3). A change in the gain of IO curve could indicate a change in sensitivity of the neuron to excitatory input. The C3H mice did not exhibit a day/night difference in IO curves measured for either the fEPSP or the PS. In contrast, slope of the IO curves for the PS recorded in the night from C57 mice were significantly larger then the slope IO curves recorded in the day (t-test, $t = 3.315$. $df = 24$, $P = 0.003$). The magnitude of the PS was larger in the night at each of the stimulus intensities between 30 and 100 μA. No diurnal differences were found in the IO curves measured from the fEPSP of C57 mice. Other electrophysiological parameters of the evoked responses were not found to vary between day and night (Table 1).

Endogenous rhythm in LTP in PS in C3H mice

Finally, two sets of experiments were performed on the C3H mice to explore the possible endogenous nature of the day/night differences observed in the PS recordings. In the first set (Fig 4A), C3H mice were kept in a LD cycle and killed in the day (1 hour prior to lights off at ZT 11) and evoked responses were recorded at night (from ZT 13 onwards). With these experiments, we sought to determine if the hippocampal slices were able to transition from the day to the night state *in vitro*. The PS of slices prepared during the day, but recorded during the night, had an LTP profile remarkably similar to the night group. As described above, the average PS slope was 334% of

baseline during the night and 177% of baseline during the day. The transition group exhibited an average PS slope of $288 \pm 4\%$ of baseline (N = 7) which was significantly greater than the day (RM ANOVA, Chi-square = 146, P < 0.001) but not the night. For control slices prepared in the day and recorded in the late day (ZT 11-12), the PS slope was $167 \pm 6\%$ of baseline (N = 3). The average PS amplitude was $180 \pm 50\%$ of baseline during the night and $74 \pm 9\%$ of baseline during the day. The transition group exhibited an average PS amplitude of $129 \pm 18\%$ of baseline which was significantly greater than the day (ANOVA, H = 9.2, P = 0.01) but not the night. Similar results were obtained with the fEPSP recordings. As described above, the average fEPSP slope was 165% of baseline during the night and 152% of baseline during the day. The transition group exhibited an average PS slope of $172 \pm 2\%$ of baseline (N = 12). The transition group also exhibited a slower rate of decay that is characteristic of the data collected during the night but not during the day. For control slices prepared in the day and recorded in the late day (ZT 11-12), the fEPSP slope was $146 \pm 12\%$ of baseline (N = 4).

<mark>Figure 4, 1 column, 6 inches</mark>

In order to determine if the previously observed diurnal rhythm in PS LTP in C3H mice was an endogenous circadian oscillation that continues in constant conditions, recordings were carried out in slices from mice that were initially entrained to a LD cycle then maintained in DD prior to experimentation. Free running activity rhythms of the mice were monitored and animals were killed at their individually calculated CT3 or CT15 (Fig. 4B). Overall, we found that the magnitude of the enhancement of the PS was significantly greater in LTP recorded from subjective night slices compared to subjective day slices (RM ANOVA, P = 0.008). The average PS slope was $243 \pm 4\%$ of baseline during the night (N = 9) and $157 \pm 2\%$ of baseline during the day (N = 9). The amplitude of the PS increased more during the subjective night (Pre-tetanus: -1.1 ± 0.1 mV; Post-

tetanus: -2.9 ± 0.3 mV) than during the subjective day (Pre-tetanus: -1.1 ± 0.1 mV; Post-tetanus: -1.6 ± 0.2 mV). A comparison of the PS slopes indicated that slopes were significantly larger in the night at the 10, 30, 40, 50, and 60 min time points (Fig. 6C; Mann-Whitney Rank sum test, $T = 50$-59, $P = 0.02$-0.004). There were no significant differences in the latency-to-peak induction or in the rate of the decay of LTP between the subjective day and night groups.

