2021

Diurnal Variation in Hippocampal Neurophysiology and Pathophysiology

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DIURNAL VARIATION IN HIPPOCAMPAL NEUROPHYSIOLOGY AND PATHOPHYSIOLOGY

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2021

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NEUROSCIENCE

ABSTRACT

Circadian rhythms are ~24-hour cycles in biological processes that are endogenously generated, entrained to light, and synchronized by the suprachiasmatic nucleus of the hypothalamus. One process that is influenced by circadian rhythms is cognitive function, which varies over the course of the day and is likely influenced by changes in neuronal physiology over the course of the day. Dysfunction in circadian rhythms has been documented in many diseases, including Alzheimer's disease (AD). AD is a neurodegenerative disease most notably characterized by dementia, amyloid beta plaques, and tau tangles. There is currently no cure for AD, and treatments only slow disease progression. Evidence for circadian rhythm dysfunction in AD includes sundowning, a worsening of dementia in the evenings, and altered rest-activity patterns. The hippocampus is a brain region crucial for learning and memory, and this region undergoes severe damage in patients with Alzheimer's disease. There has been little work done to investigate day-night differences in hippocampal neurophysiology and how it is impacted in AD. Here, we examined day-night differences in hippocampal neurophysiology in and found diurnal regulation of both inhibition and excitation onto CA1 pyramidal cells. We also found differences in excitability of CA1 pyramidal cells, and most notably, uncovered that the directionality of diurnal regulation depends on position in the hippocampus and sex. We discovered that diurnal regulation of inhibition onto and excitation of hippocampal CA1 pyramidal cells is altered, and spatial working

memory is impaired in mouse models of AD. Together, these data provide new insight into the influence of circadian regulation of hippocampal neurophysiology and emphasize the importance of tailoring studies and treatment interventions to time of day.

Keywords: hippocampus, circadian, diurnal, Alzheimer's disease, cognitive function

DEDICATION

This dissertation is dedicated to my grandmother, Nazel Guillory, who instilled the value of education in me and supported me in every way from K-PhD, to my mother, Angie, who made sure I never quit, and to my puppy Jazz, who always had a smile for me.

This dissertation is also dedicated in honor of the late Lady Vols Coach Pat Summitt whose example inspired me and led me to pursue my studies in neuroscience, and whose words remind me “Left foot, right foot, breathe.”

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Karen Gamble, for welcoming me into her lab, providing the tools I needed to grow, for trusting me, and for remembering that I am a person, not just a data generator.

I would like to thank my committee, Drs. Lori McMahon, Scott Wilson, Erik Roberson, and Douglas McMahon for their guidance throughout the development and completion of this project.

Additionally, I would like to thank Dr. Lori McMahon for providing a place for me to do science when I needed one, and for helping me find my scientific voice. I would also like to thank the McMahon lab members, especially Dr. Allie Widman, for teaching me to let go of perfection, and Nateka Jackson for helping me find the things I needed and answering every scheduling email for me.

I would like to thank Dr. Scott Wilson, for always having time to listen and for answering all of my questions with patience and kindness.

I would like to thank the Gamble Lab members for their support over the last few years, every Taco Mama day, and the good times in Studio CA1. I would especially like to thank my labmate Lacy Goode, who spent too many early mornings and late

nights in and out of lab with me, who supported me when I needed it most, provided countless spicy cookies, and who indulged my every whim, from lava lamps to Bowling for Soup and Studio basketball. I would also like to thank Natalie Remiszewski and Jacob Reeves for being excellent mentees and bringing joy to my last two years of graduate school.

I would like to thank my undergraduate mentors, Drs. Kathy Jackson, William Dees, and Anne Jerse, without whom I would not have learned that I love science and how far it could take me.

Most importantly, I would like to thank the friends I made along the way – the class of 2013, for setting the highest expectations, but helping me reach them. The Science Queens, for all of the texts (even those days of green ones), late nights, and dinner dates. My best grad school friend Matt, for making UAB feel like home. Dr. Rylie Hightower, for every trip to sonic. Dr. Jada Vaden, who was the best post-doc mentor I could have asked for. Snoop Dogg, whose Hollywood star resonates with us all. And especially Dr. Kavitha Abiraman, who stayed up too late, but read every draft, listened to every presentation, and provided endless support both in and out of science.

Lastly, I would like to thank my family for their support, especially my mom, who came to see me every time I asked her to, and even when I didn't.

TABLE OF CONTENTS

LIST OF TABLES

Table *Page*

LIST OF FIGURES

Figure *Page*

1INTRODUCTION

DIURNAL VARIATION IN SYNAPTIC PHYSIOLOGY OF HIPPOCAMPAL AREA CA1

DYSREGULATED CLOCK GENE EXPRESSION AND ABNORMAL DIURNAL REGULATION OF HIPPOCAMPAL INHIBITORY TRANSMISSION AND SPATIAL MEMORY IN AMYLOID PRECURSOR PROTEIN TRANSGENIC MICE

INTRODUCTION

Circadian Rhythms

Organisms' physiology and behavior are influenced by naturally occurring cycles, such as seasonal cycles over the course of a year or day and night cycles of over the course of a day. Because these cycles follow a rhythm, organisms can anticipate changes in their environment and adapt their physiology and behavior accordingly. In particular, organisms are strongly adapted to the cycle occurring over the course of the 24 hours, or the light-dark cycle. Biological processes that repeat over the course of 24 hours are known as circadian rhythms. Circadian rhythms can influence metabolic changes and behaviors that are evolutionarily advantageous to many species, from single-cell organisms to vertebrates. For example, photosynthetic activity in cyanobacteria[1-4], algae[5], and plants[5-7] exhibit circadian rhythmicity. Hormones associated with food intake and metabolism[8,9], social interactions[10], mating, and sleep in mammals are also under circadian influence[11].

For a process to be considered circadian, in addition to having a repeating period of nearly 24 hours, the process must have the ability to be set, or entrained, by external cues, or zeitgebers. Light can serve as a zeitgeber and is the strongest zeitgeber, but other examples of zeitgebers include social interactions and mealtimes[12,13]. The final criterion for a process to be considered a circadian rhythm is that it must persist in the absence of zeitgebers. Maintaining rhythmicity in the absence of zeitgebers is accomplished by an

intrinsic “clock”, which in mammals, is driven by a transcription-translation feedback loop (TTFL) known as the molecular clock. Molecular clocks exist across organisms, from bacteria to plants and animals, although the mechanism isn’t always the same (e.g., TTFL in invertebrates and mammals and both a post-translational oscillator and TTFL in cyanobacteria[14]). These observations lead to the idea that the molecular clock has developed multiple times over the course of evolution[15,16], an example of convergent evolution. This convergent evolution, and the fact that there are several redundancies and paralogs of clock genes within the mammalian clock stress the importance of a biological mechanism of timekeeping.

The mammalian molecular clock

The mammalian molecular clock consists of a main TTFL and an auxiliary TTFL (for review, see[17]). The main loop consists of several core clock genes considered positive regulators and negative regulators. The positive regulators are the protein products of two genes: Brain and muscle ARNT-like 1 (*Bmal1*) and Circadian locomotor output cycles kaput (*Clock*; paralog Neuronal PAS Domain Protein 2, *npas2*)[18,19]. BMAL1 and CLOCK proteins form heterodimers in the nucleus[20] and bind to the E-box regions of the negative regulator genes, *Period* (*Per1/2/3*) and *Cryptochrome* (*Cry1/2*), and of other clock-controlled genes, thus promoting their transcription. *Per* and *Cry* transcripts are then moved from the nucleus to the cytoplasm where they are translated. Once levels of PER and CRY proteins increase in the cytoplasm, they begin to heterodimerize and are then move back into the nucleus. Once in the nucleus, they interfere with the binding of BMAL1 and CLOCK to their E-box domain, thus turning

off their own transcription and completing the cycle. This process occurs over approximately 24 hours. In addition to these core clock proteins, there are several kinases and phosphatases that aid in stabilization and localization of the core clock proteins. These include casein kinase I alpha (CKIα), delta (CKIδ), and epsilon (CKIε), glycogen synthase kinase (GSK3), and protein phosphatases 1 (PP1) and 5 (PP5). The period length of the intrinsic clock is determined by the rate at which PER and CRY enter the nucleus or degrade, which is dictated by phosphorylation states via activity of CKIδ/ε and of PP1/5[21,22] and by ubiquitin tagging for degradation via the proteasome[23-27].

The auxiliary loop aids in maintaining the rhythmicity of the main loop by regulating Bmal1 transcription levels. This is accomplished through transcriptional activation by retinoid-related orphan receptors (RORa, b, and c)[28] and repression by REV-ERB(α/β)[29]. Rhythmic changes in BMAL1 levels are not necessary to drive the main loop, but the auxiliary loop leads to delayed *Cry1* mRNA expression which is important for maintaining proper circadian timing[30]. This molecular clock (**Figure 1**) exists in virtually all tissues of the body[31,32].

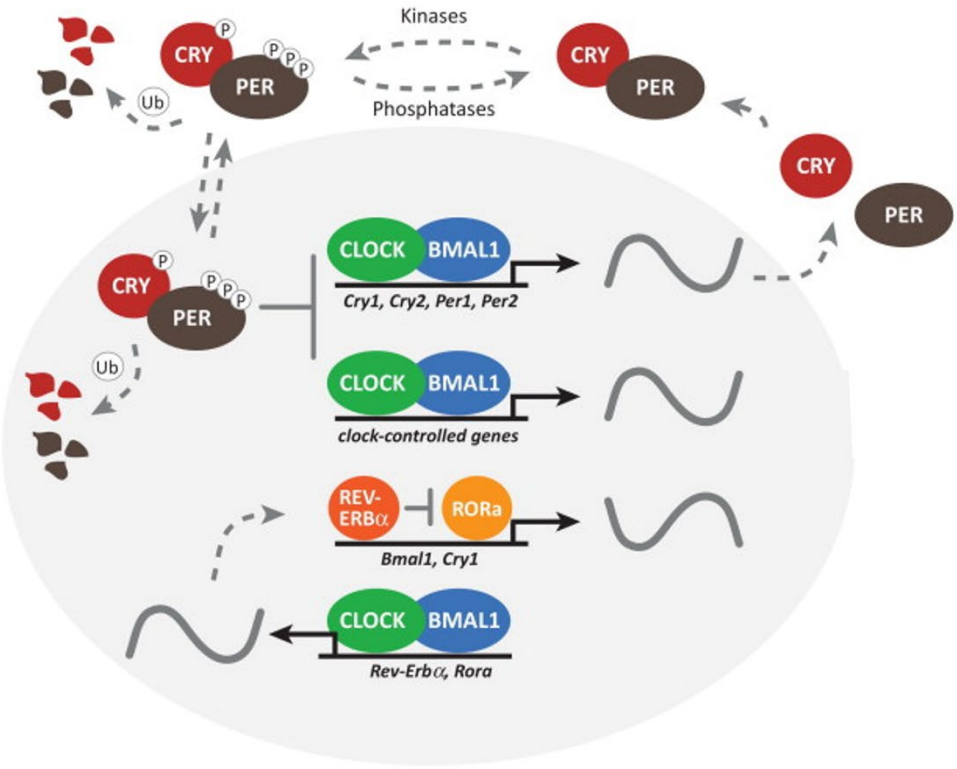

Figure 1. Mammalian molecular clock, adapted from17. Copyright 2014. Adapted with permission.

Mutations in the mammalian molecular clock

The importance of a functional molecular clock is highlighted by the redundancy of clock genes. For example, in the mammalian molecular clock, there are separate genes for *Period1/2/3*, *Cryptochrome1/2*, *Bmal1/2*, and *Npas2*. Mutation or loss of clock components typically results in changes in the period (time from peak to peak, typically ~24 hours), amplitude (magnitude of expression from highest to lowest point), or phase (where the peak and the trough of a rhythm occur) of clock-controlled gene expression, or in behavioral phenotypes that are modulated by the molecular clock. Most single gene mutations can be compensated for; however, *Bmal1* is the only single gene knock-out that

can fully eliminate circadian clock function in central and peripheral tissues[33,34]. Among other things, constitutive *Bmal1* knockout (KO) mice have arrhythmic circadian behavior/physiology and expression of clock and clock-controlled genes, decreased activity, reduced body weight, reduced lifespans, and an early aging phenotype[33,35-37].

The primary pacemaker: the suprachiasmatic nucleus

While all tissues of the body have molecular clocks, the suprachiasmatic nucleus (SCN) of the hypothalamus is considered the primary pacemaker as it is required for the synchronization of circadian rhythms throughout the body[38-40]. The SCN receives input from the retinohypothalamic tract, the pathway by which light signals are transmitted to the brain[41,42]. When light enters the eye, it activates melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs)[43-46]. Axons of the ipRGCs extend through the optic nerve and optic chiasm and synapse at the SCN where they release glutamate, resulting in excitation of GABAergic SCN neurons[47]. SCN neurons transmit time-of-day information to other brain regions by spontaneous action potential firing with a higher frequency during the day compared to night[48]. These neurons exhibit intrinsic rhythmicity in other electrophysiological properties that can affect signaling, including input resistance, resting membrane potential, and action potential waveform properties[49-51]. Expression of sodium, potassium, and calcium channels important for shaping electrical signaling also exhibit rhythmicity in SCN neurons (for review, see[52-54]). The main outputs of the SCN are to the subparaventricular zone and several nuclei of the hypothalamus. There are also projections from the SCN to the paraventricular nucleus of the thalamus, the lateral geniculate nucleus and the lateral septum[55,56]. In addition to these anatomical

connections, there is evidence that the SCN communicates indirectly with other brain regions through paracrine signaling[57].

Circadian regulation of learning and memory

The interplay between circadian rhythms and cognition is well-established through studies in both humans and animal models. Cognitive performance and memory in humans vary over the 24-hour day-night cycle with performance improving rapidly after waking and remaining high until habitual bedtime[58]. Environmental disruptions, such as shiftwork[59], and genetic disruptions in circadian rhythms also result in cognitive impairment.

In humans, studies conducted in the controlled setting of a laboratory have examined the effect of a desynchronization protocol on circadian rhythms in cognition. This was achieved by enforcing either a shorter than usual (20-hour[60]) or longer than usual (24.8 h[61], 28h[62]) day, as opposed to a 24-hour day. In these studies, cognitive performance generally improved over time (i.e., learning occurred) in the synchronized group (i.e., 24-hour day group), whereas learning was significantly impaired in the non-synchronized groups. Outside the laboratory setting, in the context of the real world, the effects of shiftwork on physiological function through disruption of circadian rhythms are well described[63]: shift workers such as nurses[59,64] and control room operators[65] exhibit a lower level of performance during the night shift in comparison to the day shift.

The earliest report of circadian regulation of learning memory described time-of-day differences in a passive avoidance task: rats learned and remembered to avoid a mild foot

shock by remaining in the lighted side of a test chamber better during the day than during the night[66]. This report was followed by numerous studies examining time-of-day differences in a variety of hippocampal-dependent memory tasks (**Table 1**). Importantly, many of these diurnally regulated effects on learning and memory efficiency persist even in the absence of external time cues[67-70].

Process	Assay	Phase (peak/nadir)	Model	Task Description	Refs.
WM	Radial arm maze	Night (21/7)	Sprague–Dawley rats	Rodents (mice or rats) are placed in the center compartment from which 8 equally spaced «arms» extend. Each arm is an elongated column of equal distance, the far end of which may contain a reward (most often food-based) not visible from the center compartment. Throughout an experiment, the animal must remember which arms have been visited to ensure that they do not repeatedly enter arms which do not contain a food reward[71].	72
WM	Radial arm maze	Day (2/14)	C3H/HeN mice		73
LTM	Radial arm maze	Night (21/7)	Sprague–Dawley rats		72
WM	Spontaneous alternation	Night (19/7)	Siberian hamsters	Rodents make alternating goal arm choices in the absence of any reinforcement, therefore considered to be spontaneous[71].	74

ITM	Novel object location	Night (20/8)	Wistar rats	Rodents are allowed to explore an arena with the four objects; in a second trial shortly thereafter, the animal again encounters the four objects, except that two of them have switched positions[71].	75
ITM	Novel object location	Night (16/4)	C57BL/6 mice		76
ITM	Novel object recognition	Night (19/3)	Siberian hamsters	Based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one; the choice to explore the novel object reflects the use of learning and recognition memory[71].	77
LTM (Acq)	Novel object recognition	Night (16/4)	C57BL/6 mice		76
LTM	Alley maze	Night (18/6)	C57BL/6 Ola mice	Rodents have to learn a complex route through a maze leading to their home cage[78].	79

LTM	Contextual fear conditioning	Day (4/16)	C57BL/6 mice	Associative learning task in which mice learn to associate a particular neutral stimulus (often a tone) with an aversive stimulus (often a mild electrical foot shock) and show a freezing response. After repeated pairings of the tone and shock, the animal learns to fear both the tone and training context[71].	69
LTM (Ret)	Contextual fear conditioning	Day (3/21)	C57BL/6J and C-3H mice	Associative learning task in which mice learn to associate a particular neutral stimulus (often a tone) with an aversive stimulus (often a mild electrical foot shock) and show a freezing response. After repeated pairings of the tone and shock, the animal learns to fear both the tone and training context[71].	67
LTM	Passive avoidance	Day (6/18)	Sprague–Dawley rats	Fear-aggravated test in which rodents learn to avoid an environment in which an aversive stimulus (such as a foot-shock) was previously delivered[71].	66
LTM	Morris Water Maze	Night (16/4)	Sprague–Dawley rats	Rodents rely on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform[71]	68

Table 1. Hippocampus-dependent memory tasks regulated by time-of-day, sorted by memory process, adapted from[80]. Copyright 2018. Adapted with permission. Key: WM: working memory; ITM: intermediate-term memory; LTM: long-term memory; Acq: acquisition; Ret: retrieval

Circadian phase disruption also impairs cognitive performance in animal models. Circadian phase can be shifted in Siberian hamsters following exposure to alternating days of either phase-advancing (shifting circadian rhythms earlier) or phase-delaying (shifting circadian rhythms later) light signals. This circadian disruption impairs performance on hippocampal dependent cognitive tasks including novel object recognition and spontaneous alternation[81]. Additionally, chronically phase advancing rats over a period of 64 days impaired performance in the Morris water maze[82]. Notably, this effect of circadian disruption on performance in the Morris water maze has also been shown in other studies[83-85]. Phase shifts of the light-dark cycle also affect the recall of contextual fear conditioning in mice[86].

A potentially less precise approach to disrupting circadian rhythms in animal models involves lesioning the SCN. Mice and rats with SCN lesions exhibit no substantial impairment in avoidance tasks, recognition memory, spatial learning, or reversal spatial learning but have modest deficits in contextual fear conditioning and in the water maze probe test[87-90]. However, SCN lesions cannot rule out the possibility of confounding factors, including damage to nearby brain regions like the hypothalamus, making the interpretation of these experiments rather complicated.

In addition to phase shifts or SCN ablation, circadian rhythm disorder is modelled through genetic mutations in animal models. To survive, animals must be able to associate a reward (such as food) with a particular place at a particular time of day. This

ability is lost in arrhythmic *Cryptochrome1/2* double knock out mice[91]. *Bmal1* knock out mice show impaired habituation to novel environments, suggesting impaired contextual memory formation[92]. Barnes maze and novel object location memory performance, hippocampal-dependent memory tasks, are impaired in a forebrain specific *Bmal1* knock out mouse model[70]. These impairments in learning and memory resulting from genetic ablation of the circadian role indicate its involvement in learning and memory.

There is also an extensive literature on circadian/diurnal rhythmicity of signaling molecules implicated in the formation of short-term and long-term memory. While a full discussion of these findings is outside the scope of this dissertation, see **Table 2** for a summary.

Signaling molecule	Short-term memory	Model organism	Long-term memory	Model organism	Memory retrieval	Model organism	Circadian/ diurnal rhythmicity	Model organism
cAMP	✓	Wistar rats	✓	C57BL/6 × 129/Ola mice	—		✓	C57/BL6 mice
PKA	X	C57BL/6 J mice Sprague-Dawley rats	✓	C57BL/6 J mice Sprague-Dawley rats	—		✓	C3H/H3 N mice
PKC	✓	Wistar rats	✓	Wistar rats	✓	CD1 mice	✓	C3H/He N mice
pMAPK	—		✓	C57/BL6	✓	C57BL/6	✓	C57/BL6 mice
pCREB	X	Wistar rats Long-Evans hooded rats	✓	Wistar rats Long-Evans hooded rats	X	C57BL/6 mice	✓	C3H/H3 N mice C57/BL6 mice
Adenylyl Cyclase	✓	C57BL/6 × 129/SV mice	✓	C57BL/6 × 129/SV mice	—		—	
CAMKI V	X	C57BL/6 N mice	✓	C57BL/6 mice	—		✓	WKY rats
CAMKII	X	129/BL6 mice	✓	129/BL6 mice	X	Wistar rats	✓	C57BL/6 mice

Table 2. Circadian modulation of memory-relevant signaling in rodents, adapted from[93]. Copyright 2018. Adapted with permission. Key: ✓: effect; X: no effect; —: unknown; cAMP: cyclic-adenosine monophosphate; PKA: protein kinase A; PKC: protein kinase C; pMAPK: phosphorylated mitogen-activated protein kinase; pCREB: phosphorylated cAMP response element-binding protein; AC: adenylyl cyclase; CAMKIV: calcium/calmodulin-dependent protein kinase type IV; CAMKII: calcium/calmodulin-dependent protein kinase type II.