Discussion:

In recent years there has been a resurgence of interest in state-dependent changes in both learned behaviors and in the cellular/molecular events that may underlie learning and memory. In particular, there has been much discussion as to the possible relationship between sleep and learning (e.g. Maquet, 2001; Siegel 2001; Stickgold et al., 2001; Walker et al., 2002; Walker et al, 2003). While the role of sleep in learning remains to be defined, sleep deprivation protocols can be shown to have a negative impact on learning as well as impact hippocampal physiology (Campbell et al., 2002; Davis et al., 2003; McDermott et al., 2003). Less studied, but perhaps equally important, is the temporal regulation imposed by the circadian timing system based in the SCN. The circadian clock is thought to broadly regulate the arousal of the nervous system. For example, the behavioral literature provides evidence for diurnal (e.g. Holloway and Wansley, 1973ab; Wansley and Holloway, 1975; Stephan and Kovacevic, 1978; Valentinuzzi et al., 2001) and circadian modulation in some learned behaviors (Chaudhury and Colwell, 2002; Fernandez et al., 2003; Valentinuzzi et al., 2004). Several *in vivo* studies have found evidence for diurnal regulation of hippocampal excitability (Barnes et al., 1977; West and Deadwyler, 1980; Cauller et al., 1985). This data also provided some indication that the rhythms may be under circadian variation. For example, Barnes and colleagues (1977) showed that a rhythm in synaptic excitability continued in one blinded rat and Cauller and coworkers (1985) reported that the rhythm in responsiveness to perforant path stimulation continued in one rat maintained in DD. While the role of the SCN in these rhythms has not yet been established, a few studies have also provided evidence that synaptic plasticity in hippocampus may also vary with the time of day (Harris and Teyler, 1983; Dana and Martinez, 1984; Ragavan et al., 1999).

In the current study, we found that the magnitude of the potentiation of the PS due to high frequency stimulation (1x100 Hz) is larger in the night than in the day. This diurnal difference was

most strikingly illustrated in C3H mice in which the night group exhibited an average potentiation that was about 70% larger than that measured during the day. Similarly, previous *in vivo* studies in the rat hippocampus found evidence for diurnal rhythms in LTP magnitude which peaked during the night (Dana and Martinez, 1984; Leung et al., 2003). Other *in vitro* LTP studies found evidence for rhythms in the magnitude of PS LTP in CA1 neurons peaked in the day in the rats (Harris and Teyler, 1983) and hamsters (Raghavan et al., 1999). So while day/night differences in LTP have now been found in several studies, the time of peak induction appears to vary perhaps because of species differences or differences in the protocols used to evoke LTP. In addition to diurnal differences in magnitude, we also found evidence to suggest that LTP induced in the night was more stable than LTP measured during the day. Specifically, our data demonstrates that the decay in LTP in the fEPSP was slower at night compared to the day. This difference in the kinetics in the decay of LTP was observed in slices from both C3H and C57 mice. Interestingly, REM sleep deprivation also causes a dramatic reduction in the stability of the maintenance phase of LTP in hippocampal slices (Davis et al., 2003). The day/night difference could be overcome with a stronger induction protocol (3x100 Hz), at least within the 60 min post-tetanus that we measured within the present study.

The circadian variation we observed in the in the magnitude of LTP recorded from the cell body layer (PS) was primarily an effect on amplitude while the fEPSP exhibited more of an effect on the stability or rate of decay of LTP. The fEPSP slope is viewed as a measure of the excitatory drive to the CA1 pyramidal neurons whereas the population spike amplitude reflects the number of pyramidal neurons producing action potentials. The CA1 cell bodies are known to receive strong GABA-mediated innervations from interneurons within the hippocampal circuit to powerfully control the excitability of CA1 pyramidal neurons (Freund & Buzsáki, 1996) and are involved in the generation of network oscillations within the hippocampus (McBrain et al., 1999). Thus the striking

diurnal variation in the magnitude of the PS may be more the result of a change in the balance between inhibition and excitation rather then purely a regulation of glutamatergic synaptic transmission (see Marder and Buonomano, 2003).