Hippocampal circuit

Excitatory synaptic transmission

As described in the section above, disrupting circadian rhythms impairs hippocampus-dependent learning and memory in animal models. The hippocampus consists of subregions including the dentate gyrus and cornu ammonis 1-3 (CA1-3, **Figure 2**). The hippocampus receives excitatory synaptic input from the entorhinal cortex (EC) in what are termed direct or indirect pathways. In the temporoammonic pathway, axons from layer III of the EC synapse directly onto apical dendrites of CA1 pyramidal cells (PCs). Alternatively, axons from layer II of the EC form the perforant pathway, also known as the trisynaptic circuit. Through this pathway, layer II EC PC axons provide glutamatergic input onto granule cells, the principal excitatory cell of the dentate gyrus. Granule cell axons, called mossy fibers, then provide excitatory synaptic transmission to CA3 PCs. CA3 PCs in turn form both recurrent glutamatergic synapses onto CA3 PCs as well as onto CA1 PCs. The CA3-CA1 axons are termed Schaffer collaterals. CA1 PCs, the main output of the hippocampus, send excitatory synaptic projections back to the EC[94].

Inhibitory synaptic transmission

While each subregion of the hippocampus is primarily composed of glutamatergic neurons that provide excitatory synaptic transmission to the next region, each subregion also contains GABAergic interneurons that provide local inhibitory synaptic transmission to the principal neurons. Activation of GABA receptors allows flux of chloride ions,

typically hyperpolarizing neurons or shunting the membrane potential away from action potential threshold[95]. GABAergic interneurons make up only 10-15% of the total neuronal population in the hippocampus[96], yet have the important role of establishing and maintaining the excitation to inhibition ratio and network oscillations for learning and memory[95]. Proper interneuron function and GABAergic inhibitory transmission is crucial for healthy brain function, and its dysfunction in implicated in several neurological/neurodegenerative diseases[97,98]. The hippocampus contains a heterogenous population of interneurons that can be classified according to anatomy, physiology, and protein expression. Interneurons synapse onto other neurons at the dendrites, soma, and even the axons[95]. Dendritic inhibition aids in shaping glutamatergic input, whereas somatic inhibition aids in controlling action potential generation[95].

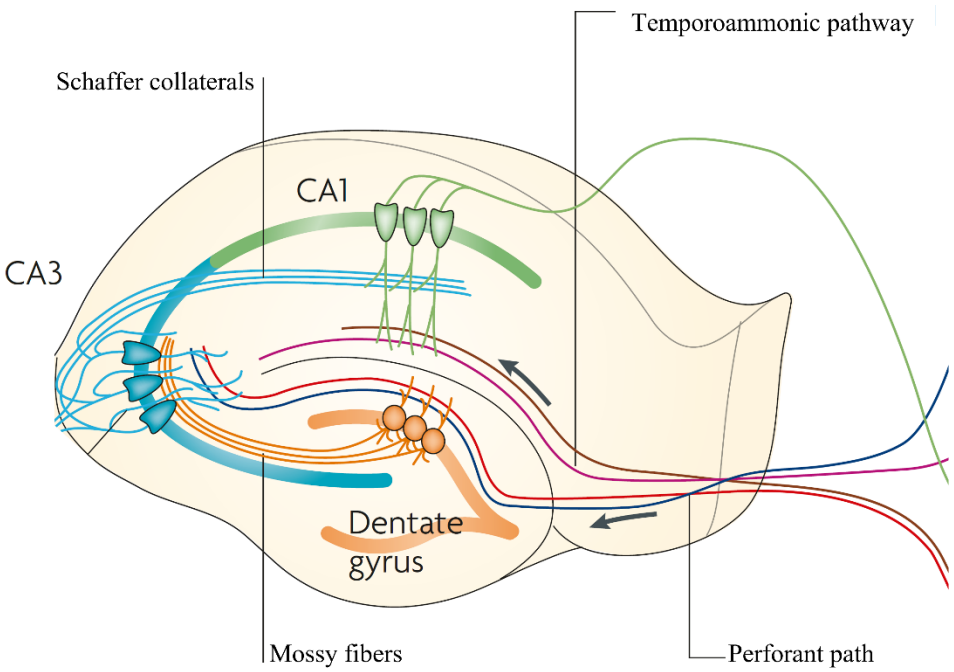

Figure 2. Circuit diagram of the hippocampal formation including the dentate gyrus (DG) and hippocampus proper (CA1-3), adapted from[94]. Copyright 2008. Adapted with permission.

Diurnal regulation of LTP

Long-term potentiation (LTP) is a fundamental and well-established property of synapses that has been long studied in the hippocampus. Considered a cellular correlate of learning and memory[99,100], investigation of the circadian regulation of LTP began as early as the early 1980s. Harris and Teyler first described variation in the incidence and magnitude of LTP across the light-dark cycle in the rat hippocampus[101]. LTP in the dentate gyrus was more likely to occur and displayed greater magnitude in the dark period. Conversely, LTP in CA1 was more likely to occur and displayed greater magnitude in the light period. This finding of increased daytime LTP in area CA1 was subsequently replicated in the Syrian hamster hippocampus[102]. In direct contrast to these

early findings are more recent studies in both mice and rats assessing LTP in the hippocampus across the light-dark cycle showing that LTP of the Schaffer collateral-CA1 synapse is greater during the night, compared to day[103,104]. Moreover, this difference is maintained in conditions of constant darkness[104], confirming the circadian nature of this variation in LTP. Similarly, studies in rats examining LTP in vivo and in slice have also found greater LTP during the night, compared to the day in both CA1 and dentate gyrus[105,106].

Recently, it has been shown that hippocampal LTP is impaired in mice with disrupted core clock genes[107,108]. Tissue non-specific, constitutive *Per2*-mutant mice exhibit a reduced magnitude of LTP as measured at the Schaffer collateral-CA1 synapse, despite intact basal excitatory synaptic transmission[107]. Similarly, in global *Bmal1*-KO mice, LTP is significantly decreased at Schaffer collateral-CA1 synapses during the day relative to wild-type mice[108].

Several kinase signaling pathways may also contribute to time-of-day dependent changes in LTP, including the diurnally regulated kinase GSK3β. Inhibition of GSK3β diminishes LTP only during the night[103]. Taken together, these data suggest that circadian regulation of other synaptic properties is likely.

Hippocampal molecular clock expression

Hippocampal neurons also express clock genes in an oscillatory manner. Studies across multiple model organisms show that the hippocampus expresses all the core components of the molecular clock (*Per1/2, Cry1/2, Clock and Bmal1*) at the transcript

and protein level[109]. *Per2* is one of the most well-characterized clock components in the hippocampus. In rats, PER2 protein expression in the dentate gyrus, assessed by immunohistochemistry, exhibits daily rhythms in expression[110-112]. In mice, PER2 protein expression is rhythmic in in areas CA3, CA2, CA1, and the dentate gyrus[107], mirrored by rhythmic expression of Cry mRNA[113]. *Per2* mRNA expression also varies with time of day in areas CA3, CA2, and CA1 of the hippocampus in Syrian hamsters[114]. In Per1-luc rats, in which luciferase is rhythmically expressed under the control of the *Per1* promoter, PER1 protein expression in the dentate gyrus shows a daily rhythm[115]. In freely moving mice, rhythmic expression of *Cry1* promoter activity in area CA1 exists in both a light-dark cycle and constant darkness[116]. *Bmal1* mRNA expression exhibits time of day variations in the areas CA3, CA2, and CA1 of the Syrian hamster's hippocampus[114]. This finding is preserved in the dentate gyrus and CA1 of the Siberian hamster[117] and the hippocampal formation of mice[118]. BMAL protein and CLOCK protein also undergo diurnal patterns of expression in the areas CA3 and CA1 of the mouse brain[119]. Time-of-day-dependent rhythmic expression of clock genes *Per1, Per2, Cry1, Cry2, Clock,* and *Bmal1* and their corresponding protein products have been detected in hippocampal neurons of mice, including parvalbumin positive interneurons, but not in glia[109]. Notably, hippocampal slices continue to oscillate in culture[107], where presumably any influence of the SCN has been removed. This suggests that independent oscillations may exist, potentially synchronized by the SCN in vivo. The dentate gyrus rhythm of PER2 in rats persists even after electrolytic lesion of the SCN[111], further suggesting the hippocampus functions as an extra-SCN oscillator. Together, these studies suggest the existence of a robust molecular clock in principal neurons of the hippocampus.

Alzheimer's disease

The hippocampus undergoes strong neurodegeneration in Alzheimer's disease. Dr. Alois Alzheimer is credited with describing the first patient characterized with what he called "presenile dementia". His patient was a 51-year-old woman who presented with progressive memory loss and confusion, mania, sleeplessness, and agitation. Within 4 years, she was unable to perform daily activities independently and died at age 55. To attempt to find a cause for her neuropsychiatric symptoms, her brain was examined post-mortem by Emil Kraepelin. Kraepelin noted that her cerebral cortex was thinned, neurons were riddled with plaques, and tangles were found in nerve fibers. While plaques had been described, they were typically in more elderly patients, and neurofibrillary tangles were a new finding[120]. This disease would later become known as Alzheimer's disease (AD), characterized by dementia, and two pathological hallmarks, amyloid beta plaques and tau tangles.

Today, AD is the leading cause of dementia and affects roughly 44 million people worldwide. It accounts for 60-80% of dementia cases and is likely underdiagnosed[121]. In the United States, it affects over 6 million people over the age of 65, two thirds of which are women[121]. Age is the greatest risk factor for AD, with the likelihood of developing AD increasing after age 65. This is followed by genetic and lifestyle factors[121]. Some genetic factors increase the likelihood of developing AD, including *apolipoprotein E4* (*APOE4*), *bridging integrator-1* (*BIN1*), and *triggering receptor expressed on myeloid cells 2* (*TREM2*), among others[122]. Other genetic factors are determinate in developing AD, or autosomal dominant, including mutations in *amyloid precursor protein* (*APP*),

and *presenilin 1*/*2* (*PSEN1, PSEN2*)[121,123]. These autosomal dominant genes account for only 1% of AD cases. While age and genetics are unchangeable factors, there are modifiable lifestyle behaviors that increase the risk of AD that can be adjusted to reduce the risk of AD such as poor diet, lack of exercise, and incidence of head injury[121]. Further, sleep disturbances may precede the onset of more typical symptoms, in some cases by decades[124]. AD patients typically survive 4–8 years after diagnosis, but some survive for twenty more, underscoring the uncertainty of disease[121]. While mortality due to other major causes is declining, deaths due to AD are increasing, highlighting the need for better treatments or a cure.

Clinical manifestation of AD

Clinical symptoms are those that are detected in the clinic and can be identified by the patient or family members[121]. AD is divided into four stages: preclinical, early (mild), middle (moderate), and late stage (severe) AD. In the preclinical stage, changes in the brain typically start occurring before clinical presentation of symptoms. While patients in the preclinical stage of AD have no symptoms, the stage is defined by evidence of biomarkers or genetic risk factors[121]. Patients with mild AD typically function independently but may have clinically diagnosable mild cognitive impairment, also noticeable by friends and family[121]. Moderate AD is characterized by more pronounced dementia and dependence on assistance for daily activities[121]. Patients with late-stage AD have severe dementia, difficulty communicating, and need extensive care with daily activity[121]. These stages exist on a continuum and progression varies between patients.

Pathological manifestations of AD

In addition to neurodegeneration in AD, neuropathological changes include build-up of amyloid beta plaques and neurofibrillary tau tangles. These follow a pattern of regional spread throughout the brain, starting at the medial temporal lobe and gradually affecting other parts of the cerebral cortex in later stages[125].

Amyloid beta is a product of enzymatic cleavage of amyloid precursor protein (APP). APP undergoes proteolytic processing by three secretases including α-secretase, β-secretase, and γ-secretase. This processing occurs in two pathways: an amyloidogenic pathway and nonamyloidogenic pathway[126]. In amyloidogenic processing, the first cleavage is made in the extracellular domain by β-secretase, resulting in the release of a soluble fragment (sAPPβ). The second cleavage is in the transmembrane domain by γ-secretase and results in the production of the amyloid beta peptide, a small peptide called p3, and the release of the APP intracellular domain (AICD). Under typical physiological conditions, however, APP undergoes non-amyloidogenic processing more frequently. In this pathway, α-secretase makes the initial cleavage releasing sAPPα and preventing the formation of amyloid beta. It is thought that an imbalance in APP cleavage through these pathways leads to increased amyloid beta production seen in AD[127]. Amyloid beta chain length varies depending on the γ-secretase cleavage site. The two major products of amyloid beta cleavage in the brain are amyloid beta40 (80-90%) and amyloid beta42 (5-10%)[128]. Although amyloid beta40 is several-fold more abundant than amyloid beta42 in soluble form, plaques tend to consist of the longer form, amyloid beta42, which has two additional hydrophobic amino acids[129]. *In vitro*, amyloid beta42 has lower solubility than

amyloid beta40 and tends to form protofibrils and fibrillar aggregates at lower concentrations and higher rates than amyloid beta40 or other amyloid beta variants[130-133]. Amyloid beta can have wide-ranging effects on the brain. For example, plaque aggregation results in activation of microglia and inflammation in a mouse model of AD[134], and amyloid beta can drive tau propagation[135,136]. There is also evidence of amyloid beta oligomers binding to glutamatergic receptors, altering receptor expression at the membrane, calcium homeostasis, and overall synaptic function[137-142]. Amyloid beta oligomers also inhibit long-term potentiation and synaptic function in several rodent models[143-148].

Tau is a microtubule-associated protein, the product of the gene *microtubule-associated protein tau* (*mapt*). Neuronal microtubules guide intracellular transport and induce morphological changes during the various phases of neuronal development and synapse formation[149]. They function in stabilization of microtubules primarily in axons[150,151]. Neurofibrillary tangles in AD are composed of abnormally hyperphosphorylated tau aggregated into bundles of filaments[152]. The mechanisms by which tau becomes hyperphosphorylated in AD are not fully understood but include altered activity of several kinases and phosphatases including glycogen synthase kinase-3β (GSK-3β), cyclin dependent kinase 5, and protein phosphatase 2A[153-156]. While hyperphosphorylated tau is the primary component of neurofibrillary tangles, cognitive decline begins before neurofibrillary tangle formation in mouse models, suggesting that soluble oligomeric pathologic tau is the more likely cause for neuronal dysfunction[157,158]. It is also unclear how soluble tau induces neurotoxicity, although there is evidence of

soluble tau causing impairment of synaptic function, mitochondrial function, and axonal transport[159].

Network Hyperexcitability in AD

Abnormal neuronal network excitability may lead to hypersynchrony, altered oscillatory rhythmic activity and abnormal interneuron function, which may contribute to cognitive dysfunction AD[160]. Network hyperexcitability, as evidenced by epileptiform activity or seizures, is thought to be a comorbidity of AD that occurs in later stages of the disease[161]. However, epileptiform activity is more common in AD than other forms of dementia[162] and can occur throughout disease progression[163]. The incidence of unprovoked seizures is greater in patients with sporadic AD than age-matched control subjects[161,164-166], especially in cases with early disease onset (before age 60)[164,165,167,168]. An 87-fold higher incidence of seizures occurs in patients with autosomal dominant forms of AD[168,169], and convulsive seizures occur in 50–80% of those patients[167,170,171]. Functional magnetic resonance imaging studies have detected hyperactivation of several brain regions in AD patients, including the hippocampus[172]. Cognitive decline begins 5–7 years earlier in patients with both AD and mild cognitive impairment who experience epilepsy than in patients who do not, again suggesting that hyperexcitability worsens disease state, especially cognitive function[173]. In several mouse models of AD, network hyperexcitability are reported before the onset of amyloid beta plaque deposition and cognitive impairment[174-178]. Studies in mice have shown that network hyperexcitability contributes to the spread of pathological tau, contributing to disease progression[179,180]. When treating hyperexcitability, evidence for improved cognitive function and pathology

are conflicting. An antiepileptic drug, levetiracetam, improved memory task performance in patients with MCI[181], but not AD patients[182]. Several studies in rodent models showed improved cognitive performance with levetiracetam[183-187]. Some of those studies found reductions in amyloid beta plaque pathology and improved clearance[186], while others found no change[185].

Circadian dysfunction in AD

While circadian disruptions are typical with increased age, people with AD have more severe circadian disruptions compared to healthy adults of the same age, and these disruptions are present even in pre-symptomatic stages[188-194]. Specifically, people with AD show more fragmented sleep-wake cycles and experience sundowning, or late day confusion[188]. Seizures and epileptiform activity are more common in both the general population and AD patients at night[195-197]. Cerebral spinal fluid (CSF) levels of amyloid beta exhibit diurnal variation in humans, though the difference wanes with age[198-200]. In mice, interstitial fluid (ISF) levels of amyloid beta also oscillate with the course of the day[201], and diurnal variation was lost in AD mice[202]. Disrupting the molecular clock in the forebrain of an AD mouse model resulted in altered amyloid beta oscillations and increased amyloid beta plaque accumulation, demonstrating a critical role of circadian rhythms in AD pathogenesis[203]. CSF tau in humans and ISF tau in mice also exhibit diurnal fluctuation, and those fluctuations are altered in cases of sleep deprivation[204]. In addition, expression of core circadian clock genes is altered in AD mouse models. *Per2* mRNA and/or protein levels in the SCN or hypothalamus in APP/PS1 and 5XFAD mice are reduced compared to age-matched controls[205,206]. Intracranial injection of amyloid

beta31-35 in the hippocampus of WT mice reduced the nocturnal peak in PER2 protein levels[207]. In young APP/PS1 mice, hippocampal clock gene expression was not different from WT controls, with both genotypes showing a time-of-day difference[208]. However, by 12-15 months, APP/PS1 mice showed reduced diurnal rhythms in hippocampal clock gene mRNA expression[209]. AD mouse models also exhibit altered neurophysiology. In the SCN, action potential firing rate of neurons is higher during the day compared to night[51,52], but in Tg-SwDI mice, the daytime firing rate of SCN neurons is reduced, resulting in a dampening of the day/night difference[210]. A better understanding of how circadian rhythms influence hippocampal physiology and pathophysiology could aid in both treatment of disease and the search for biomarkers for earlier detection.

Current treatments of AD

As of today, there are few treatments for AD. The available treatments only slow disease progression, and only temporarily. Some nonpharmacological therapies that improve quality of life or slow the decline of cognitive function include physical exercise, memory training, and light therapy targeting sleep improvement[211-216]. Currently, there are four drugs that target neurotransmission, and one FDA approved treatment that targets amyloid beta.

In the 1970s, post-mortem studies of brains of AD patients revealed earliest dysfunction in the cholinergic system of neurotransmission in neocortex and hippocampus. These studies found reductions of acetylcholinesterase and choline acetyltransferase, enzymes involved in the breakdown and synthesis of the

neurotransmitter acetylcholine (ACh)[217-219]. During this time, ACh was found to play a large role in learning and memory in the CNS[220]. Together, this evidence led to the cholinergic hypothesis in AD and an attempt to discover treatments targeting this system. Rivastigmine, galantamine, and donepezil are acetylcholinesterase inhibitors whose use typically result in improved cognitive function; however, this improvement typically only lasts for a year, depends on disease severity, and is not effective in all AD patients[221-231]. In the 1980s, progress was made in understanding the role of NMDA receptors in long-term potentiation and their modulation of hippocampal-dependent learning and memory[232-236]. Thus, NMDA receptors made a good target for treatment of learning and memory disorders[237-239]. In 2003, memantine, an NMDA receptor antagonist, was approved for treatment of AD. While memantine does improve cognitive function and daily living, this improvement also lasts about a year before symptoms are not different from baseline[240]. The acetylcholinesterase inhibitors are typically used to treat mild to moderate AD, while memantine is used in moderate to severe cases, and sometimes combined with donepezil[221-231].

In 1992, another hypothesis was put forth – the amyloid cascade hypothesis. Hardy and Higgins hypothesized that amyloid beta deposits are the cause of AD and that other pathologies follow[241]. Over the years, several approaches to reducing amyloid beta have been attempted with little success. One approach to this is using the immune system to produce antibodies to amyloid beta to increase clearance of amyloid beta from the central nervous system. Aducanumab is a monoclonal antibody treatment that was FDA approved in 2021. While aducanumab has thus far shown improved amyloid beta

clearance in high-dose groups, there is no correlation of clearance with the meaningful benefit of improved cognitive function[242].

Finally, melatonin and light therapy are two methods of synchronizing the circadian clock and enforcing a consistent diurnal rhythm of sleep and activity that have been studied extensively as potential treatments for AD. Melatonin administration at bedtime simulates the body's natural signal that it is night and exerts direct effects on clock gene expression in the SCN and other tissues. Similarly, ensuring darkness at night and then applying high-intensity white or blue light in the morning has similar effects on the circadian system. Unfortunately, trials employing melatonin or light at the appropriate time of day have shown little, if any, effect on sleep in AD patients[243-245]. Leveraging the circadian clock in other ways as a target is an ongoing area of study, providing a unique therapeutic opportunity for the treatment of AD and other neurodegenerative diseases.

Outstanding questions

- Cognitive function that relies – at least in part – on the hippocampus varies as a function of time of day, but the underlying time of day variations in hippocampal neural circuit function have not been examined in detail. While changes in hippocampal LTP have been shown across time of day, an understanding of other measures of electrical activity at the level of the single neuron is lacking. I address the question "Do synaptic inputs, intrinsic properties, and resulting excitability of CA1 pyramidal cells vary diurnally?" in chapter 2, "Diurnal Variation in Synaptic Physiology of Hippocampal Area CA1".

- One of the most notable symptoms in patients with AD is cognitive dysfunction. Along with disruption of sleep-wake cycles, worsening of cognitive dysfunction in humans with AD occurs in the evenings, pointing to impaired circadian function in the hippocampus. I address the question “Is diurnal regulation of hippocampal-dependent cognitive function and neuronal physiology impaired in Alzheimer’s disease?” in chapter 3, “Dysregulated clock gene expression and abnormal diurnal regulation of hippocampal inhibitory transmission and spatial memory in amyloid precursor protein transgenic mice” and chapter 4 “Absence of diurnal rhythm in spatial memory and inhibitory transmission in the hippocampus of the J20 model of Alzheimer’s disease”.

DIURNAL VARIATION IN SYNAPTIC PHYSIOLOGY OF HIPPOCAMPAL AREA CA1

by

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In Preparation for Submission to *Journal of Physiology*

Format adapted for dissertation

ABSTRACT

Circadian rhythms are biological processes that cycle across 24-hours and are known to regulate many aspects of behavior and physiology, including cognitive function. Time of day changes in neuronal physiology likely contribute to the circadian rhythms of cognitive function, such as hippocampal-dependent memory tasks. In the hippocampus, long term potentiation, the synaptic correlate of learning and memory, exhibits day-night differences with increased magnitude during the active period in rodents (night). However, how time of day influences synaptic drive onto and membrane properties of CA1 pyramidal cells is an open question. In this study, we used electrophysiology to examine diurnal variation in synaptic transmission and neuronal excitability. We found that inhibition and excitation onto CA1 pyramidal cells vary across time of day, along with CA1 pyramidal cell excitability, and found that these time of day-dependent differences vary by sex. Overall, we reveal diurnal variation in neuronal and synaptic function in the hippocampus and, importantly, underscore the importance of considering sex and neuronal heterogeneity in the study of neural circuits.

INTRODUCTION

The hippocampus is the seat of learning and memory in the brain, and its primary output is generated by the principal cells in area CA1, called CA1 pyramidal cells[1]. Firing of a CA1 pyramidal cell, like any other neuron, is a function of synaptic excitatory and inhibitory drive, membrane properties of the neuron that determine excitability, and the effect of neuromodulators[1]. Another variable that is relatively unexplored in the hippocampus is how CA1 pyramidal cell excitability is modulated by time of day. Time of day variations in biological function are generated by a circadian rhythm network, which regulates the timing of 24-h rhythms in physiology and behavior, enabling animals to adapt to and anticipate regularly occurring events in the environment[2]. Circadian regulation of normal physiological processes is advantageous, and dysregulation of circadian rhythms has been shown to exacerbate disease onset and symptoms. Therefore, understanding circadian influence on physiology is crucial for designing interventions for diseases with circadian dysfunction, such as neurodegenerative diseases.

Circadian rhythms are maintained at the molecular level in all tissues of the body by a transcription-translation feedback loop known as the molecular clock[2]. The suprachiasmatic nucleus (SCN) of the hypothalamus is the principal orchestrator of this endogenous clock, and its electrical properties vary across time of day. In fact, circadian regulation of neuronal excitability has been observed in a range of species, including

rodents[3], drosophila[4,5], zebrafish[6], and is widespread in the brain[7]. Although the SCN is the principal clock, multiple studies have documented the existence of autonomous circadian clocks in other brain regions including the hippocampus[7]. At the molecular level, subregions of the hippocampus rhythmically express core clock proteins, with the cell body layer of area CA1 having the strongest expression of PER2[8]. Moreover, over 600 genes, including those encoding ion channels and synaptic proteins show circadian expression in the hippocampus[9,10]. At the cellular level, long term potentiation (LTP), a phenomenon in which certain types of synaptic stimulation result in a long-lasting increase in the strength of synaptic transmission, is expressed at a greater magnitude at night compared to day[11,12]. However, a detailed understanding of how time of day regulates synaptic drive onto and membrane properties of CA1 pyramidal cells, is lacking. Here we found that hippocampal synaptic transmission and neuronal excitability vary as a function of time of day and additionally uncovered that these changes depend on sex.

METHODS

Animals All animal procedures followed the Guide for the Care and Use of Laboratory Animals, U.S. Public Health Service, and were approved by the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee. All experiments were done in 6–12-week-old C57BL/6J mice of both sexes obtained from Jackson Laboratories (stock #000664) or from the C57BL/6J colony at UAB. Mice were maintained on a 12:12 light/dark cycle with ad libitum access to food (LabDiet Rodent 5001 by Purina) and water.

Electrophysiology

Slice preparation. Mice were euthanized with cervical dislocation and rapid decapitation between ZT 0-1 or ZT 11-12 for day and night experiments, respectively. Both sex and time of day were interleaved. For extracellular field experiments, brains were removed and 350 μm coronal slices were prepared using a VT1200 S vibratome (Leica Biosystems) in an ice-cold solution containing the following (in mM): 85 NaCl, 2.5 KCl, 4 $MgSO_4$ * 7 H_2O, 0.5 $CaCl_2$ * $2H_2O$, 1.25 NaH_2PO_4, 75 Sucrose, 25 $NaHCO_3$, 25 Glucose saturated in 95% O_2 and 5% CO_2. Slices were allowed to rest for at least one hour in a recovery solution of standard ACSF containing the following (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$ * $7H_2O$, 2.5 $CaCl_2$ * $2H_2O$, 1 NaH_2PO_4, 26 $NaHCO_3$, and 11 glucose, bubbled with 95% O_2/5% CO_2. For whole-cell patch clamp experiments, brains were removed and 300 μm

coronal slices were prepared using a VT1200 S vibratome (Leica Biosystems) in an ice-cold solution containing the following (in mM): 110 choline chloride, 25 glucose, 7 $MgCl_2$, 2.5 KCl, 1.25 Na_2PO_4, 0.5 $CaCl_2$, 1.3 Na-ascorbate, 3 Na-pyruvate, and 25 $NaHCO_3$, bubbled with 95% O2/5% CO_2. Slices were allowed to rest for at least one hour at room temperature in a recovery solution containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 Na2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 25 glucose, bubbled with 95% O_2/5% CO_2. For experiments measuring inhibitory synaptic events, 2 mM kynurenic acid was added to the recovery solution.

Whole-cell patch clamp recordings. All whole-cell recordings were made from CA1 pyramidal neurons using the blind patch technique between ZT 2-6 (day) or ZT 13-17 (night) at 32°C in standard artificial cerebrospinal fluid (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 Na_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, and 25 glucose, bubbled with 95% O2/5% CO_2. Data were acquired using a Multiclamp 700B amplifier, Axon Digidata 1440A and 1550B digitizer, and pClamp10/11 software (Molecular Devices, San Jose, CA). Patch pipettes (BF150–086; Sutter Instruments, Novato, CA) were pulled on a Sutter P-97 horizontal puller (Sutter Instruments, Novato, CA) to a resistance between 2.5-5 MΩ. All cells were dialyzed for 5 min prior to experimental recording. Cells used for analysis had access resistance less than 30 MΩ that did not change by more than 20% for the duration of recordings.

Inhibitory postsynaptic currents. For inhibitory postsynaptic currents, patch pipette solution contained (in mM): 140 CsCl, 10 EGTA, 5 $MgCl_2$, 2 Na-ATP, 0.3 Na-GTP, 10 HEPES, and 0.2% biocytin (pH 7.3, 290 mOsm), and 5 QX-314 (sodium channel antagonist) added at time of use. Spontaneous inhibitory postsynaptic currents (sIPSCs)

were pharmacologically isolated with bath perfusion of 10μM NBQX (AMPAR antagonist, Hello Bio) and 5μM CPP (NDMAR antagonist, Hello Bio). Miniature IPSCs (mIPSCs) were recorded as above with the addition 0.5μM TTX (voltage-gated sodium channel inhibitor, Tocris). All cells were dialyzed for five minutes prior to experimental recordings and held at −70 mV. Signals were filtered at 5 kHz and digitized at 10 kHz.

Excitatory postsynaptic currents. For excitatory postsynaptic currents, patch pipette solution contained (in mM): 100 CsOH, 100 Gluconic acid (50%), 0.6 EGTA, 5 $MgCl_2$, 2 Na-ATP*3H_2O, 0.3 Na-GTP, 40 HEPES, 7 Phosphocreatine, biocytin (0.2%), and 5 QX-314 added at time of use. Spontaneous excitatory postsynaptic currents (sEPSCs) were pharmacologically isolated with bath perfusion of 10 μM Gabazine ($GABA_AR$ antagonist, Hello Bio). Miniature EPSCs (mEPSCs) were recorded as above with the addition 0.5 μM TTX. All cells were dialyzed for five minutes prior to experimental recordings and held at −70 mV. Signals were filtered at 5 kHz and digitized at 10 kHz.

Current Clamp recordings. For current clamp experiments, patch pipette solution contained (in mM): 135 K-Gluconate, 2 $MgCl_2$, 0.1 EGTA, 10 HEPES, 4 KCl, 2 Mg-ATP, 0.5 Na-GTP, and 10 Phosphocreatine (pH 7.3, 310 mOsm, and 2–4 MΩ). Signals were filtered at 10 kHz and digitized at 20 kHz. Neuronal excitability was examined by injecting progressive steps of depolarizing current from rest (0 pA to 400 pA at 20 pA increments) and counting the number of action potentials firing during each 1000 ms current step. Sag ratio was examined by injecting progressive steps of hyperpolarizing current from rest (0 pA to -400 pA at -50 pA increments). Sag ratio was calculated by dividing the sag voltage by the maximum voltage deflection from baseline. Sag voltage was calculated by measuring the difference between steady state of the hyperpolarizing injection and the peak

negative voltage. To measure intrinsic properties, NBQX, CPP, and Gabazine were added to recording ACSF at 5μM, 10μM, and 10μM, respectively.

Field Recordings. Recordings were obtained between ZT 1-6 or ZT 12-18 for day and night recordings, respectively. Field excitatory postsynaptic potentials (fEPSPs) were recorded from slices in a submersion recording chamber continuously perfused with standard ACSF at 3-5 ml/min and 26–28°C. To stimulate Schaffer collateral axons, a bipolar stimulating electrode was placed in stratum radiatum within 200–300 μm of a recording electrode. Data acquisition and analysis was performed through pCLAMP10/11. Data were recorded using a Kerr Scientific S2 amplifier (Kerr Tissue Recording System, Kerr Scientific Instruments, Auckland, NZ) and pClamp10 acquisition software (Molecular Devices, San Jose, CA). Signals were digitized at 10 kHz (Digidata 1550B).

Input-output (I/O) curves. Input-output curves were generated by increasing the stimulus intensity from 0.2 μA to 200 μA in 10 μA increments. Baseline fEPSPs were obtained by delivering a 0.1Hz stimulation to elicit fEPSPs of approximately -0.20 mV/ms for 20 min.

Long term potentiation (LTP). Briefly, after a stable 20 minute baseline recording, LTP was induced by delivering a high-frequency stimulus (HFS; 100Hz) of 2 trains (0.5 s duration; 15 s interval) and field excitatory postsynaptic potential (fEPSP) slopes were recorded for 40 min following HFS, as in[11,13]. This weaker stimulation protocol was used in order to avoid masking a day/night difference in LTP magnitude.

Immunohistochemistry

Biocytin. To confirm that cells recorded to measure postsynaptic currents were CA1 pyramidal cells, all cells were filled with biocytin for at least 20 minutes. Slices containing

filled cells were fixed in 4% PFA for at least 24 hours, then washed for 3 x 10 min in PBS, and incubated for 2-3 hours at RT in a TBS solution containing 10% NDS, 3% BSA, 1% Glycine, 0.4% Triton X, and streptavidin-488 (1:1000). Slices were then washed for 3 x 10 min in PBS and mounted on glass slides and coverslips with ProLong Gold Antifade mounting media containing DAPI. Slides were visualized on a BZ-X700 fluorescence microscope (Keyence). Any cells that could not be classified as CA1 pyramidal cells based on location and morphology were excluded from analysis.

Analysis and Statistics Data were analyzed and visualized using SPSS Statistics and Prism-GraphPad. All results from statistical analyses are included as Tables 1-4.

Field Recordings. Input-output data were analyzed using a linear mixed model with field excitatory postsynaptic potential (fEPSP) slope as a function of Time of Day, Sex, and Stimulation Intensity. LTP data were normalized to baseline and the last 10 minutes of LTP were used for analysis. Data were analyzed using a linear mixed model with normalized fEPSP slope as a function of Time of Day, Sex, and Time.

Whole-cell Electrophysiology. Postsynaptic currents (inhibitory and excitatory) were automatically detected using pClamp's template search analysis then manually inspected for false event detection. The amplitudes and interevent interval (IEI) were analyzed using a generalized estimating equation (GEE) with an unstructured working correlation matrix structure, a subject effect of cell, and a within-subject effect of postsynaptic events to account for multiple events from each cell. The data were non-normally distributed and were thus trimmed of upper and lower outliers (10%) then transformed using either a log transformation in the case of the amplitude data, or a log plus one transformation in the

case of IEI data, to follow approximately normal distributions before analysis. Current clamp recordings were split into anterior and posterior groups based on their position along the anterior-posterior axis. Cells were considered “anterior” if recorded in hippocampal slices that matched panel 48 or lower in the Allen brain atlas, and “posterior” if they matched 49 or higher (**Supplemental Figure 1**). By group, recordings were analyzed using a linear mixed model with current injection as a repeated measures factor. Sidak post hoc tests were used for significant interactions.

RESULTS

Inhibition onto CA1 pyramidal cells is greater during the day compared to night, regardless of sex

Given that hippocampal-dependent behavior varies as a function of time of day, and that CA1 pyramidal cells exhibit rhythmic expression of ion channels, we first asked if synaptic transmission varies with time of day. First, to examine synaptic inhibition onto CA1 pyramidal cells, we measured spontaneous inhibitory postsynaptic currents (sIPSCs; **Figure 1**, **Supplementary Table 1**), a result of both action-potential dependent and independent neurotransmitter release, using whole-cell voltage clamp (**Figure 1A**, **B**). There was a significant main effect of Time of Day on both sIPSC interevent interval (IEI) (**Figure 1C**; $p = 0.033$) and amplitude (**Figure 1D**; $p = 0.008$). The sIPSC IEI was shorter, and the amplitude was larger during the day regardless of sex, indicating greater inhibition during the day compared to night.

To determine if this increased daytime inhibition onto CA1 pyramidal cells is due to presynaptic or postsynaptic changes, we measured miniature IPSCS (**Fig 1E**, mIPSCs) in the presence of the voltage-gated sodium channel blocker tetrodotoxin (TTX). There was no significant main effect of Time of Day ($p = 0.760$) or Sex ($p = 0.392$) on mIPSC IEI, indicating that the day-night difference seen in sIPSCs IEI is likely action potential-driven (**Figure 1F**, **Supplementary Table 1**). While there was no significant interaction between Time of Day and Sex ($p = 0.068$), the mean values between male day and night

differed by ~12.ms (mean and SEM: Male day, 98.24 +/-1.05ms; Male night, 85.78 +/-1.04ms).

We unexpectedly found a significant interaction between Time of Day and Sex on mIPSC amplitude (**Figure 1G**; p = 0.038), with larger amplitudes in females compared to males during the day (p = 0.006), however, this ~2pA difference is likely not biologically relevant (mean and SEM: Female day, 34.68+/-1.01pA; Male day, 32.67 +/- 1.02pA).

Taken together, these data suggest that action potential-dependent inhibition onto CA1 pyramidal cells is greater during the day compared to night.

Excitation onto CA1 pyramidal cells depends on sex

Next, we examined if synaptic excitation onto CA1 pyramidal cells varies with time-of-day by measuring spontaneous excitatory postsynaptic currents (EPSCs, **Figure 2A**) using whole-cell voltage clamp. There was a trend toward a main effect of Time of Day (**Figure 2B**; p = 0.052) on sEPSC IEI, with longer IEIs during the day compared to night, suggesting greater excitation at night. There was no significant main effect of Time of Day on sEPSC amplitude (**Figure 2C**, **Supplementary Table 1**; p = 0.371). Unexpectedly, there was a significant main effect of Sex on both sEPSC IEI (**Figure 2B**, p = 0.022) and amplitude (**Figure 2C**; p = 0.020). There were shorter IEIs in females compared to males, suggesting a higher frequency of excitatory events at night. While the difference in event amplitude was significant, it is likely not biologically relevant as it is a difference of ~1.5pA (mean and SEM: Female, 24.95 +/- 1.02pA; Male, 23.58 +/- 1.01pA). Overall, females had more excitatory synaptic input, with shorter IEI values compared to males.

We repeated these experiments in TTX-isolated mEPSCs (**Figure 2D**) to determine if the sex difference in excitation and the nighttime increase in sEPSC frequency is due to presynaptic or postsynaptic changes. There was a significant interaction between Time of Day and Sex on mEPSC IEI (**Figure 2E**; $p = 0.021$). IEIs recorded from males were shorter at night than during the day ($p = .002$), indicating a greater frequency of excitatory events at night in male mice, while there was no statistical difference in time of day in females. There was no significant main effect of Sex ($p = 0.227$) or Time of Day ($p = 0.150$) on mEPSC amplitude (**Figure 2F**, **Supplementary Table 1**), suggesting that mEPSC amplitudes were not different during the day compared to night or between male and female mice.

These data suggest that: (i) spontaneous excitatory input onto CA1 pyramidal cells is numerically greater ($p=0.052$) at night compared to day; (ii) in males, this increased nighttime frequency is preserved in the absence of action potentials; (iii) in females, this increased nighttime frequency is action potential-dependent. Further, in female mice, excitation is overall greater than in males.

Ventral-like CA1 pyramidal cells are more excitable at night in male mice

Given that broadly, inhibition is greater at night and excitation is greater during the day, we wondered if this opposing diurnal variation in synaptic excitatory and inhibitory input results in diurnal variation in CA1 pyramidal cell excitability. To this end, we patched CA1 pyramidal cells in current clamp with the circuit intact (i.e, in the absence of synaptic antagonists), and without clamping cell membrane potential. We also accounted for known differences in excitability of CA1 pyramidal cells across the dorsal-ventral axis[14-17] that could occlude time-of-day differences, and divided cells into anterior (dorsal-like) and

posterior (ventral-like) groups based on slice anatomy (**Supplementary Figure 1**). To assess excitability, we injected increasing amounts of depolarizing current into pyramidal cells and measured the number of action potentials elicited (spikes per step; **Figure 3**). There were no time of day or sex differences in pyramidal cells recorded from anterior slices (**Figure 3A**, **B**, **Supplementary Table 2**). However, in cells recorded from posterior slices, there was a significant interaction between sex and time of day. CA1 pyramidal cells generate more spikes per step at night compared to day, but only in male mice (**Figure 3C**, **D**; $p < 0.001$). Cells recorded from male mice elicit more spikes per step than those from female mice ($p = 0.006$) only at night.