Previous *in vivo* recordings from the hippocampal region have found some evidence for diurnal changes in basic neural excitability. An earlier study investigating variation in IO relationship *in vivo* found that evoked responses in the dentate gyrus and CA1 of rat hippocampi varied as a function of the behavioral state of the animals (Winson and Abzug, 1977). Later work by West and Deadwyler (1980) showed that PS amplitude was greater in the light then in the dark period of the rat and that spike amplitude was greater when the animals were in their slow wave sleep state compared to other states. Additionally, a report by Cauller et al. (1985) using *in vivo* recordings found that fEPSP magnitude peaked in the night while PS magnitude peaked in the day. These rhythms in hippocampal excitability may continue in the brain slice preparation. For example, in CA3 neurons, both depolarization-induced excitability and the magnitude of high-voltage activated Ca^{2+} currents vary with the time of day with peaks during the night (Kole et al., 2001). Similarly, a study by Liu et al., (2000) showed the existence of diurnal differences in synaptic excitability in the dentate gyrus and that blocking adenosine receptors eliminated the diurnal variation. In the present study, we investigated whether there was a diurnal variation in baseline excitability in dendritic (fEPSP) and somatic (PS) regions of the post-synaptic CA1 pyramidal cells of hippocampal slices from C57 and C3H mice. We only found significant differences with the PS recorded from C57 mice. At least one previous study found that the IO curves are larger in C3H compared to C57 BL/6 mice (Bampton et al., 1999) and our data confirms their observations.

Two experiments were performed to explore the possible endogenous nature of the day/night differences observed in the PS recordings in the C3H mice. In one, in order to determine

if the previously observed diurnal rhythm in PS LTP in C3H mice is endogenous and, presumably circadian in nature, recordings were carried out in slices from mice maintained in DD prior to experimentation. Free running activity rhythms of the mice were monitored and animals were killed at their individually calculated subjective day or night. Again, we found that the magnitude of the enhancement of the PS was significantly greater in LTP recorded from brain slices in the subjective night compared to subjective day. Acute effects of light or the endogenous timing system can drive a rhythm seen in mice held in an LD cycle. The finding that the rhythm continues in mice held in DD strongly implicates an endogenous timing system as being responsible for the rhythm in LTP. Ideal circadian rhythm studies follow a measured variable in an individual through time and show that the magnitude varies with the circadian cycle. This is not possible for this type of LTP measured in acute hippocampal slices but instead we are able to demonstrate that the magnitude of the potentiation across a population varies as a function of time even when the organisms are held in constant conditions. In the other, mice were killed in the light portion of an LD cycle while the evoked responses were recorded at night. We reasoned that if the isolated hippocampus has some oscillatory properties, then the time of slice preparation should not be as important as the time that LTP was recorded.   We investigated whether slices prepared in the day but recorded in the night would show potentiated evoked responses similar to those from slices prepared and recorded in the day or in the night. The resulting LTP was strikingly similar in both the higher magnitude and slower decay kinetics to the previous night group. Control slices recorded in the late day (ZT 11-12) still exhibited typical "day" magnitudes and kinetics of LTP. To us, this observation suggests that the hippocampal slices are not "frozen" at the time of slice preparation but instead continue to change from the day to the night state *in vitro*.

These experiments do not allow us to know the anatomical location of the oscillator responsible for driving rhythms in hippocampal synaptic plasticity. One possibility, that we favor,

is that this rhythm is due to some type of oscillator mechanism within the hippocampus itself. Our finding that the isolated hippocampal slice appears to transition from day to night supports this possibility. Interestingly, at least one study has found expression of circadian clock genes mPer1 and mPer2 mRNA in the hippocampus may be rhythmic with peak expression in the night (Wakamatsu et al., 2001). However, it is also possible that an oscillator in the SCN or another brain region has long-lasting influences on the rhythmicity of hippocampal neurons. A slow-acting signal from another location could already have reached the hippocampus prior to the preparation of the brain slice. While this signaling seems less likely then a direct hippocampal circadian oscillator, it is also hard to exclude.

In the current study, we took advantage of strain differences between C57 and C3H mice to examine the possible role of MEL as a signal responsible for driving these rhythms in synaptic plasticity. MEL is a rhythmically synthesized hormone that has been suggested to be an output molecule by which the circadian system transmits temporal information. MEL mediates its effects via high-affinity receptors found throughout the nervous system including the hippocampus, the subiculum, and entorhinal cortex (Weaver et al., 1989; Siuciak et al., 1990; Musshoff et al., 2002). Electrophysiological studies of CA1 neurons have reported that MEL increases the spontaneous firing rate (Musshoff et al., 2002) as well as inhibits $GABA_A$ mediated currents (Wan et al., 1999). Furthermore, previous studies have found that MEL can regulate synaptic plasticity measured in hippocampal neurons (Collins and Davies, 1997; El-Sherif et al., 2003). Thus, it is possible that the rhythms in LTP are driven by a rhythm in the secretion of MEL. We addressed this hypothesis by comparing LTP in fEPSP and PS in a strain of mice that secretes MEL rhythmically (C3H) to another strain (C57) that carries a mutation that does not allow the production of MEL (Ebihara et al., 1986). The diurnal rhythm was more robust in the MEL-secreting C3H mice and suggests that more work is needed to clarify the possible role of MEL as a regulator of LTP and learning. It is

also clear that the rhythms were still present in C57 mice that do not secrete MEL. Thus, while MEL may be an important regulator of hippocampal physiology and synaptic plasticity, this hormone cannot be solely responsible for the rhythms observed in the current study.