As the observed time-of-day variation in excitability could be driven by synaptic and/or intrinsic factors, we assessed intrinsic excitability in a separate cohort of animals by including synaptic antagonists in the recording ACSF to isolate the cell from the circuit. In this case, in cells recorded from anterior slices, there was a main effect of Sex, with increased spikes per step in males compared to females (**Figure 3E**, **F**; $p = 0.021$). In cells recorded from posterior slices, there was a significant interaction between Sex and Time of Day. Cells recorded from male mice had more spikes per step at night compared to day (**Figure 3G**, **H**; $p = 0.002$), but this day-night difference was absent in recordings from females. Cells recorded from male mice had more spikes per step than from female mice ($p = 0.015$), but only at night.

Overall, these data suggest that excitability of ventral-like CA1 pyramidal cells in male mice is diurnally regulated, with increased excitability at night, both intrinsically and within the intact circuit. Further, intrinsic excitability is greater in male mice than female

mice at night. In dorsal-like CA1 pyramidal cells, there is a sex-difference in intrinsic excitability, with males more excitable than females regardless of time of day.

CA1 pyramidal cell sag ratio depends on time of day and sex

Activation of hyperpolarization-activated cyclic nucleotide-gated ion channel (HCN) channels at hyperpolarized membrane potentials results in a characteristic depolarizing voltage change called "sag" voltage. In the SCN, HCN channel function varies across time of day[18], but diurnal regulation of sag in the hippocampus is unexplored. In the hippocampus, HCN channel expression in CA1 pyramidal cells varies along the dorsal-ventral axis[14,15,19,20]. For these reasons, we wanted to determine whether sag voltage also exhibits diurnal variation in the hippocampus. To examine sag voltage, we injected hyperpolarizing current steps into CA1 pyramidal cells and measured the subsequent peak hyperpolarized voltage (**Figure 4**, **Supplementary Table 3**). To account for differences in input resistance, we then calculated sag ratio by dividing this peak hyperpolarized voltage by the steady state voltage, thereby normalizing for input resistance differences between cells. Statistically significant observations were once again restricted to the ventral-like posterior cells. We found a significant interaction between Time of Day and Sex (**Figure 4B**; $p < 0.001$). Recordings from CA1 pyramidal cells from female mice had a lower sag ratio at night compared to day ($p < 0.001$), and at night, cells from female mice had a lower sag ratio than males ($p < 0.001$).

Again, we repeated the experiment in separate animals with the addition of synaptic antagonists in the recording ACSF to determine if diurnal differences in sag are dependent on synaptic input or are intrinsic to CA1 pyramidal cells. Interestingly, with the addition of synaptic antagonists, the patterns of diurnal variation in the anteriorly (dorsal-like)

versus posteriorly (ventral-like) recorded CA1 pyramidal cells were reversed with respect to both time-of-day and sex. There was a significant interaction between sex and Time of Day (**Figure 4C**; $p < 0.001$) in anterior, dorsal-like CA1 pyramidal cells. Sag ratio in pyramidal cells recorded from male mice is greater during the day than night ($p < 0.001$), while there is no day-night difference in females. Additionally, during the day, pyramidal cells in male mice have greater sag than those in female mice ($p < 0.001$), but at night, this is reversed with female mice having greater sag than males ($p < 0.001$). In posterior, ventral-like CA1 pyramidal cells, the main effect of time of day was trending toward significance (**Figure 4D**; $p = 0.059$) with greater sag at night compared to day.

These data suggest that time-of-day variation in sag is dependent on synaptic input, spatial factors (anterior/posterior) and sex (male/female).

LTP is greater at night compared to day, regardless of sex

There is evidence that changes in LTP can be attributed to both synaptic mechanisms and intrinsic changes in excitability. Long term potentiation (LTP) is considered the cellular correlate of learning and memory[21]. LTP at the CA3 to CA1 synapse is higher at night compared to day in male mice[11,12], but to our knowledge there are no published reports of the effects of time of day on LTP magnitude in female mice. First, to test if basal synaptic transmission varies diurnally, we stimulated the Schaffer Collaterals and recorded dendritic excitatory field synaptic potentials (fEPSPs) of CA1 pyramidal cells in stratum radiatum in response to increasing stimulation intensities (0.2-200 μA, Δ10μA). We measured fEPSP slope, which represents the excitatory postsynaptic response, as a function of stimulation intensity in male and female mice across both times of day (**Figure 5**, **Supplementary Table 4**). There was no significant main effect of Sex (**Figure 5A, B**;

p = 0.552) or Time of Day (p = 0.981). There was a significant interaction between Sex*Stimulation Intensity ($p < 0.001$), indicating that males have larger fEPSP slopes than females, but this is only at the highest stimulation intensities (180µA, 190µA, 200µA, p = 0.041, 0.043, and 0.035, respectively) and is likely not biologically relevant. Together, these data suggest basal synaptic transmission is unaffected by time of day and largely does not vary with sex (at stimulation intensities relevant for LTP induction). To assess day-night differences in synaptic plasticity, we stimulated the Schaffer Collaterals and recorded fEPSPs from CA1 stratum radiatum before and after using a high frequency stimulation protocol. As expected, there was a main effect of Time of Day, with greater LTP at night compared to day (**Figure 5C, D**; $p < 0.001$). However, there was no significant main effect of Sex or interaction between Time of Day and Sex, indicating LTP is magnitude is not different between male and female mice.

DISCUSSION

While we know that circadian rhythms play a role in learning and memory, the underlying mechanisms by which they shape synaptic transmission and membrane function in the hippocampus are largely unexplored. In this study, we show that diurnal regulation of these processes depends on multiple factors, including synaptic transmission, sex, and position along the hippocampal longitudinal axis.

We first examined whether synaptic inhibition and excitation were regulated by time of day. Our sIPSC and mIPSC data suggest that that action potential-dependent inhibition onto CA1 pyramidal cells is greater during the day compared to night. Given that these inhibitory currents were pharmacologically isolated with glutamate receptor antagonists, it is likely that increased day-time interneuron activity is spontaneously generated. Indeed, reports in the literature suggest that some interneurons in area CA1 are spontaneously active[22-26]; however, definitive evidence of time-of-day variation in spontaneous interneuron firing in the hippocampus is lacking.

Next, we found that excitatory input onto CA1 pyramidal cells is numerically greater during the night compared to day, and that in the absence of action-potential dependent neurotransmitter release, this difference persists in males, but is absent in females. This suggests that the increased excitation at night in females is likely action-potential driven. Major excitatory input onto CA1 pyramidal cells arrives from the axons of principal neurons of the downstream area CA3 (Schaffer collaterals; CA3 pyramidal

cells) or from the entorhinal cortex (Temperoammonic pathway). CA3 pyramidal cells also exhibit circadian rhythms in excitability, with larger calcium current, decreased afterhyperpolarization, and reduced spike frequency adaptation at night compared to day[27]. This increased night time CA3 pyramidal cell excitability could translate to increased sEPSC onto CA1 pyramidal cells at night compared to day.

That excitation and inhibition onto CA1 pyramidal cells peak at opposite times of day is in line with previously published work[28,29], however, those reports find increased excitation during the day and increased inhibition at night. Multiple factors can account for the discrepancies in these results. First, while previous studies used both male and female animals, neither study examined differences between the sexes, which, as we have shown, can have a dramatic impact on results and overall conclusions. Second, the specific hours examined across the dark and light cycles are different across studies. Third, the anterior-posterior axis of the hippocampus heavily influences neurophysiology[14,15,19,20], and it is unclear whether this factor was accounted for. These differences across studies further emphasize the profound complexity of circadian regulation of neuronal physiology and emphasize the need for further, rigorous assessment of circadian impact physiology.

Because of the diurnal variation in excitatory and inhibitory input onto CA1 pyramidal cells, we next wanted to determine whether CA1 pyramidal cell excitability varied across time of day. We found that cells from posterior slices of male mice fired more action potentials at night compared to day, and that this was maintained when synaptic transmission was blocked. However, in cells from anterior slices, there was no difference in excitability until synaptic transmission was blocked, indicating that anterior CA1 pyramidal cell excitability over the time of day is intrinsically regulated. Several factors

could contribute to the diurnal differences we observed in neuronal excitability, including diurnal variation in synaptic drive (excitatory and inhibitory input) and/or diurnal variation in intrinsic excitability. Additionally, these mechanisms underlying increased excitability may not be mutually exclusive. In posterior slices where diurnal variation in excitability was tied to synaptic input, one approach to differentiate between synaptic versus intrinsic factors is to assess the excitation to inhibition ratio following Schaffer collateral stimulation onto individual CA1 pyramidal cells. In anterior slices where diurnal variation in excitability was seemingly intrinsically generated, any number of voltage gated ion channels may be contributing. It will be interesting to narrow down ion channels that are differentially expressed as a function of both time of day and location along the longitudinal axis.

When examining sag ratio across time of day, we again found no differences in cells recorded from anterior slices but significant differences in cells recorded from posterior cells. In the presence of synaptic antagonists, the differences observed in sag in cells recorded from posterior slices were eliminated, while differences in anterior slices were uncovered. To our knowledge, this is the first studying examining a measure of HCN channel function (sag) between day and night in the hippocampus. Given that in the SCN, HCN channel function varies with time of day[18], and in the hippocampus, HCN channel expression varies along the dorsoventral axis[15], it is not surprising that we found that diurnal regulation of sag depends on both these factors in addition to sex. Intrinsic properties of CA1 pyramidal cells vary along the dorsoventral axis, at least partially due to differences in receptor composition. Most reports examining intrinsic properties, including sag, do so in the presence of synaptic antagonists, therefore, a suitable comparison of our

results in the in-tact circuit is difficult. Overall, we found that diurnal regulation of sag in anterior cells seems to be a product of intrinsic excitability, while in posterior cells, it seems to be driven by consequence of synaptic transmission.

When taken together, the measures of excitability and sag suggest that diurnal variation is intrinsic to anterior, dorsal-like CA1 pyramidal cells, but dependent on synaptic transmission for ventral-like pyramidal cells.

As diurnal variation depends on sex, we next sought to determine whether increased night-time LTP depends on sex. When assessing basal synaptic transmission across time of day, we found no difference between males and females, and no day-night difference, indicating that evoked basal synaptic transmission does not vary across time of day. While we have replicated the finding that LTP is greater at night compared to day[11-13], we show for the first time that despite differences in circadian regulation of synaptic transmission and excitability, the sex difference does not extend to day-night differences in LTP. An important note for this experiment is that we were underpowered to account for differences along the anterior-posterior axis. The lack of segregation of these data according to slice position along the anterior-posterior axis may have occluded detection of a sex difference in circadian regulation of LTP; this possibility cannot be entirely ruled out. Other factors that might have influenced our results is the use of a high frequency stimulation (HFS; 100 Hz for 0.5s, 2X).

Overall, we reveal diurnal variation in neuronal and synaptic function in the hippocampus and, importantly, underscore the importance of considering sex and neuronal heterogeneity within a brain region in the study of neural circuits.

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FIGURES

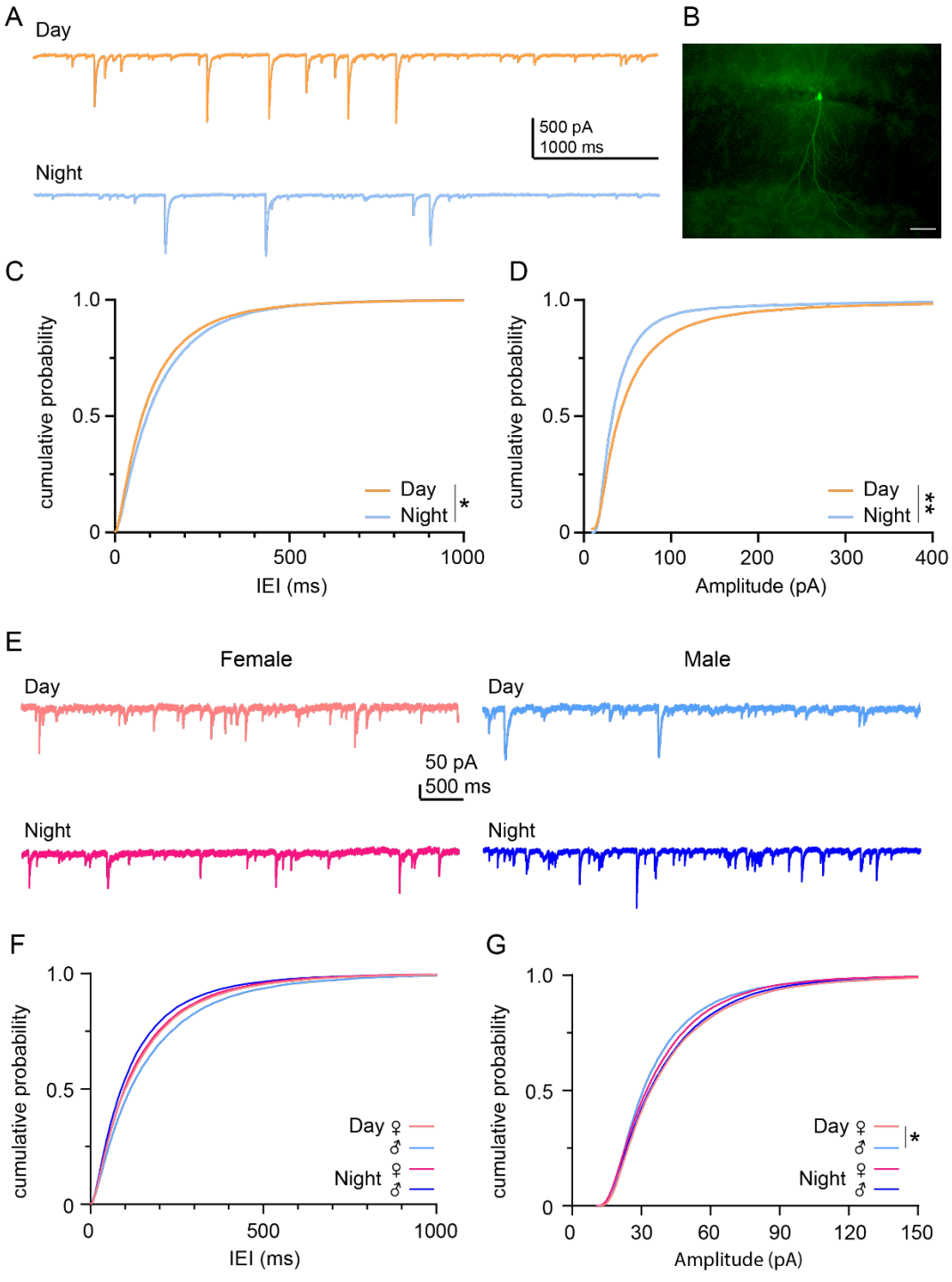
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Figure 1. Inhibition onto CA1 pyramidal cells is greater during the day compared to night, regardless of sex. Whole cell patch clamp was used to measure interevent interval (IEI) and amplitude of sIPSCs and mIPSCs onto CA1 pyramidal cells. Image of biocytin filled cell, scale bar is 200 microns (B). Pipette recording solution contained CsCl and QX-314, and cells were held at -70mV. Representative sIPSC traces (A) from pyramidal cells at day (orange) and night (blue). sIPSC IEI was shorter (C), and the amplitude was larger (D) during the day regardless of sex. Representative mIPSC traces (E) from pyramidal cells from female (pink) and male (blue) mice at day (light) and night (dark). mIPSC IEI did not vary with sex or time of day (F), but amplitude with larger amplitudes in females compared to males during the day (G). See Supplementary Table 1 for statistical results.

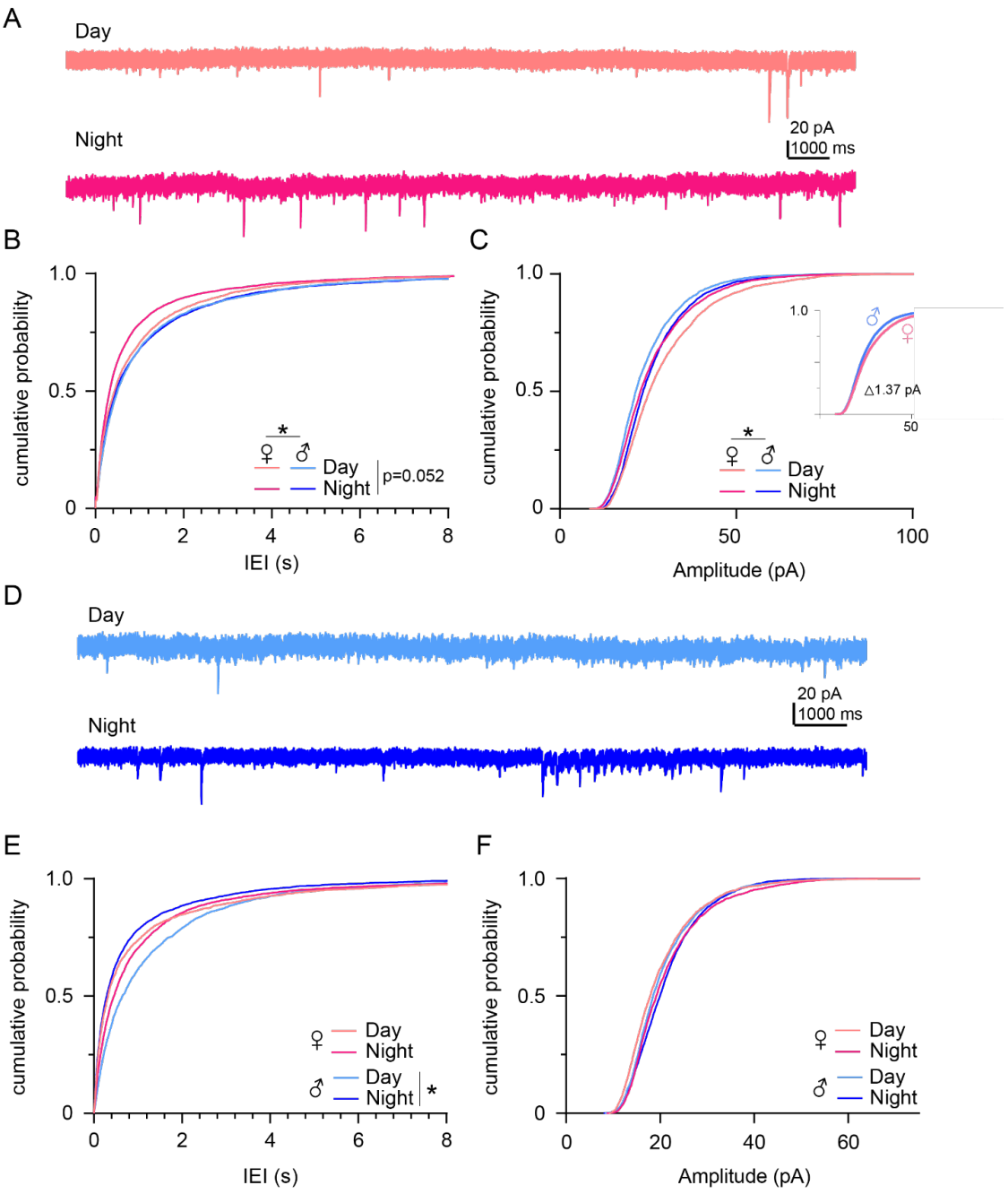


Figure 2. Excitation onto CA1 pyramidal cells depends on sex. Whole cell patch clamp was used to measure interevent interval (IEI) and amplitude of sEPSCs and mEPSCs onto CA1 pyramidal cells. Pipette recording solution contained CsOH and QX-314, and cells were held at -70mV. Representative sEPSC traces (A) from pyramidal cells from female mice at day (light pink) and at night (dark pink). sEPSC IEI trended toward longer IEIs during the day compared to night and were shorter in females compared to males (B). There was no effect of time of day on sEPSC amplitude (C). Representative mEPSC traces (D) onto pyramidal cells from male mice at day (light blue) and at night (dark blue). mEPSC IEIs in males were shorter at night than during the day,

with no statistical difference in time of day in females (E). mEPSC amplitudes were not different during the day compared to night or between male and female mice (F). See Supplementary Table 1 for statistical results.