Circadian rhythms in hippocampal function could be directly, or indirectly, driven by a variety of factors whose levels change with the daily circadian cycle. For example, the circadian system has a profound regulatory effect on activity levels in animals and changes in activity levels may alter hippocampal physiology. Even access to running wheel enhances performance on learning tasks as well as the magnitude of LTP recorded from hippocampal brain slices during the day (e.g. Van Praag et al., 1999; Farmer et al., 2004). A recent *in vivo* study demonstrated that hippocampal LTP was larger when rats were active (night) than when sleeping or awake but immobilized, and provided evidence that cholinergic activity may be responsible (Leung et al., 2003). There have been reports of daily activity-driven fluctuations of adenosine in the hippocampus *in vivo* (Huston et al., 1996; Portas et al., 1997) and adenosine also modulates hippocampal synaptic transmission (Liu et al., 2000). Besides activity driven changes, the levels of many hormones are also known to vary rhythmically. For example, the levels of circulating cortisol vary with a strong diurnal rhythm and a variety of evidence suggests that this hormone can impact hippocampal physiology and LTP (Lupien and McEwen, 1997; Pavlides et al., 2002). Dana and Martinez (1984) previously showed that *in vivo* PS LTP was greater at night in control animals while in adrenalectomized rats the effect was reversed. Taken as a whole, previous work has identified a large number of signaling molecules that alter the magnitude of LTP (Lisman et al., 2003; Silva, 2003) and the levels of some of these molecules are known to be rhythmic within the hippocampus. Thus, while it may be premature to speculate on the underlying mechanisms responsible for the circadian variation in hippocampal LTP, there are a number of candidate processes that can be investigated.

Finally, while it is always difficult to link cellular/molecular research to behavioral endpoints, we would like to consider the possible functional significance of these rhythms in synaptic plasticity within the hippocampus. Among other functions, the circuitry in the hippocampus has been clearly linked to memory formation especially declarative memories i.e. the encoding, storage, and recall of memories for specific events. We do not yet know to what extent our findings at the SC/CA1 synapse will apply to the other synapses in the hippocampus nor is the relationship between LTP at a single synapse and learning always clear (e.g. Martin et al., 2000; Sweatt, 2001). Nevertheless, in the simplest case, we would speculate that the rhythms that we see in LTP at the SC/CA1 synapse would lead to rhythms in hippocampal-dependent learned behaviors. Our finding that LTP peaked during the active phase of the mouse's activity cycle is consistent with previous data indicating that performance in hippocampal-dependent learned behaviors peaked during the night for rodents (Haffmann and Balschun, 1992; Valentinuzzi et al., 2001; Valentinuzzi et al., 2004; but also see Chaudhury and Colwell, 2002). Our finding that the strong tetanus protocol overcame the diurnal regulation is also consistent with behavioral data indicating the strongly trained rodents are less likely to express rhythms in learning (Chaudhury and Colwell, 2002; Valentinuzzi et al., 2004). More broadly, there is evidence that the time of day is an aspect of the physical environment that can be learned much in the same way that an organism can learn spatial parameters. A number of studies have documented the ability of organisms to learn to go to specific locations at specific times of day (see Daan, 2000). This type of time-place learning (called "Zeitgedächtnis" or time memory) in which the time of day is used as a discriminatory cue for choosing among two or more food locations has been described in several species (e.g. Biebach et al., 1991; Mistlberger et al., 1996; Carr and Wilkie 1997; Means et al., 2000). These learned associations of time and place may not involve the SCN-based circadian system but would be expected to be dependent upon an intact hippocampus. In general, time of day variation in learning and memory has been described in many

species including humans (e.g. Dijk et al., 1992). Unfortunately, the underlying mechanisms,

including the possible involvement of the circadian system, have not been carefully investigated. To

our mind, the work in the present study certainly supports the hypothesis that the circadian system

can regulate the ability of animals to learn and demonstrates that this regulation can occur at the

level of cellular mechanisms that underlie learning.