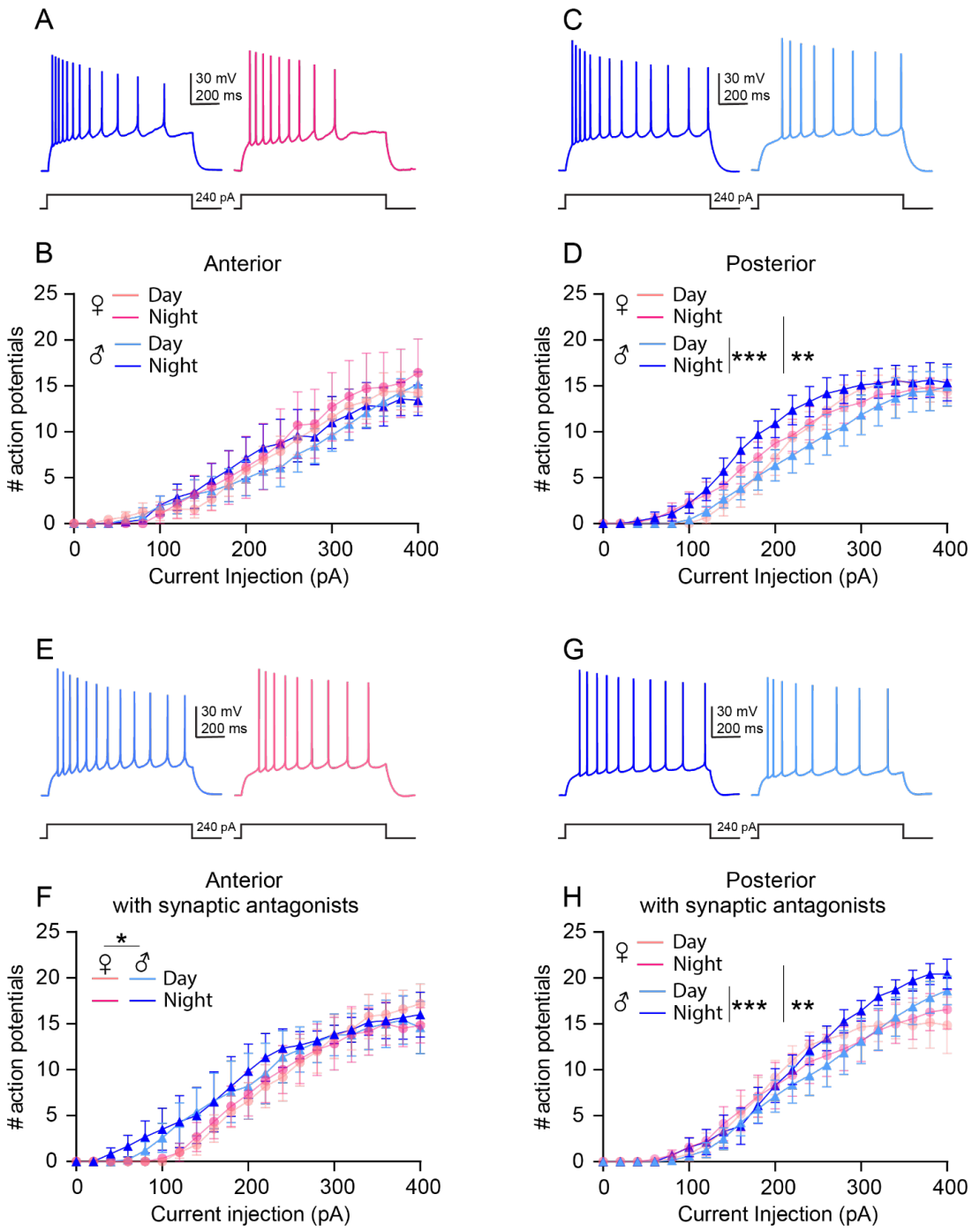


Figure 3. CA1 pyramidal cells are more excitable at night in posterior slices from male mice. Whole cell patch clamp was used to measure spikes per step with increasing current injections in CA1 pyramidal cells. Pipette recording solution contained KGlu, and cell voltage was not clamped. Whole-cell current clamp recordings of the number of spikes per step when recorded during the Day (light) or Night (dark) in Anterior or Posterior slices from Female (pink) or Male (blue) mice. When spikes per step were recorded from anterior slices in the intact circuit (A-B), there were no time of day or sex

differences. In posterior slices (C-D), CA1 pyramidal cells from male mice generate more spikes per step at night (dark blue) compared to day (light blue) and current injection at night in male mice (D, dark blue) elicit more spikes per step than those from female mice (D, dark pink). When spikes per step were recorded from anterior slices in synaptic blockers (E-F), spikes per step were greater in males (blue) compared to females (pink). In posterior slices (G-H), CA1 pyramidal cells from male mice generate more spikes per step at night (dark blue) compared to day (light blue) and current injection at night in male mice (H, dark blue) elicit more spikes per step than those from female mice (H, dark pink). See Supplementary Table 2 for statistical results.

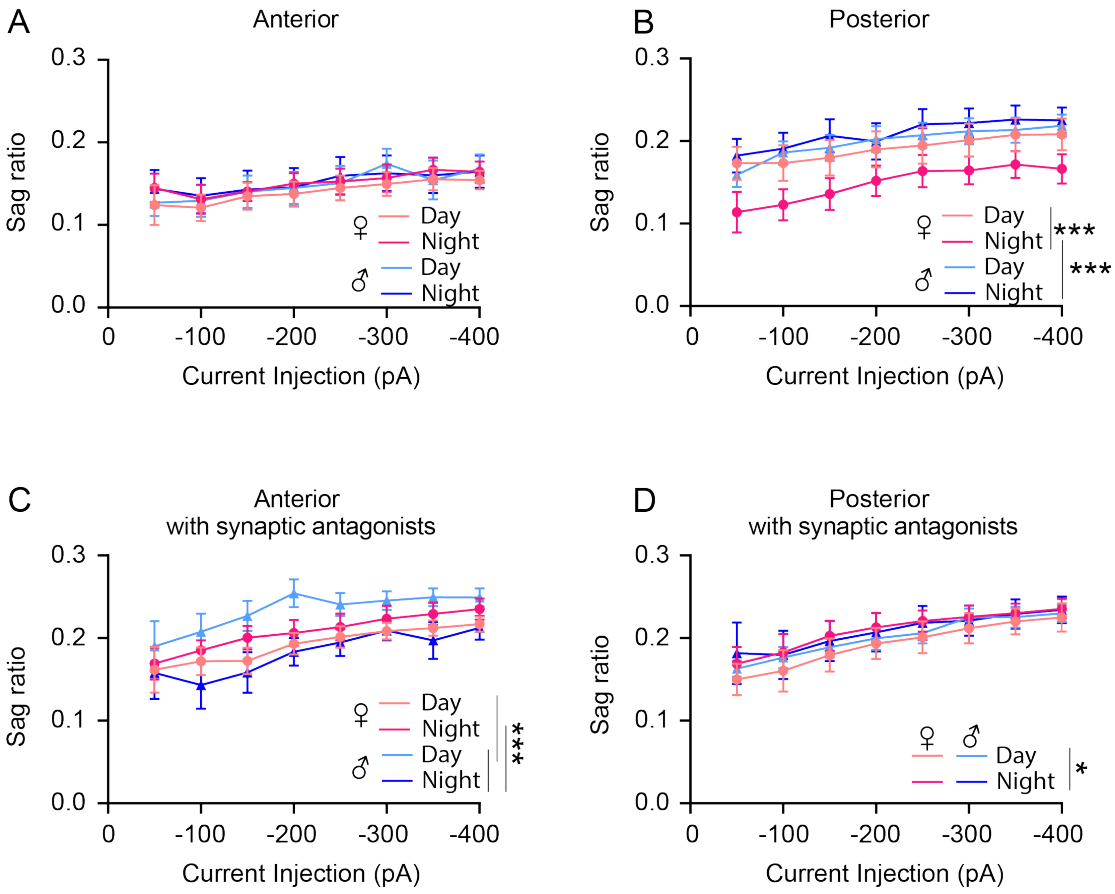


Figure 4. CA1 pyramidal cell sag ratio depends on time of day and sex. Whole cell patch clamp was used to measure sag with hyperpolarizing current injections in CA1 pyramidal cells. Pipette recording solution contained KGlu, and cell voltage was not clamped. Whole-cell current clamp recordings of the sag ratio when recorded during the Day (light) or Night (dark) in Anterior or Posterior slices from Female (pink) or Male (blue) mice. When sag ratio was recorded from anterior slices in the intact circuit (A), there were no time of day or sex differences. In posterior slices (B), CA1 pyramidal cells from female mice had a lower sag ratio at night (dark pink) compared to day (light pink), and at night, cells from female mice (dark pink) had a lower sag ratio than males (dark blue). When sag was recorded from anterior slices in synaptic blockers (C), CA1 pyramidal cells from male mice had greater sag ratios during the day (light blue) compared to night (dark blue). During the day, sag ratio in pyramidal cells was greater in male mice (light blue) than female mice (light pink), but at night, females (dark pink) had greater sag than males (dark blue). When sag was recorded from posterior slices synaptic blockers (D) there was a trend towards significance ($p = 0.059$) with greater sag at night (dark lines) compared to day (light lines). See Supplementary Table 3 for statistical results.

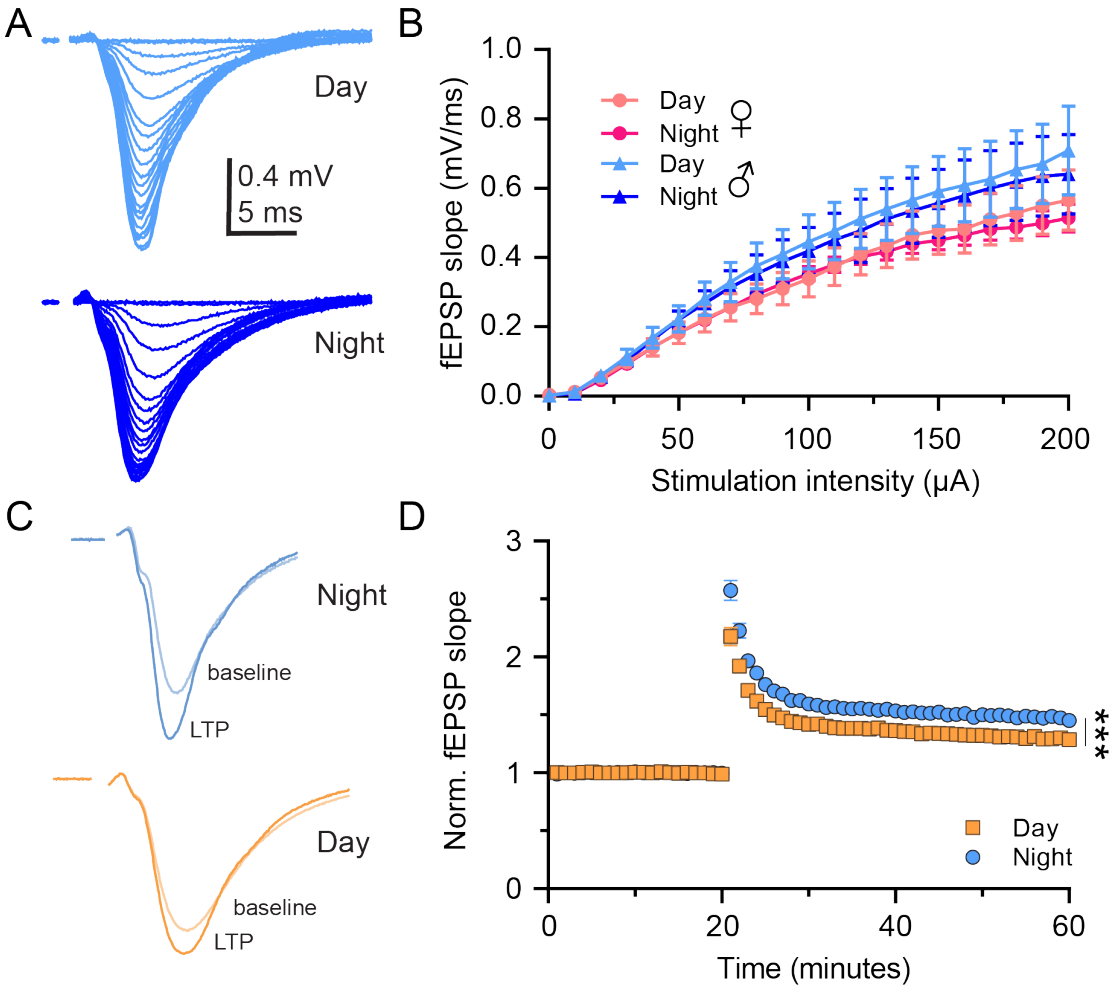
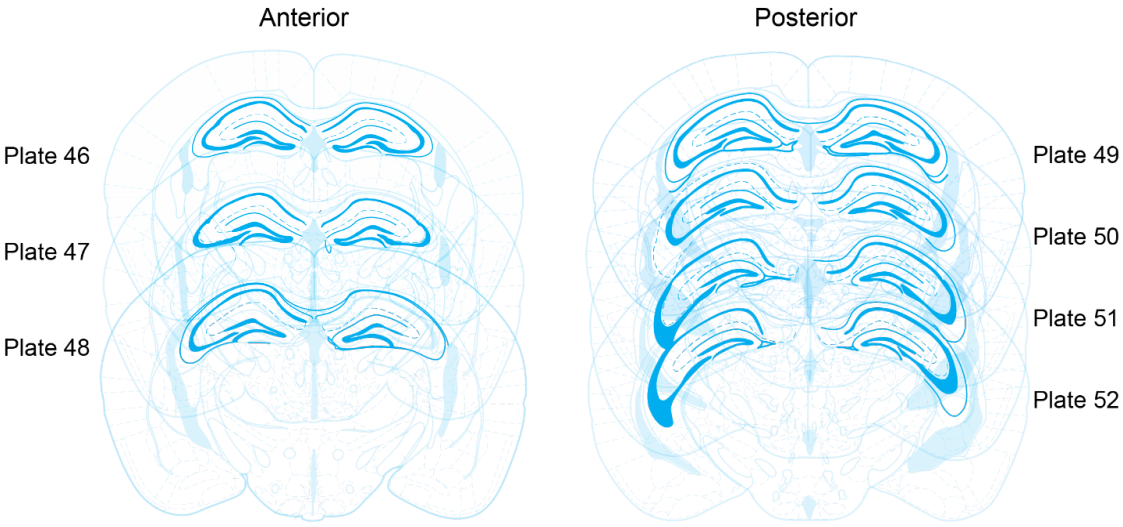


Figure 5. LTP is greater at night compared to day, regardless of sex. Field recordings in the dendritic field of CA1 pyramidal cells in response to stimulation of Schaffer collaterals was used to generate input-output curves (A-B). Relationship between the fEPSP slope and stimulation intensity, i.e. basal synaptic transmission, was unaffected by time of day and sex. Field recordings in the dendritic field of CA1 pyramidal cells before and after a high frequency tetanus (HFS; 100 Hz, 0.5s, 2X; t=20 mins) to CA3 Schaffer collaterals was used to produce LTP (C-D). LTP at night (blue) was greater compared to day (orange) but was not different between females and males.



Supplementary Figure 1. Anterior-Posterior Segregation of slices per Allen Brain Atlas. Cells were considered "anterior" if recorded in hippocampal slices that matched panel 48 or lower in the Allen brain atlas, and "posterior" if they matched 49 or higher.

Experiment	Measure	Transformation	Sex	Time of Day	Interaction
sIPSC	**IEI**	Log10(amp)	$\chi2$ (1) = 2.367, p = 0.124	$\chi2$ (1) = 4.551, p = 0.033	$\chi2$ (1) = 1.693, p = 0.193
	Amp	Log10(IEI+1)	$\chi2$ (1) = 1.179, p = 0.277	$\chi2$ (1) = 7.070, p = 0.008	$\chi2$ (1) = 0.553 p = 0.457
mIPSC	**IEI**	Log10(amp)	$\chi2$ (1) = 0.731, p = 0.392	$\chi2$ (1) = 0.093, p = 0.760	$\chi2$ (1) = 3.339, p = 0.068
	Amp	Log10(IEI+1)	$\chi2$ (1) = 1.493, p = 0.222	$\chi2$ (1) = 0.281, p = 0.596	$\chi2$ (1) = 4.325, p = 0.038
sEPSC	**IEI**	Log10(amp)	$\chi2$ (1) = 5.211, p = 0.022	$\chi2$ (1) = 3.786, p = 0.052	$\chi2$ (1) = 3.341, p = 0.068
	Amp	Log10(IEI+1)	$\chi2$ (1) = 5.424, p = 0.020	$\chi2$ (1) = 0.799, p = 0.371	$\chi2$ (1) = 0.483 p = 0.487
mEPSC	**IEI**	Log10(amp)	$\chi2$ (1) = 0.365, p = 0.546	$\chi2$ (1) = 3.415, p = 0.065	$\chi2$ (1) = 5.286, p = 0.021
	Amp	Log10(IEI+1)	$\chi2$ (1) = 1.457, p = 0.227	$\chi2$ (1) = 2.071, p = 0.150	$\chi2$ (1) = 1.691, p = 0.194

Supplementary Table 1. Statistical analyses of IPSCs and EPSCs.

	Anterior	**Posterior**
Time of Day	$F_{1, 429.350}$ = 2.727, p = 0.099	$F_{1, 560.259}$ = 16.764, p < 0.001
Sex	$F_{1, 429.350}$ = 1.093, p = 0.269	$F_{1, 560.259}$ = 0.411, p = 0.522
Current step	$F_{15, 55.715}$ = 25.160, p < 0.001	$F_{15, 64.175}$ = 46.064, p < 0.001
Time of Day X Sex	$F_{1, 429.350}$ = 0.001, p = 0.997	$F_{1, 560.259}$ = 8.219, p = 0.004
Time of Day X Current step	$F_{15, 55.715}$ = 0.106, p > 1.000	$F_{15, 64.175}$ = 0.403, p = 0.973
Sex X Current step	$F_{15, 55.715}$ = 0.310, p = 0.992	$F_{15, 64.175}$ = 0.124, p > 1.000
Current step X Time of Day X Sex	$F_{15, 55.715}$ = 0.118, p > 1.000	$F_{15, 64.175}$ = 0.384, p = 0.979
	Anterior with Antagonists	**Posterior with Antagonists**
Time of Day	$F_{1, 271.518}$ = 0.100, p = 0.753	$F_{1, 355.663}$ = 3.443, p = 0.064
Sex	$F_{1, 271.518}$ = 5.405, p = 0.021	$F_{1, 355.663}$ = 1.182, p = 0.268
Current step	$F_{15, 33.436}$ = 23.245, p < 0.001	$F_{15, 42.155}$ = 59.628, p < 0.001
Time of Day X Sex	$F_{1, 271.518}$ = 0.428, p = 0.514	$F_{1, 355.663}$ = 5.607, p = 0.018
Time of Day X Current step	$F_{15, 33.436}$ = 0.066, p > 1.000	$F_{15, 42.155}$ = 0.100, p > 1.000
Sex X Current step	$F_{15, 33.436}$ = 0.373, p = 0.977	$F_{15, 42.155}$ = 0.830, p = 0.641
Current step X Time of Day X Sex	$F_{15, 33.436}$ = 0.071, p > 1.000	$F_{15, 42.155}$ = 0.343, p = 0.986

Supplementary Table 2. Statistical analyses of spikes per step in response to depolarizing current injections.

	Anterior	Posterior
Time of Day	$F_{1, 227.212} = 1.070$, $p = 0.302$	$F_{1, 305.374} = 5.727$, $p = 0.017$
Sex	$F_{1, 227.212} = 0.467$, $p = 0.495$	$F_{1, 305.374} = 26.122$, $p < 0.001$
Current step	$F_{7, 48.979} = 1.810$, $p = 0.107$	$F_{7, 72.856} = 3.461$, $p = 0.003$
Time of Day X Sex	$F_{1, 227.212} = 0.291$, $p = 0.590$	$F_{1, 305.374} = 15.480$, $p < 0.001$
Time of Day X Current step	$F_{7, 48.979} = 0.090$, $p = 0.999$	$F_{7, 72.856} = 0.062$, $p > 1.000$
Sex X Current step	$F_{7, 48.979} = 0.085$, $p = 0.999$	$F_{7, 72.856} = 0.065$, $p > 1.000$
Current step X Time of Day X Sex	$F_{7, 48.979} = 0.030$, $p > 1.000$	$F_{7, 72.856} = 0.120$, $p = 0.997$
	Anterior with Antagonists	**Posterior with Antagonists**
Time of Day	$F_{1, 105.282} = 10.435$, $p = 0.002$	$F_{1, 163.214} = 3.628$, $p = 0.059$
Sex	$F_{1, 105.282} = 2.808$, $p = 0.097$	$F_{1, 163.214} = 0.378$, $p = 0.540$
Current step	$F_{7, 29.822} = 4.548$, $p = 0.001$	$F_{7, 41.798} = 6.731$, $p < 0.001$
Time of Day X Sex	$F_{1, 105.282} = 31.372$, $p < 0.001$	$F_{1, 163.214} = 0.716$, $p = 0.399$
Time of Day X Current step	$F_{7, 29.822} = 0.165$, $p = 0.990$	$F_{7, 41.798} = 0.089$, $p = 0.999$
Sex X Current step	$F_{7, 29.822} = 0.078$, $p = 0.999$	$F_{7, 41.798} = 0.040$, $p > 1.000$
Current step X Time of Day X Sex	$F_{7, 29.822} = 0.243$, $p = 0.970$	$F_{7, 41.798} = 0.032$, $p > 1.000$

Supplementary Table 3. Statistical analyses of sag ratio in response to hyperpolarizing current injections.

	Input-Output	LTP
Time of Day	$F_{1, 132.001} = 0.001$, $p = 0.981$	$F_{1, 335.447} = 108.681$, $p < 0.001$
Sex	$F_{1, 132.001} = 0.356$, $p = 0.552$	$F_{1, 335.447} = 0.030$, $p = 0.864$
Time of Day X Sex	$F_{1, 132.001} = 0.002$, $p = 0.961$	$F_{1, 335.447} = 1.292$, $p = 0.256$
Stimulation Intensity	$F_{1, 544.476} = 1380.736$, $p < 0.001$	
Time of Day X Stimulation Intensity	$F_{1, 544.476} = 0.726$, $p = 0.394$	
Sex X Stimulation Intensity	$F_{1, 544.476} = 17.958$, $p < 0.001$	
Time of Day X Sex X Stimulation Intensity	$F_{1, 544.476} = 0.134$, $p = 0.715$	

Supplementary Table 4. Statistical analyses of field input-output curves and LTP.

DYSREGULATED CLOCK GENE EXPRESSION AND ABNORMAL DIURNAL REGULATION OF HIPPOCAMPAL INHIBITORY TRANSMISSION AND SPATIAL MEMORY IN AMYLOID PRECURSOR PROTEIN TRANSGENIC MICE

by

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Neurobiology of Disease 2021 Jul 30;158:105454.doi: 10.1016/j.nbd.2021.105454

Format adapted for dissertation

ABSTRACT

Patients with Alzheimer's disease (AD) often have fragmentation of sleep/wake cycles and disrupted 24-h (circadian) activity. Despite this, little work has investigated the potential underlying day/night disruptions in cognition and neuronal physiology in the hippocampus. The molecular clock, an intrinsic transcription-translation feedback loop that regulates circadian behavior, may also regulate hippocampal neurophysiological activity. We hypothesized that disrupted diurnal variation in clock gene expression in the hippocampus corresponds with loss of normal day/night differences in membrane excitability, synaptic physiology, and cognition. We previously reported that the Tg-SwDI mouse model of AD has disrupted circadian locomotor rhythms and neurophysiological output of the suprachiasmatic nucleus (the primary circadian clock). Here, we report that Tg-SwDI mice failed to show day-night differences in a spatial working memory task, unlike wild-type controls that exhibited enhanced spatial working memory at night. Moreover, Tg-SwDI mice had lower levels of Per2, one of the core components of the molecular clock, at both mRNA and protein levels when compared to age-matched controls. Interestingly, we discovered neurophysiological impairments in area CA1 of the Tg-SwDI hippocampus. In controls, spontaneous inhibitory post-synaptic currents (sIPSCs) in pyramidal cells showed greater amplitude and lower inter-event interval during the day than the night. However, the normal day/night differences in sIPSCs were absent (amplitude) or reversed (inter-event interval) in pyramidal cells from Tg-SwDI mice. In control mice, current injection

into CA1 pyramidal cells produced more firing during the night than during the day, but no day/night difference in excitability was observed in Tg-SwDI mice. The normal day/night difference in excitability in controls was blocked by GABA receptor inhibition. Together, these results demonstrate that the normal diurnal regulation of inhibitory transmission in the hippocampus is diminished in a mouse model of AD, leading to decreased daytime inhibition onto hippocampal CA1 pyramidal cells. Uncovering disrupted day/night differences in circadian gene regulation, hippocampal physiology, and memory in AD mouse models may provide insight into possible chronotherapeutic strategies to ameliorate Alzheimer's disease symptoms or delay pathological onset.

INTRODUCTION

Alzheimer's disease (AD), the most prevalent cause of dementia worldwide [1], is characterized by development of pathological amyloid beta plaques and tau tangles throughout the brain, and regions associated with memory such as the hippocampus are particularly vulnerable. Patients with AD also exhibit subclinical epileptiform activity or seizures, indicative of network hyperexcitability [2]. This network hyperexcitability is seen in early, even preclinical, stages of AD (defined by normal cognition with positive biomarkers for amyloid pathology). In general, epileptiform activity is greater and seizure thresholds are lower during sleep/rest, resulting in increased frequency of seizures during that time of day [3,4]. Thus, network hyperexcitability may be exacerbated in AD patients because of documented disruptions in sleep-wake cycles, misaligned body temperature rhythms, and altered patterns of activity, all of which are driven by the circadian clock [5,6].

Circadian rhythms are endogenous, ~24-h oscillations in physiology and behavior that are driven by the molecular clock. The molecular clock is a cellular transcription-translation feedback loop, involving the proteins BMAL1, PER1/2, and CRY1/2, present in nearly all cells of the body [7]. The principal circadian pacemaker located in the hypothalamus, the suprachiasmatic nucleus (SCN), is responsible for coordinating rhythms throughout the body and in different regions of the brain [8 {Paul, 2019 #1128}]. In addition to physiological rhythms, cognitive performance also fluctuates across the course of the day, peaking shortly after waking and declining after several hours [9]. Environmental

disruptions, such as fragmented sleep or altered light cycles, and genetic manipulations to components of the molecular clock impair cognition [9-13]. Similar to rhythms of behavior and cognition, electrophysiological properties fluctuate across the course of the day in many brain regions, including the hippocampus [14,15]. Rodent studies have shown that hippocampal long-term potentiation (LTP), a synaptic correlate of learning and memory [16], is enhanced during the active period compared to the inactive period [17,18]. In fact, whole-body deletion of *Bmal1* impairs LTP [19]. At the cellular level, rat CA1 pyramidal cell resting membrane potential is more depolarized at night relative to day [15]. Clock-controlled genes expressed in the hippocampus may underlie this link given that over 600 transcripts show circadian variation in the hippocampus, including those encoding ion channels and synaptic proteins such as *Kcna4*, *Clca2*, *Trpc7*, *Kcnk3*, *Gria3*, and *Nlgn1* [20].

We recently found circadian impairment in the central SCN clock in the Tg-SwDI model of AD [21] – these mice express the human *APP* gene with the Swedish (K670N/M671L), Dutch (E693Q), and Iowa (D694N) mutations at levels similar to the levels of mouse APP [22]. We showed that these mice do not have hyperactive locomotor activity frequently observed in other mouse models of AD [23] but do show a significantly shorter free-running period than age-matched controls at 3, 6, and 10 months of age. In addition, Tg-SwDI mice at 6-8 months of age have greatly reduced day-night differences in SCN action potential firing rate (compared to control mice), primarily due to a lower daytime firing rate [21]. Here, we asked whether previous findings extend to other brain regions, particularly the hippocampus. Tg-SwDI mice develop fibrillar amyloid deposition specifically in the cerebral microvasculature rather than in the parenchyma, and this pattern of deposition is associated with impaired spatial learning and memory [22,24,25]. To our

knowledge, examination of day/night differences in hippocampal function or physiology has yet to be assessed in this model or other models of AD. Therefore, in the present study we sought to determine whether altered expression of key molecular clock components in the hippocampus corresponds with loss of day/night differences in membrane excitability, synaptic physiology, and cognition.

MATERIALS AND METHODS

Animals. All animal procedures followed the Guide for the Care and Use of Laboratory Animals, U.S. Public Health Service, and were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Mice of both sexes were maintained on a 12:12 light/dark cycle with ad libitum access to food (LabDiet Rodent 5001 by Purina) and water. Mice expressing the human amyloid precursor protein with the familial Swedish (K670N/M671L), Dutch (E693Q), Iowa (D694N) homozygous mutations (Tg-SwDI; Davis et al. 2004) were bred at the University of Alabama at Birmingham (UAB) on a congenic on a C57BL/6 background. Mice were compared to age matched C57BL/6 wild type (WT) mice from the National Institute on Aging Aged Rodent Colony or from the C57BL/6 colony at UAB. Control, C57BL/6 mice used in the electrophysiology experiment in Figure 4 also carried the *Per1*::GFP transgene [26].

Spatial working memory. Spatial working memory was tested during the day (Zeitgeber time (ZT) 2) and night (ZT 14) with a T-maze, spontaneous alternation protocol (adapted from [27])[28]. Mice were handled for 4 consecutive days prior to testing to reduce anxiety from being handled during the test. Mice were placed in the maze at the base of the T arm and allowed to freely choose an arm of the T. Upon entry to an arm, a gate was closed, trapping the mouse in the arm for 30 seconds. The mouse was removed, the gate opened, and the mouse returned to the base of the maze. An alternation was recorded if the

mouse entered the opposite arm as the last entry. Each mouse performed 5 trials, and an alternation percentage was calculated as (# alternations / # of trials). Males and females were tested separately, and WT and AD were interleaved during trials. During night testing, dim red light (< 5.0 lux) was used for visualization. Animals were only excluded from analysis if there was significant cage disruption during the time period of the test (i.e., excessive fighting in the animal cage) or if there was a notable directional bias in arm choices (i.e. animals only used the left arm in all 10 entries).

Quantitative real-time PCR (qRT-PCR). Quantitative real-time PCR was performed as previously described (Lucas et al. 2012). At 4 months of age, mice were euthanized via cervical dislocation at ZT 2, 8, 14, and 20 with the aid of night vision goggles for enucleation at dark time points. Brains were removed and hippocampus was extracted and stored at -80ºC. Tissue was homogenized in TRIzol (Thermo Fisher Scientific) using the Omni Bead Ruptor Homogenizer (OMNI International, Kennesaw, GA, USA). RNA was then isolated using the choloform-isopropanol method following the manufacturer's instructions (Invitrogen). RNA concentration and purity were assessed using a NanoDrop One (Thermo Fisher Scientific). Equivalent amounts of RNA (1 µg) were treated with DNase I (Promega) at 37°C for 30 minutes, DNase Stop solution at 65°C for 15 minutes and then RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) at 37°C for two hours. Samples were then stored at -20ºC. Transcripts were measured using mouse-specific primers from Applied Biosystems and JumpStart Taq Readymix (Sigma, St. Louis, MO, USA) using a protocol with an initial ramp for 10 min at 95°C and 40 subsequent cycles of 95°C for 15 sec and

60°C for one min. Relative concentration of transcript was calculated in comparison to a standard curve generated from pooled cDNA samples and then diluted (1:5, 1:10, 1:20, 1:40; calibrator method). These values were then normalized to 18S (Hs99999901_s1) and expressed as mean +/- SEM. 18S values were analyzed to ensure no difference across time of day or genotype prior to normalization. The Applied Biosystems primer/probe sets used are as follows: beta-actin (Mm00607939_s1), Per1 (Mm00501813_m1), Per2 (Mm00478099_m1), Rev-erbα (Mm00520708_m1), Cry1 (Mm00514392_m1), Cry2 (Mm01331539_m1), Bmal1 (Mm00500223_m1), Bmal2 (Mm00549497_m1).

Thioflavin-S staining and Immunostaining.

Thioflavin-S staining At 6 months of age, mice were anesthetized with isoflurane and transcardially perfused with ice-cold PBS. The whole brain was extracted and fixed in 4% paraformaldehyde for ~24 h at 4°C, and then cryoprotected in 15% sucrose in PBS for ~24 h followed by 30% sucrose in PBS for another ~24 h at 4°C. The brains were then sectioned into 40 μm slices using a Leica SM 2010R microtome. Hippocampal slices were mounted on glass slides with 8-10 slices per slide. The slices were then allowed to dry completely before staining (about 2-3 hours). Thioflavin-S staining followed a standard staining procedure with a 7-chamber staining apparatus. The slides were first washed with 70% EtOH for one minute followed by a wash in 80% EtOH for one minute. The slides were then incubated in filtered Thio-S stain (Sigma-Aldrich, 1% in 80% EtOH) for 15 minutes. The slides were protected from light. Following the staining, the slides were washed in 80% EtOH for one minute and then 70% EtOH for one minute. Finally, the slices were washed twice with distilled water. The slices were allowed to dry in a light-tight

container for at least two hours then coverslipped using VECTASHEILD HardSet Antifade Mounting Medium with DAPI. Thio-S-stained beta-pleated sheets were visualized using a Keyence BZ-X series All-in-One Fluorescence Microscope.

Western Blots At 4 and 9 months of age, mice were euthanized via cervical dislocation at ZT 2, 8, 14, and 20 with the aid of night vision goggles for enucleation at dark time points. Brains were removed and whole hippocampus was extracted. Hippocampi were placed in ice-cold sucrose homogenization buffer (5 mM Tris-HCl (pH 7.4), 0.32 M sucrose, with a protease inhibitor tablet (Complete Mini, Roche Diagnostics, Mannheim, Germany)), minced using a scalpel blade, snap frozen in liquid nitrogen, and stored at -80°C until ready for homogenization. Samples were homogenized in ice-sold sucrose homogenization buffer using a Power Gen 125 homogenizer (Thermo Fisher Scientific, Waltham, MA). Protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Pierce™ BCA Protein Assay Kit) and 15 µg homogenate were loaded into 4-12% Bis-tris gels (Invitrogen). Gels were transferred onto nitrocellulose immunoblotting paper using Bio-Rad Trans-Blot Turbo transfer system at 1.3 Amp and 25 V for 12 min. Blots were blocked in blocking buffer for 1 hour at room temperature and probed overnight at 4°C for PER2 (1:1000 in 5% BSA and TBST; Alpha Diagnostic Intl., Inc., #PER21-A), BMAL1 (1:1000 in 5% BSA and TBST, Signalway Antibody, LLC, #21415,), GSK3β (3D10) (1:1000 in 5% milk and TBST, Cell Signaling Technology, #9832), phospho-GSK3β (Ser9) (1:1000 in 5% BSA and TBST, Cell Signaling Technology, #93360), and GAPDH (1:5000, Cell Signaling, #2118). The appropriate IRDye secondary (Li-Cor Biosciences) was applied, and then blots were imaged and analyzed using Li-Cor Image Studio Lite version 5.2. Comparisons across

immunoblots were achieved using an average of two or three inter-blot control samples on each blot. For every blot, all bands were normalized to the inter-blot average then to the loading control (GAPDH or GSK3β).

Immunohistochemistry At 4 months of age, mice were anesthetized with isoflurane and transcardially perfused with ice-cold PBS between ZT 3 and ZT 6. The whole brain was extracted and fixed in 4% paraformaldehyde for ~24 h at 4°C, and then cryoprotected in 15% sucrose in PBS for ~24 h followed by 30% sucrose in PBS for another 24 h at 4°C. Fixed, cryoprotected brains were then frozen on dry ice and stored at -80°C until 40-µm serial coronal slices were sectioned with a cryostat (Leica CM1850). For antibody labeling, sections were washed in PBS at RT for 3 X 10 min before blocking for 30 min in a PBS solution containing 5% Normal Donkey Serum (NDS) and 0.25% Triton X-100, followed by overnight incubation at 4°C with primary antibodies against GAD-67 (Millipore, 1:2000) and parvalbumin (Swant, 1:2000) in PBS containing 1% NDS and 0.1% Triton X-100. Sections were washed in 0.1% Triton X-100 PBS for 3 X 10 min before incubation with either donkey anti-rabbit Alexa fluor 488 (Invitrogen, 1:1000) or donkey anti-mouse Alexa fluor 555 (Invitrogen, 1:1000) secondary antibody in PBS containing 1% NDS and 0.1% Triton X-100. Sections were then washed in PBS and mounted on glass slides and coverslips with ProLong Gold Antifade Mounting media containing DAPI. Slides were imaged with a Nikon A1R confocal microscope using 10X and 20X objectives. Images were analyzed using ImageJ. For GAD analysis, background noise was automatically subtracted in ImageJ and three, identical rectangular regions of interest (ROI) were drawn in stratum oriens, stratum pyramidale, and stratum moleculare of area CA1 in dorsal

hippocampus. The mean intensity of each ROI was measured in the channel of interest. For PV image analysis, PV^+ cell counts were made in ImageJ using the multi-point tool.

Electrophysiology. *Voltage clamp recordings* at 4 months of age, mice were euthanized with cervical dislocation and rapid decapitation, brains were removed, and 350 μm coronal slices were prepared using a VT1000P vibratome (Leica Biosystems). Slices were cut in ice cold cutting solution containing the following (in mM): 110 choline chloride, 25 glucose, 7 MgCl2, 2.5 KCl, 1.25 Na2PO4, 0.5 CaCl2, 1.3 Na-ascorbate, 3 Na-pyruvate, and 25 NaHCO3, bubbled with 95% O2/5% CO2. Slices were rested at room temperature for at least one hour in recovery solution containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 Na2PO4, 2 CaCl2, 1 MgCl2, 25 NaHCO3, 25 glucose and 2 kynurenic acid bubbled with 95% O2/5% CO2. Recordings were made in standard artificial cerebral spinal fluid (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 Na2PO4, 2 CaCl2, 1 MgCl2, 25 NaHCO3, and 25 glucose bubbled with 95% O2/5% CO2. Patch pipette solution contained (in mM): 140.0 CsCl, 10.0 EGTA, 5.0 MgCl2, 2.0 Na-ATP, 0.3 Na-GTP, 10.0 HEPES, and 0.2% biocytin (pH 7.3, 290 mOsm), and QX-314 (sodium channel antagonist, 5 mM) added at time of use. Examples of biocytin filled pyramidal neurons from both WT and Tg-SwDI mice are shown in Supplementary Figure 1. Patch pipettes (BF150-086; Sutter Instruments, Novato, CA) were pulled on a Sutter P-97 horizontal puller (Sutter Instruments, Novato, CA) to a resistance between 3-5 MΩ. Recordings were performed in a submersion chamber with continuous perfusion of oxygenated ACSF at 2.5 mL/min. The blind patch technique was used to acquire whole-cell recordings from CA1 pyramidal neurons. Neuronal activity was recorded using an

Axopatch 200B amplifier and pClamp10 acquisition software (Molecular Devices, Sunnyvale, CA). Signals were filtered at 5 kHz and digitized at 10 kHz (Digidata 1440). Spontaneous inhibitory post-synaptic currents (sIPSCs) were recorded between ZT 4-10 or ZT 13-19 and pharmacologically isolated with bath perfusion of NBQX (AMPAR antagonist, 10 μM, Hello Bio) and CPP (NDMAR antagonist, 5 μM, Hello Bio). All cells were dialyzed for 5 min prior to experimental recordings and held at -70 mV. Cells used for analysis had access resistance less than 30 MOhms that did not change by more than 20% for the duration of recording.

Current Clamp Recordings At 6-7 months of age, mice were euthanized via cervical dislocation at ZT 0-1 for day or 11-12 for night recordings and coronal hippocampal slices (300 μm) were prepared using a Campden 7000SMZ (World Precision Instruments, Lafayette, IN). Slices were cut in cold cutting solution containing the following (in mM): 250 sucrose, 26 NaHCO3, 1.25 Na2HPO4-7H2O, 1.2 MgSO4-7H2O, 10 glucose, 2.5 MgCl2, 3.5 KCl bubbled with 95% O2/5% CO2. Slices were transferred to a beaker containing 50% sucrose saline and 50% normal saline (in mM: 130 NaCl, 20 NaHCO3, 1 Na2HPO4-7H2O, 1.3 MgSO4-7H2O, 10 Glucose, 3.5 KCl, 2.5 CaCl2) at room temperature for 20 min and then transferred to an open recording chamber (Warner Instruments, Hambden, CT) that was continuously perfused at a rate of 2.0 ml/min with normal saline, bubbled with 5% CO2 / 95% O2 and heated to 34 ± 0.5 °C. Patch pipette solution contained (in mM): 135 K-gluconate, 10 KCl, 10 HEPES, 0.5 EGTA; pH 7.4. Patch pipettes (BF150-110-10; Sutter Instruments, Novato, CA) were pulled on a Sutter P-97 horizontal puller (Sutter Instruments, Novato, CA) to a resistance between 4-6 MΩ. Neuronal activity was recorded using an Multiclamp 700B amplifier and pClamp10

acquisition software (Molecular Devices, Sunnyvale, CA). Signals were filtered at 10 kHz and digitized at 20 kHz (Digidata 1440A). Whole-cell current clamp recordings were made from CA1 pyramidal cells using blind patch technique between ZT 2-8 or ZT 13-19 for day and night recordings, respectively. Resting membrane potential was measured in gap free current clamp mode (as in (Paul et al., 2016). Neuronal excitability was studied by injecting progressive steps of depolarizing current from rest (0 pA to 280 pA at 20 pA increments) and counting the number of action potentials firing during each 800ms current step. For recordings in *Per1::GFP* mice, slices were treated with GABAR antagonists (10μM bicuculline and 1μM CGP55848) or vehicle (0.002% DMSO) continuously in the bath for the duration of the recording. Cells were included if they exhibited stable recordings with resting membrane potential of < -50mV, action potential amplitude of > 55mV, and had holding (leak) current less than -130pA when held at -60mV.