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Fig. 1: Schematic of experimental design. Panel A shows schematic of hippocampus illustrating electrode placement for stimulating as well as recording electrodes. Panel B shows examples of fEPSP and PS before and after tetanus. Panel C illustrates the experimental design in which day/night comparisons were made by preparing brain slices at ZT 3 and recording between ZT 4-10 (Day) or preparing tissue at ZT 15 and recording between ZT 16-22 (Night).

Fig. 2: Diurnal variation in LTP measured in cell body region of CA1 neurons in C3H and C57 mice. Panel A shows plots of PS slope (normalized as a % of baseline) as function of time measured in the day and night in the C3H mice. Panel B shows plots of PS slope (normalized as a % of baseline) as function of time measured in the day and night in the C57 mice. The tetanus of 1x100 Hz stimulation was given at time = 0.

Fig. 3: Input-output (IO) curves illustrating the relationship between the magnitudes of stimulation current and evoked response for PS recorded from C3H (top panel) and C57 (bottom panel) mice. The C3H mice did not exhibit a day/night difference in IO curves measured for the PS (top panel). In contrast, the IO curves for the PS recorded in the night from C57 mice were significantly larger then the IO curves recorded in the day (bottom panel). The magnitude of the PS was larger in the night at each of the stimulus intensities between 30 and 100 μA. No diurnal differences were found in the IO curves measured from the fEPSP of C3H or C57 mice (data not shown).
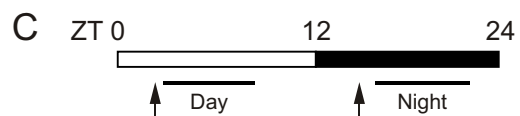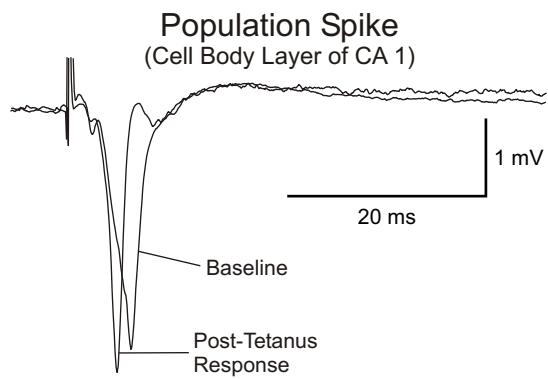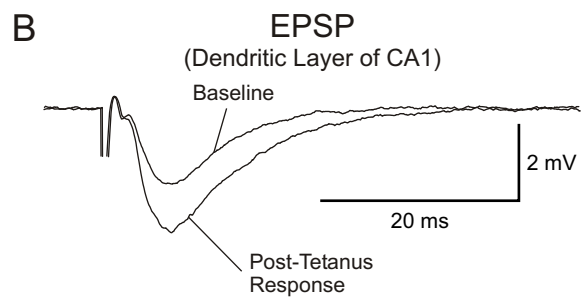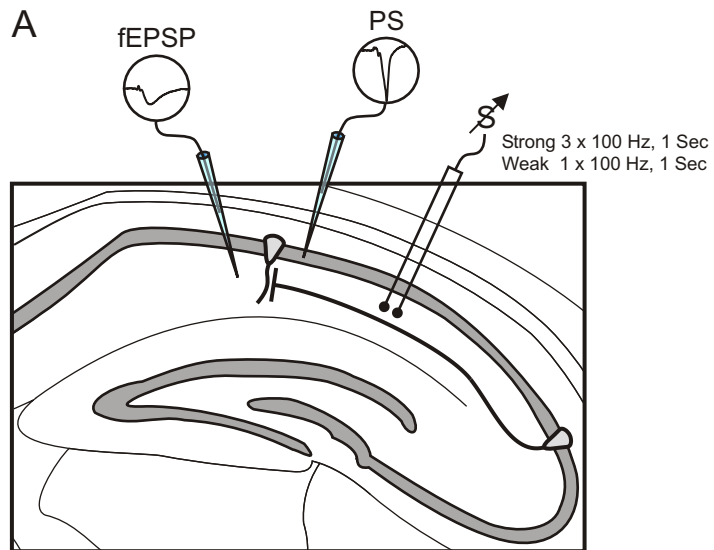
Fig. 4: Endogenous rhythms recorded from isolated hippocampal slices. **A)** C3H mice were kept in a LD cycle and killed in the day (1 hour prior to lights off at ZT 11) and evoked responses were