Statistical Analysis. Statistics were calculated using SPSS software (version 25) and GraphPad prism 6 software. Assumptions of parametric tests, including normality and homogeneity of variance were determined, and if violated, nonparametric or ordinal tests were done. Ordinal regression was used for behavioral data analysis, stratified by sex (see behavioral methods above), with post hoc comparisons and Holmes corrected alpha. RNA and protein expression data were analyzed via two-way ANOVA because four times of day are not sufficient to ascertain "rhythmicity" (e.g., via Cosinor or JTK_Cycle analysis). Thus, data were first assessed for a main effect of sex, and if significant, two-way ANOVAs were performed on data stratified by sex. In the absence of sex differences, two-way ANOVAs were performed with both sexes pooled. All significant interactions were

followed by Tukey's post hoc comparison. Voltage clamp data were analyzed with Kolmogorov-Smirnov tests with Bonferroni correction, and histology data were compared with independent samples t-tests. Unless otherwise specified, significance was ascribed at $p < 0.05$. Current clamp data were analyzed with three- way ANOVAs with repeated measures followed by planned comparisons of each current step. Although data included both males and females in each group, the groups were insufficiently powered for a four-way ANOVA (Sex X Time X Genotype X Current Step). The data that support the findings of this study are available from the corresponding author.

RESULTS

Tg-SwDI mice lack normal diurnal patterns in spatial working memory

As previously mentioned, cognitive performance fluctuates across the day-night cycle, typically peaking during the time an animal is awake [29]. As rodents are nocturnal, most cognitive performance is better during the night than during the day [30,31], although contextual fear memory is better during the day than night [32]. These differences persist even if mice are housed in constant darkness [33,34]. Thus, testing at both times of day is important when assessing cognitive function. Tg-SwDI mice have spatial memory deficits (i.e., Barnes maze performance) when vascular amyloid deposits are first detected in the subiculum (~3 months of age; [24,25]), although deficits in other cognitive tasks such as novel object recognition are not detected even at older ages [35,36]. Importantly, day/night differences have yet to be assessed in Tg-SwDI mice on any memory task at any age. To address this question, we examined spontaneous alternation in a T-maze, a task with demonstrated day/night differences in mice [37] and other rodents [38,39]. This task employs trials, thus avoiding the confound of diurnal differences in locomotor activity that could affect the number of arm entries [36]. Moreover, better performance at night persists in rodents housed in constant darkness [38] and does not require multiple days of training that could disrupt sleep and circadian rhythms. Male and female Tg-SwDI mice (4 months and 9 months) were compared to control mice (day vs night) in four separate experiments. While both male and female mice were examined, they were done so in separate experiments, therefore, we are unable to examine differences in sex. Day/night differences

in alternation scores for female, 4-month-old mice depended upon genotype (Figure 1A and B, Supplementary Table 1; two-way ANOVA, interaction, $p < 0.05$). Control mice had significantly higher alternation at night compared to day ($p < 0.05$), but Tg-SwDI mice had no day/night difference due to significantly lower alternation at night compared to alternation of WT mice at night ($p < 0.05$). Males at 4 months of age showed better performance at night than during the day (Figure 1B main effect, $p < 0.05$); however, the interaction with genotype failed to reach significance ($p = 0.20$). At 9 months, day-night differences for both the male and female mice depended upon genotype (Figure 1C and D, $p < 0.05$). Although male and female control mice exhibited higher alternation at night ($p < 0.05$), neither male nor female Tg-SwDI animals showed a significant day/night difference in alternation ($p > 0.38$). These results show that, in general, diurnal rhythms in spatial working memory are impaired in this AD model. Notably, as late as 6 months of age, we detected fibrillar amyloid depositions in the subiculum but not in the hippocampus (Supplementary Figure 2), suggesting that dysregulation in diurnal rhythms in spatial working memory occurs before plaque deposition.

Alterations to the hippocampal molecular clock in Tg-SwDI mice

A functional circadian system is important for learning and memory [33,40], and the Tg-SwDI AD model shows impaired spatial learning and memory (Figure 1; [24]) along with disrupted circadian wheel running behavior and altered SCN activity [21]. Thus, we sought to determine whether Tg-SwDI mice also have disruptions of the hippocampal molecular clock. To test this hypothesis, we extracted whole hippocampus from 4-month Tg-SwDI males and females and age-matched WT controls at ZT 2, 8, 14, and 20, and performed qRT-PCR to examine mRNA expression of *Bmal1*, *Bmal2*, *Cry1*, *Cry2*, *Per1*, *Per2*, and

Rev-erbα. All data for gene transcripts and proteins were stratified or pooled by sex based on a significant or non-significant (respectively) main effect of sex. All results of the two-way ANOVAs (Genotype X Time-of-Day) are reported in Supplementary Table 2.

There were no sex differences in mRNA expression of *Bmal1*, *Bmal2*, *Cry1*, and *Per1*, and thus, samples from males and females were pooled. Tg-SwDI mice had overall reduced expression of these genes compared to controls (Genotype main effect, $p < 0.05$; Figure 2A-C and E). Although diurnal variation was not evident for expression of these genes, *Cry1* expression appeared to show variation over time of day, but this effect failed to reach statistical significance (main effect of Time of Day, $p = 0.089$). Gene expression of *Cry2, Per2, and Rev-erbα* was stratified by sex for analysis due to a significant main effect of sex. Compared to control females, *Cry2* mRNA expression from Tg-SwDI females was generally increased ($p < 0.05$, Figure 2D; for males, see Supplementary Figure 3B), and *Per2* and *Rev-erbα* expression was decreased ($p < 0.05$, Figure 2F, and G). Expression of *Per2* showed diurnal variation in both females (Figure 2F) and males (Supplementary Figure 3A) with expression higher at both night time points (ZT 14 and ZT 20) compared to one or both of the day time points (females, ZT 2 and 8 vs ZT 14 and 20, $p < 0.05$; males, ZT 2 vs ZT 14 and 20, $p < 0.05$). Finally, *Rev-erbα* mRNA expression from male Tg-SwDI mice were significantly lower (regardless of time) than that of control mice ($p < 0.05$, Figure 2H). Expression of the control gene, 18s, did not vary across time of day (Supplementary Table 2).

To test if the changes in circadian clock gene mRNA expression also manifested at the protein level, we assessed hippocampal protein levels of two core clock components, PER2 and BMAL1, at both 4 months and 9 months. Given no significant effect of Sex,

data from males and females were pooled for two-way ANOVAs (Supplementary Table 3). At 4 months, PER2 levels were significantly reduced in Tg-SwDI mice compared to controls ($p < 0.05$, Figure 3A). Regardless of genotype, PER2 levels showed diurnal variation with higher expression at ZT 20 than ZT 8 (Tukey, $p < 0.05$). For BMAL1, diurnal variation was dependent upon genotype, such that at ZT20, BMAL1 levels were higher in Tg-SwDI mice than in control mice (interaction, $p < 0.05$; Tukey's HSD, $p < 0.001$; Figure 3B). At ZT14, there was a trend for BMAL1 levels from Tg-SwDi to be lower than WT ($p = 0.57$). By 9 months, overall levels of both PER2 and BMAL1 were significantly lower compared to control samples (main effect, $p < 0.05$; Figure 3C-D). At this age, there was a trend for diurnal variation in PER2 and BMAL1 levels ($p = 0.079$ and $p = 0.088$, respectively.

Phosphorylation of glycogen synthase kinase 3 beta (GSK3β), a second messenger protein important for spatial memory [41], is regulated in a circadian manner in the hippocampus [17]. Moreover, GSK3β phosphorylates various components of the molecular clock [42-44]. Beyond involvement in both circadian rhythms and memory, phosphorylated (inactive) GSK3β is reduced in AD [45]. At 4 months, phosphorylation of GSK3β (Supplementary Figure 4A) peaked at ZT 8 in both genotypes, although no Genotype difference was observed at this age (data for males and female pooled given no effect of Sex; Time-of-Day main effect, $p < 0.05$; Genotype main effect, $p = 0.14$; Supplementary Table 3). At 9 months, we detected no diurnal variation in GSK3β phosphorylation (data stratified by Sex, for both males and females, $p > 0.19$, Supplementary Figure 4B and C). Only female, 9-month Tg-SwDI mice had overall reduced GSK3β phosphorylation

compared to controls across all times of day (main effect of Genotype, $p < 0.05$). These data suggest that aged, female Tg-SwDI mice have increased levels of active GSK3β.

Day/night differences in synaptic and intrinsic properties of hippocampal CA1 pyramidal cells are lost in Tg-SwDI mice

Brain circuits crucial for learning and memory reside in the hippocampus, which is one of the earliest regions impacted by AD [46-48]. Post-mortem tissue analysis from patients with AD shows reduction of interneuron populations in the hippocampus [49,50], and mouse models of AD have demonstrated the importance of interneuron function in cognitive performance [2]. Parvalbumin-expressing interneurons, in particular, are necessary for spatial working memory [51]. Studies in multiple AD mouse models suggest that deficits in synaptic transmission occur early in the hippocampus [52-54]. Given that hippocampal neurophysiology also varies across time-of-day (for review, see [55]), we hypothesized that diurnal hippocampal neuronal physiology may be disrupted in the Tg-SwDI mouse model in a manner that results in decreased inhibition.

To test this hypothesis, we recorded pharmacologically isolated spontaneous inhibitory postsynaptic currents (sIPSCs) using whole-cell voltage clamp of CA1 pyramidal cells (PCs) from 4-month-old male and female WT controls and Tg-SwDI mice (Figure 4A). In slices from WT control mice, sIPSC interevent interval (IEI) was significantly shorter during the day than at night ($p < 0.001$; Figure 4B (top)), and amplitude was significantly greater during the day than at night ($p < 0.001$; Figure 4B (bottom)). Because IEI is the inverse of frequency, this result indicates that the inhibition onto CA1 PCs was greater during the day than at night in controls, with more frequent and larger IPSCs. In recordings from CA1 PCs of Tg-SwDI mice, sIPSC amplitude was also

greater during the day than at night ($p < 0.001$; Figure 4B (bottom)), similar to control mice. However, the day/night difference in IEI was reversed in slices from Tg-SwDI mice, with increased IEI during the day ($p < 0.001$; Figure 4B (top)), suggesting that TgSwDI mice have reduced inhibitory input onto excitatory PCs during the day.

Next, we examined day/night differences in the excitability of CA1 PCs. Similar to data previously published in rats [15], whole-cell current clamp recordings in 4-month-old control mice indicated a significant day/night difference in RMP, with greater depolarization at night (-67.51 ± 0.74 mV) compared to day (-71.58 ± 0.55 mV; $t_{43.7} = -4.20$, $p < 0.001$). To determine whether there were additional day/night differences in excitability, we assessed the number of action potentials generated in response to current injections of increasing magnitude from resting membrane potential, in the presence of vehicle (0.002% DMSO) (Figure 5A, right) or GABA receptor (GABAR) antagonists (Figure 5A, left) (10μM bicuculline and 1μM CGP55848). Three-way ANOVA of vehicle-treated slices indicated a significant Current Step X Treatment X Time of Day interaction ($p < 0.05$, Supplementary Table 4). In the vehicle-treated group, CA1 PCs produced more action potentials at night than during the day in response to increasing current injections (for Steps 160 to 280 pA, $p < 0.05$; Figure 5A, left) and were thus more excitable at night.

In these vehicle-treated slices, both excitation and inhibition were present, constituting an intact local circuit. Therefore, to determine the role of inhibition in these day/night excitability differences, we also performed this experiment in the presence of GABAR antagonists. Blocking GABARs eliminated the day/night difference in CA1 PC excitability (simple main effects of day vs night at all current steps, $p > 0.435$; Figure 5A, right). Simple main effects comparing GABAR antagonist-treated cells to vehicle-treated

cells during the day indicated statistical trends for the GABAR antagonist group to have more spikes at 180, 200, and 220 pA steps ($p = 0.071$, 0.055, and 0.067, respectively). Blocking GABARs had no effect on spikes per step at night.

We next asked if the day/night difference observed in vehicle-treated PCs was diminished in Tg-SwDI mice. There was no day/night difference in spikes per step in CA1 PCs in Tg-SwDI mice ($p > 0.105$; Figure 5B). Taken together, these data indicate that there is normally a day/night difference in CA1 PC excitability that requires inhibitory GABAergic transmission, as it is blocked by GABA receptor antagonists. Interestingly, the effect of blocking GABA receptors was very similar to the excitability phenotype observed in Tg-SwDI mice, with loss of the normal day/night difference in excitability in both conditions.

Finally, to determine if the overall decrease in inhibition irrespective of time of day could be due to a reduction in the absolute number of interneurons in this mouse model of neurodegenerative disease, we probed for markers of interneurons, including GAD-67 and parvalbumin (PV), in the hippocampus of 4-month WT and Tg-SwDI mice. There was no significant difference in GAD^+ fluorescence ($t_{11} = 1.671$, $p > 0.05$; Figure 6A-B) or in PV^+ cell numbers ($t_{40} = 1.135$, $p > 0.05$; Figure 6C-D) between WT and Tg-SwDI mice.

DISCUSSION

Altered rest and activity patterns are commonly reported symptoms in patients with AD [5,56-64]. This circadian disruption may exacerbate AD pathology and vice versa [65]. In the APP-PS1 mouse model of AD, adult-specific genetic ablation of the molecular clock accelerates amyloid beta pathology [66]. Sleep deprivation, in both wild type and AD models, potentiates amyloid plaque formation [67,68]. In the Tg-SwDI model, utilized in this paper, circadian locomotor activity is disrupted and day/night differences in excitability of SCN neurons are diminished due to reduced daytime firing [21]. Additionally, cerebral microvascular amyloid is first detected in the hippocampus, subiculum, and cortex at 3 months in Tg-SwDI mice with pronounced spread by 6 months, covering the entire forebrain by 12 months [22,25]. In the present study, we show lack of the normal diurnal differences in spatial working memory in this AD model, which develop relatively early in parallel with a general reduction in hippocampal molecular clock gene expression. Importantly, we also provide the first evidence that normal day/night differences in hippocampal neurophysiology, including inhibitory synaptic input, are impaired in a mouse model of AD.

Given the reported circadian behavior and SCN disturbances in the Tg-SwDI model, we aimed to investigate whether this model also had disrupted rhythms in spatial working memory. While the Tg-SwDI model has been shown to develop deficits in Barnes maze performance beginning at 3 months of age [24,25], there had been no published studies

of day/night differences in performance of any tasks at any age. Additionally, the Barnes maze requires multiple daily trials over the course of multiple days [69], introducing the possible confounds of light exposure or sleep deprivation. Therefore we chose to assess spatial working memory with T-maze Spontaneous Alternation, a task that can be measured at two different times of day with minimal training and normally shows strong day/night difference in performance [34,37,40]. As previously shown, we observed a significant day/night difference in performance in controls, with high alternation at night. However, female Tg-SwDI animals had lower alternation than controls at night with no significant difference between day and night performance. At 9 months, both male and female wild-type mice had significantly higher performance at night than during the day, while the Tg-SwDI mice had no significant day/night difference. In healthy individuals, performance in hippocampal-dependent tasks is best after waking and declines after several hours [9]. Given the significant circadian disruption seen in AD patients, daily patterns of cognition are an important consideration for treatment of AD. Approximately 25% of patients with AD exhibit sundowning, a clinical phenomenon in which neuropsychiatric symptoms manifest in the late afternoon, evening or at night [70]. Additionally, patients in the preclinical stage of Alzheimer's disease are more likely to have increased rest/activity rhythm fragmentation [5]. Exploring circadian deficits present in AD underlines a growing need to design therapies for AD that could be administered at specific times of day for optimal benefit.

Because the day/night difference in spatial working memory is linked to the molecular clock [10,31,33], we hypothesized that expression of circadian clock genes and clock-controlled genes may be altered in AD. The molecular clock consists of several core clock proteins, including positive regulators CLOCK and BMAL1, which dimerize in the

nucleus and bind to E-box regions of clock genes *Period (Per)* and *Cryptochrome (Cry),* the negative regulators, allowing their transcription. The transcripts are then translocated to the cytoplasm where they are translated into proteins. While in the cytoplasm, PER and CRY accumulate and are either marked for degradation or re-entry to the nucleus by phosphorylation via casein kinase 1 or GSK3β, with these phosphorylation events setting the period length [42,71]. Eventually, PER and CRY dimerize and are shuttled back to the nucleus where they interfere with the binding of CLOCK and BMAL1, thus turning off their own transcription. This entire process takes ~24 hours. A second, auxiliary loop exists with the activator retinoid-related orphan receptor (ROR) and repressor REV-ERBαβ, which drives the transcription of *Bmal1* [7,72]. In addition to these core clock components, the molecular clock also drives rhythmic expression of clock-controlled genes in a region-specific manner throughout the brain, including the hippocampus [20,55]. Rhythmic gene transcripts that could affect hippocampal plasticity include *Arc*, *Gria3*, *Nlgn1*, *Shisa9*, *Grid2*, and *Trpc7* [20].

Reductions in several core clock components as measured by both mRNA and protein have been reported in multiple AD models. Our findings are consistent with other studies showing reduced clock gene expression and protein levels in both cell culture and mouse models. Two other AD mouse models (APP/PS1 and 5XFAD) showed reduced *Per2* mRNA and/or protein levels in the SCN or hypothalamus, compared to age-matched controls [73,74]. In the hippocampus, intracranial injection of $A\beta_{31-35}$ in WT mice reduced the nocturnal peak in PER2 protein levels [75]. In young APP/PS1 mice, hippocampal clock gene expression was not different from WT controls, with both genotypes showing a time of day difference [76]. However, by 12-15 months, APP/PS1 mice showed reduced diurnal rhythms

in hippocampal clock gene mRNA expression [77]. Our present study shows overall reduction in gene expression of *Bmal1, Bmal2, Cry1, Per1,* and *Reverbα,* across both males and females, and reduced *Per2* expression specific to female mice at 4 months of age. Thus, expression of many but not all molecular clock genes and protein levels (PER2) were lower overall in the Tg-SwDI hippocampus than in controls (with the exception of *Cry2* mRNA and BMAL1 protein levels). Although we were surprised to observe an increase in BMAL1 protein levels at ZT20 in Tg-SwDI mice (compared to controls), it is important to note that there is much complexity in the molecular clock regulation, whereby stability of BMAL1 may be important for transcriptional activity of clock-controlled genes [72]. We do not yet understand the downstream implications of reduced clock gene expression in AD mouse models.

In addition to reduced expression of core components of the molecular clock, we observed reduced levels of phosphorylated (inactive) GSK3β, which phosphorylates various components of the molecular clock [42-44], in older female Tg-SwDI mice. Younger mice (both Tg-SwDI and WT) and older males had normal diurnal variation of pGSK3β in the hippocampus as previously reported [17]. Because the GSK3 de-phosphorylated state is the active state, reduced phosphorylation levels would suggest that the AD females have increased activity of GSK3β in the hippocampus. This activity can lead to increased Aβ and tau in the brain and alter protein homeostasis [78]. However, reduced pGSK3β was not observed in 4 month old Tg-SwDI mice, suggesting that the early deficits observed here and by others (Aβ accumulation, cognitive deficits, and reduced molecular clock components) are not due to altered GSK3β rhythms, and there must be another currently unidentified mechanism at this age. Some evidence suggests reduced clock function may

lead to several issues including Aβ accumulation, synaptic dysfunction, oxidative stress, altered proteostasis, immune dysfunction, and neuroinflammation [78]. Interestingly, neuroinflammation may be a mediator of early cognitive dysfunction in Tg-SwDI such that microvascular amyloid in hippocampal-associated regions (i.e., the subiculum) may lead to hippocampal neuroinflammation and impaired function [24,36].

Importantly, the molecular clock exerts transcriptional regulation of excitable membranes. Many ion channel-related genes examined in CircaDB database show significant 24-hour rhythms in expression in the SCN [79]. Therefore, it is possible that deficits in the molecular clock alter neuronal membrane physiology. A substantial literature illustrates the existence of daily rhythms in neuronal physiology in multiple regions of the rodent brain (for review, see [55]). The ratio of excitation to inhibition is crucial for normal brain function and modelling based on human EEG data suggests that this ratio exhibits circadian differences [80]. Notably, the ratio of excitation to inhibition is disrupted early in AD models [52,81]. In the J20 mouse model of AD, daytime recordings of sIPSCs onto PCs of the parietal cortex showed a reduction in frequency compared to NT littermate controls [82]. When inhibitory PV interneuron excitability in the J20 model was restored, sIPSC amplitude and frequency was similar to NT controls, and epileptiform activity was reduced [82]. Our study is the first to report the lack of day/night differences in sIPSCs in a mouse model of AD.