recorded at night (from ZT 13-18). The PS LTP of slices prepared during the day but recorded

during the night had the profile remarkably similar to the night group. Similar results were obtained

with the fEPSP recordings (data not shown). **B)** Animals were maintained in DD and the onset of

activity was taken as CT 12. Plots of PS slope (normalized as a % of baseline) as function of time

measured in the subjective day and subjective night. The PS LTP was larger in the subjective night.

For both sets of experiments the tetanus of 1x100 Hz stimulation was given at time = 0..
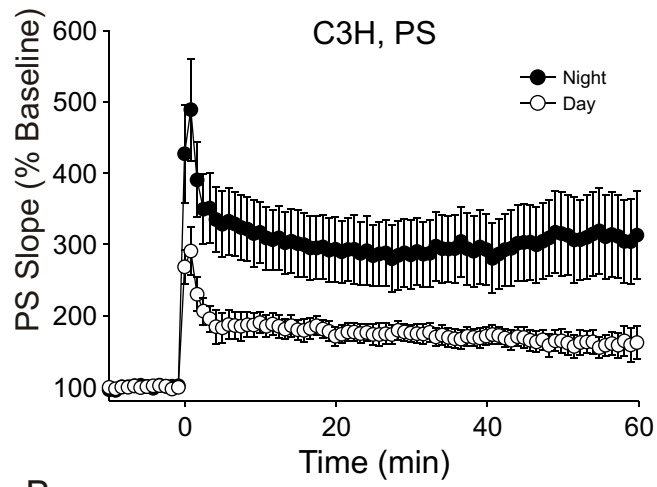
C3H	PS, Day	PS, Night	fEPSP, Day	fEPSP, Night
Amp (mV)	2.0 ± 0.2	1.3 ± 0.2	2.1 ± 0.2	2.0 ± 0.2
Slope(mV/msec)	-0.5 ± 0.1	-0.6 ± 0.1	-0.5 ± 0.1	-0.5 ± 0.1
Duration (msec)	6.8 ± 0.2	5.9 ± 0.7	8.3 ± 0.2	7.7 ± 0.3
50% stimulus (µA)	60 ± 3.5	59.4 ± 4.0	56.8 ± 2.2	53.9 ± 2.4

C57	PS, Day	PS, Night	fEPSP, Day	fEPSP, Night
Amp (mV)	1.4 ± 0.1	1.5 ± 0.2	2.1 ± 0.13	1.6 ± 0.1
Slope(mV/msec)	-0.5 ± 0.1	-0.8 ± 0.1	-0.3 ± 0.1	-0.4 ± 0.1
Duration (msec)	7.1 ± 0.3	6.7 ± 0.5	7.0 ± 0.3	7.3 ± 0.2
50% stimulus (µA)	63.8 ± 2.4	57.7 ± 2.25	64.0 ± 3.2	72.3 ± 1.5

Table 1: Comparison of measured parameters of PS and fEPSP recorded during day (ZT 4-6) and night (ZT 16-18). Before each experiment, we measured amplitude, slope, duration at ½ max, and current to evoke 50% response. Values shown are mean ± SEM.

A

fEPSP

PS

Strong 3 x 100 Hz, 1 Sec
Weak 1 x 100 Hz, 1 Sec

B

EPSP
(Dendritic Layer of CA1)

Baseline

2 mV

20 ms

Post-Tetanus
Response

Population Spike
(Cell Body Layer of CA 1)

1 mV

20 ms

Baseline

Post-Tetanus
Response

C

ZT 0   12   24

Day   Night

A

C3H, PS

● Night
○ Day

B

C57, PS

▼ Night
▽ Day

A

ZT 0   12   24

C3H PS

- ● Night
- ○ Day
- ◇ Day to Night

PS Slope (% Baseline)

Time (min)

B

CT 0   12   24

Sub Day   Sub Night

C3H PS

- ● Subjective Night
- ○ Subjective Day

PS Slope (% Baseline)

Time (min)