Circadian rhythms in neurophysiology have been best described in the SCN, where the action potential firing rate of neurons is higher during the day compared to night [26,83]. In Tg-SwDI mice, the daytime firing rate of SCN neurons is reduced, resulting in a dampening of the day/night difference [21]. Thus far, a single report has shown that rat

hippocampal neurons exhibit day/night differences in one measure of excitability, with CA1 pyramidal cell RMP being more depolarized during the night compared to day [15]. In control mice, we demonstrated a similar day/night difference in excitability, with CA1 pyramidal cells resting at more hyperpolarized potentials during the day. Additionally, we showed that current injections of increasing magnitude into the soma of CA1 pyramidal cells produce more action potentials during the night, suggesting an overall increase in CA1 PC excitability at night. Importantly, we showed for the first time an absence of day/night difference in excitability in an AD mouse model, suggesting a circadian dysregulation of excitability. In future studies, it will be important to investigate if the lack of the day/night difference in CA1 PC excitability that we report here is tied to the dysregulation of the molecular clock. Notably, in control slices, blocking GABAR signaling abolished the day/night difference in CA1 PC excitability. Altogether, our data suggest that both cell autonomous (potential molecular clock-mediated rhythms in ion channel function) and non-autonomous (diurnal regulation of tonic or phasic inhibition) mechanisms may work in concert to maintain temporal control of excitability, which is lost in pathophysiological conditions such as AD. One caveat in the interpretation of our results is that we assessed both neuronal physiology and behavior in Tg-SwDI mice at a single day- and night-time point and thus cannot differentiate a shift in phase from a loss of rhythm.

The lack of day/night differences in CA1 PC excitability and sIPSCs in TgSWDI mice align with several pieces of evidence supporting the hypothesis of hyperexcitability in AD. Epileptiform activity has been documented in AD patients and is more likely to be observed at night, when patients are asleep [84,85]. Several populations of interneurons,

including parvalbumin-expressing interneurons, are reduced in post-mortem tissue of AD patients [49,50]. Fast-spiking parvalbumin interneurons are necessary for generation of oscillations in the gamma frequency, which are important in learning and memory [86-89]. Deficits in gamma oscillatory activity and reduced activity of fast-spiking interneurons are observed in both mouse models of AD and AD patients [82,85,90,91]. Thus, future work should investigate circadian dysregulation of specific interneuron sub-types in the context of AD.

Our present findings highlight the importance of diurnal regulation of neuronal physiology and the potential for chronotherapeutic interventions. Clarifying how AD progression impacts day/night neurophysiological differences could lead to chronotherapeutic strategies, whereby hyperexcitability is treated at a specific time of day. In the future, it will be important to dissect roles of somatic-targeting and dendritic-targeting interneurons to determine where the loss of inhibition is originating. Because CSF Aβ and tau exhibit a diurnal pattern [68,92], other AD biomarkers are also likely to differ in the day versus night. Thus, the circadian clock is an important consideration for all future studies of AD pathogenesis and therapeutics.

ACKNOWLEDGEMENTS

This work was supported by NIH grants R01NS082413 (KLG), R56AG061785 (EDR and KLG), RF1AG059405 (EDR), R01NS108713 (KLG and RMC), T32NS061788 (ARF), and F31NS115299 (LKG). We would like to thank Allie Widman for technical assistance with electrophysiology and Courtney Rogers for assistance with animal behavior experiments. We would like to thank Dr. William van Nostrand (University of Rhode Island) for providing helpful insight regarding the Tg-SwDI model. We would also like to thank Kavitha Abiraman for helpful comments in the writing of the manuscript.

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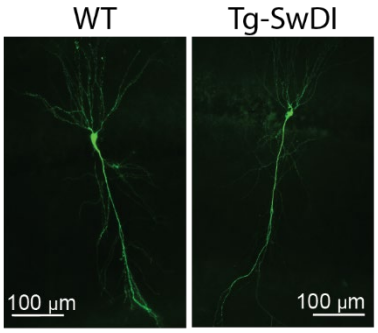
FIGURES



Supplementary Figure 1. Biocytin filled CA1 PCs from WT (left) and Tg-SwDI (right) mice.

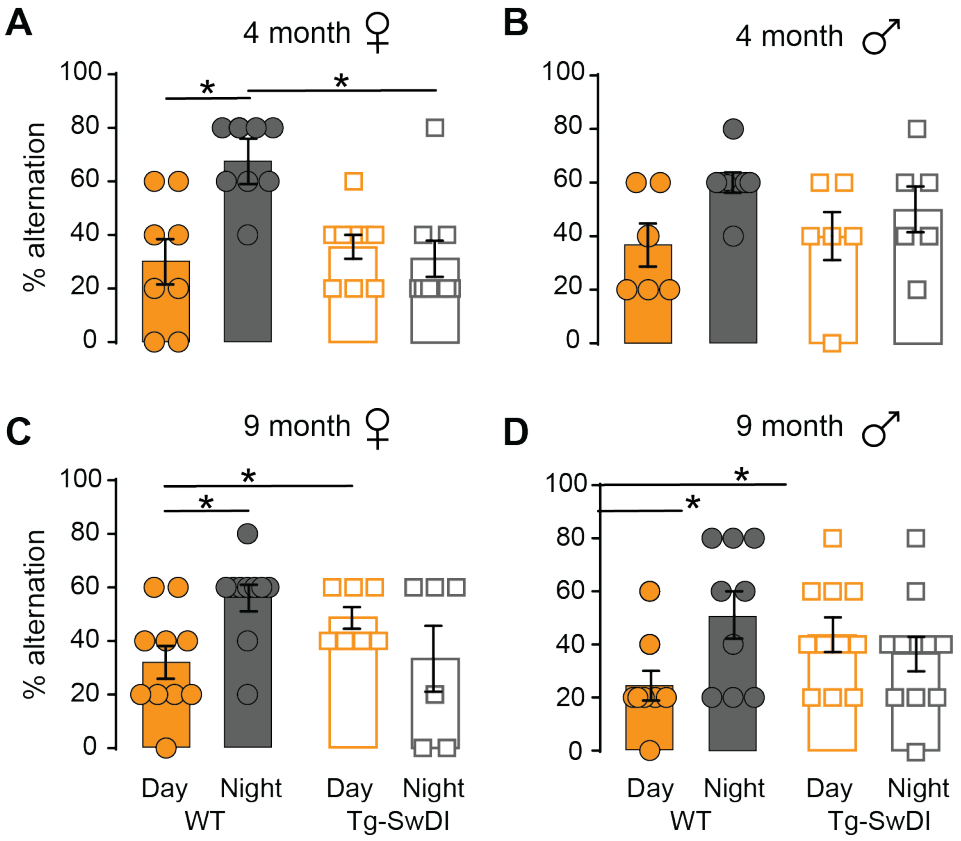
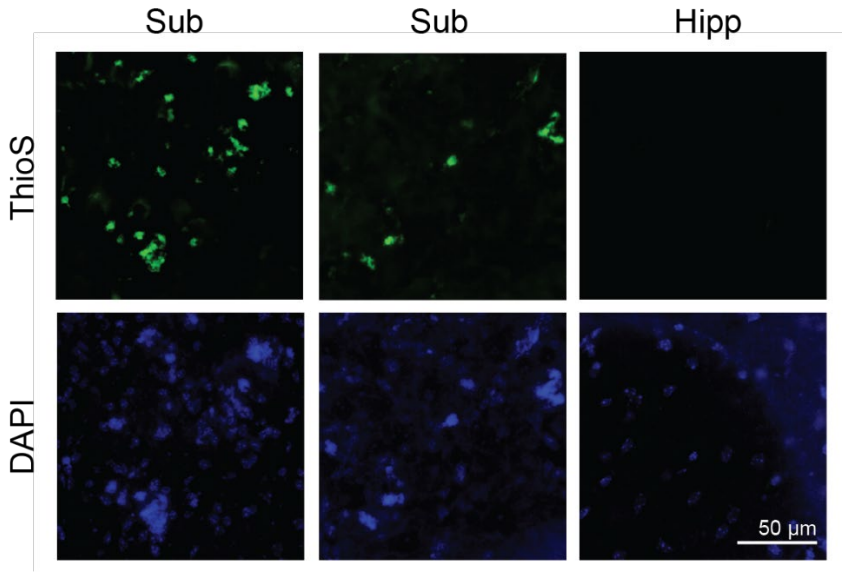


Figure 1. Loss of day/night differences in spatial working memory in 4-month and 9-month AD mice. Percent alternation at ZT 2 vs ZT 14 in WT and AD mice. 4-month WT and Tg-SwDI female (A) and male (B) mice tested during day (orange) and night (grey). WT mice had significant day/night difference in percent alternation ($p < 0.05$), and at night, Tg-SwDI mice had lower alternation percentages than WT mice ($p < 0.01$). 9-month WT and Tg-SwDI female (C) and male (D) mice tested during day (orange) and night (grey). Post-hoc analysis indicated a loss of day/night differences in both male and female Tg-SwDI mice ($p = 0.386$ and 0.521, respectively) and slightly higher alternation of older Tg-SwDI mice (males and females) compared to WT during the day ($p < 0.02$) and a tendency for lower alternation compared to WT females during the night ($p = 0.076$). For A, B, C, and D, a mixed model ordinal regression indicated significant main effects for time of day for all 4-month mice ($p < 0.05$), and significant Time of Day X Genotype interactions for 4-month females and all 9-month mice ($p < 0.05$, n = 34 (4-month females), 26 (4-month males), 33 (9-month females), 40 (9-month males)). See Supplementary Table 1 for statistical results of the regression.



Supplementary Figure 2. ThioS staining in 6-month Tg-SwDI mice. Mouse brain sections from Tg-SwDI mice (6 months) were stained for fibrillar amyloid using thioflavin S (green). Fibrillar amyloid were detected in the subiculum (Left and Middle) but not hippocampus (Right). Scale bar, 50 µm.

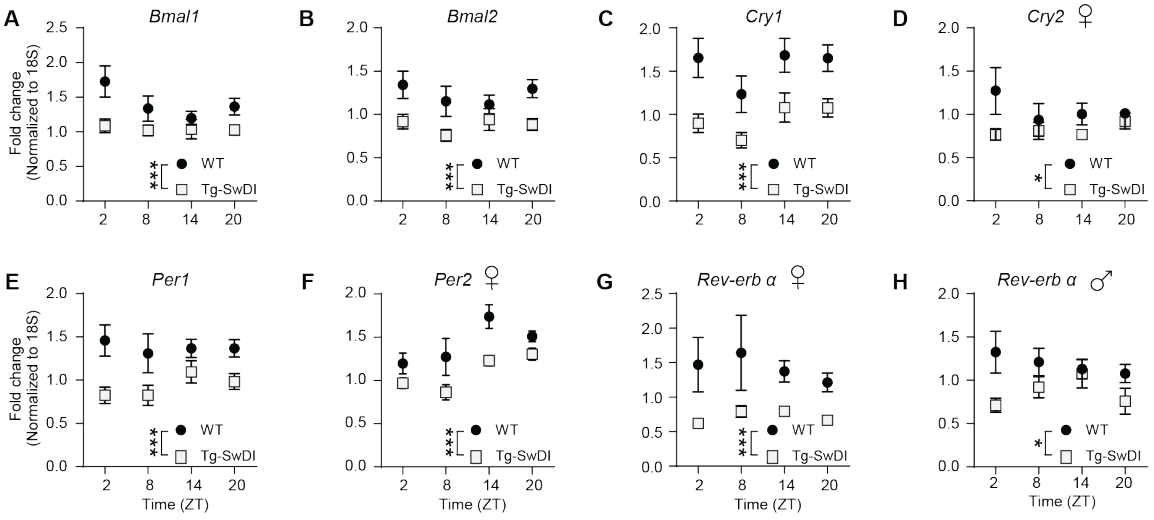
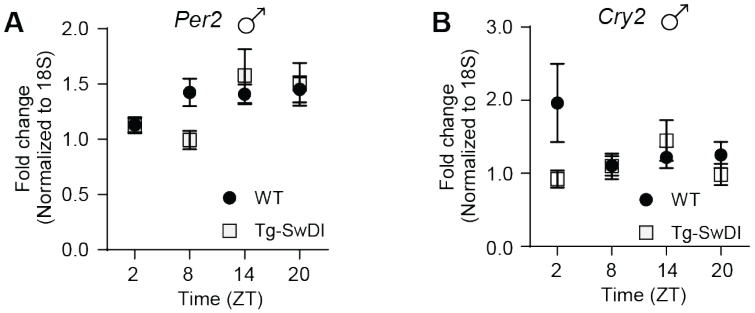


Figure 2. Reduced circadian gene mRNA expression over the day/night cycle in 4-month AD mice. Transcript levels from WT (filled circles) and Tg-SwDI (open squares) mice at ZT 2, 8, 14, and 20 for *Bmal1* (A), *Bmal2* (B), *Cry1* (C), *Cry2* females (D), *Per1* (E), *Per2* females (F), *Rev-erbα* females (G) and males (H). Two-way ANOVA indicated significant main effects of Genotype for *Bmal1*, *Cry1*, and *Cry 2* females, *Per1*, *Per2* females, and *Rev-erbα*. A significant Time of Day main effect was observed for *Per2* females ($p < 0.05$). No significant Time of Day X Genotype interactions were observed. Males and females were combined unless indicated (n = 5-10 mice per Sex/Time/Genotype group). See Supplementary Table 2 for statistical results.



Supplementary Figure 3. *Per2* mRNA expression in 4-month male mice. Transcript levels from WT (filled circles) and Tg-SwDI (open squares) male mice at ZT 2, 8, 14, and 20 for *Per2* (A) and *Cry2* (B). Two-way ANOVA indicated no significant effects. See Supplementary Table 2 for statistical results.

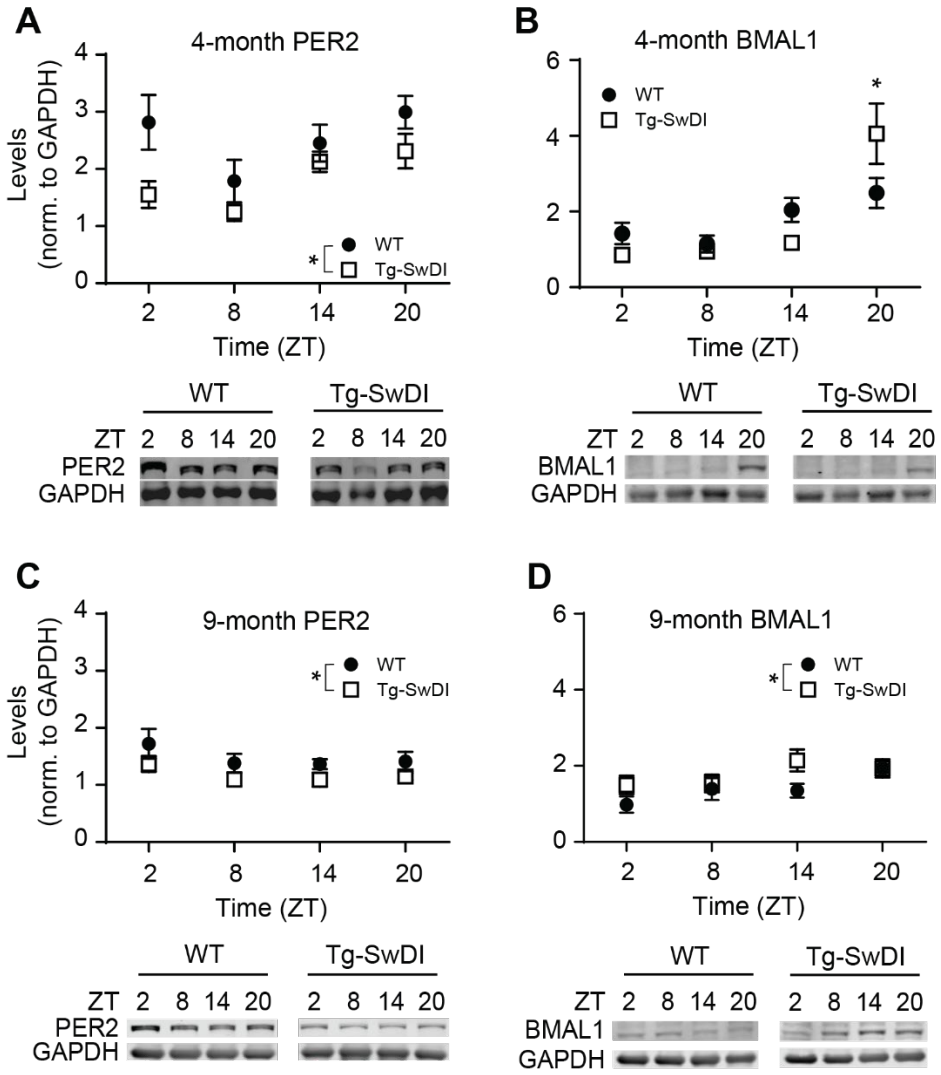
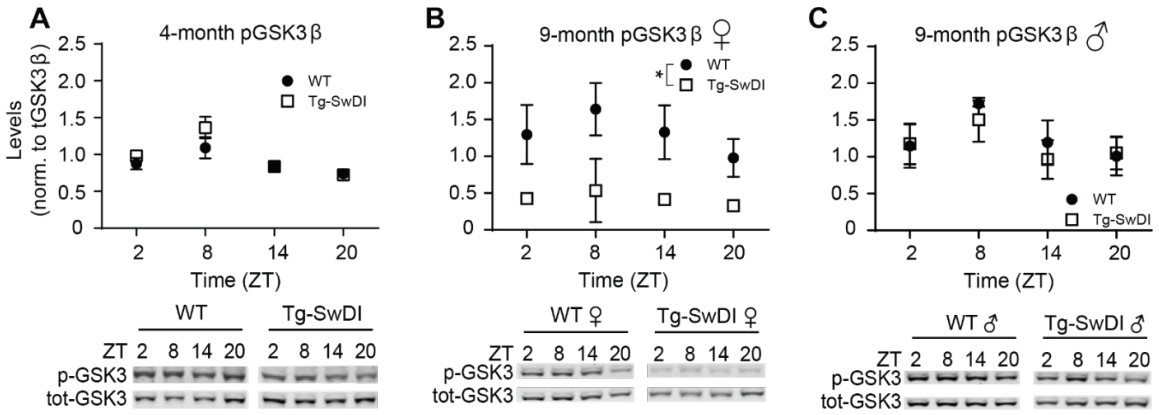


Figure 3. Loss of day/night variation in clock protein expression in Tg-SwDI mice. Normalized expression for PER2 and BMAL1 in 4-month (A and B) and 9-month (C and D) Tg-SwDI mice (open squares) and age-matched WT controls (solid circles). For 4-month mice, two-way ANOVA indicated significant main effects of Genotype and Time of Day for PER2 (A; $p < 0.05$) but no interaction (n = 9-15 mice/group). Levels of BMAL1 (B) varied by both Time-of-Day and Genotype (interaction, $p < 0.01$) with higher levels in AD mice than WT mice at ZT 20 ($p < 0.05$) and a trend for AD mice to have lower levels than WT mice at ZT 14 (p 0.057). Both Genotypes showed highest expression at ZT 20 (WT: ZT 20 vs ZT 8, $p < 0.05$; AD, ZT 20 vs all other ZTs, $p < 0.05$; n = 9-16 mice/group). For 9-month mice, two-way ANOVA indicated a significant main effect of Genotype for PER2 (C; $p < 0.05$) and a trend for Time of Day ($p = 0.08$), and no interaction (n = 10-16 mice/group). For BMAL1 (D), a significant main effect of Genotype ($p < 0.05$) revealed higher levels in AD mice than WT mice with a trend for a Time-of-Day effect ($p = 0.088$) but no significant interaction ($p = 0.523$). See Supplementary Table 3 for statistical results.



Supplementary Figure 4. Loss of day/night variation pGSK3β expression in Tg-SwDI mice. Normalized expression for pGSK3β in Tg-SwDI mice (open squares) and age-matched WT controls (solid circles). For levels of p-GSK3β (normalized to total GSK3β), ZT 8 levels were greater than all other time points (significant main effect of Time-of-Day and post hoc, $p < 0.01$), with no effect of Genotype or significant interaction (n = 8-15/group). At 9-months females (B) showed a significant effect of Genotype ($p < 0.05$; no other effects) but no genotype difference was detected in male (C) mice (n = 5-9 mice/group). See Supplementary Table 3 for statistical results.

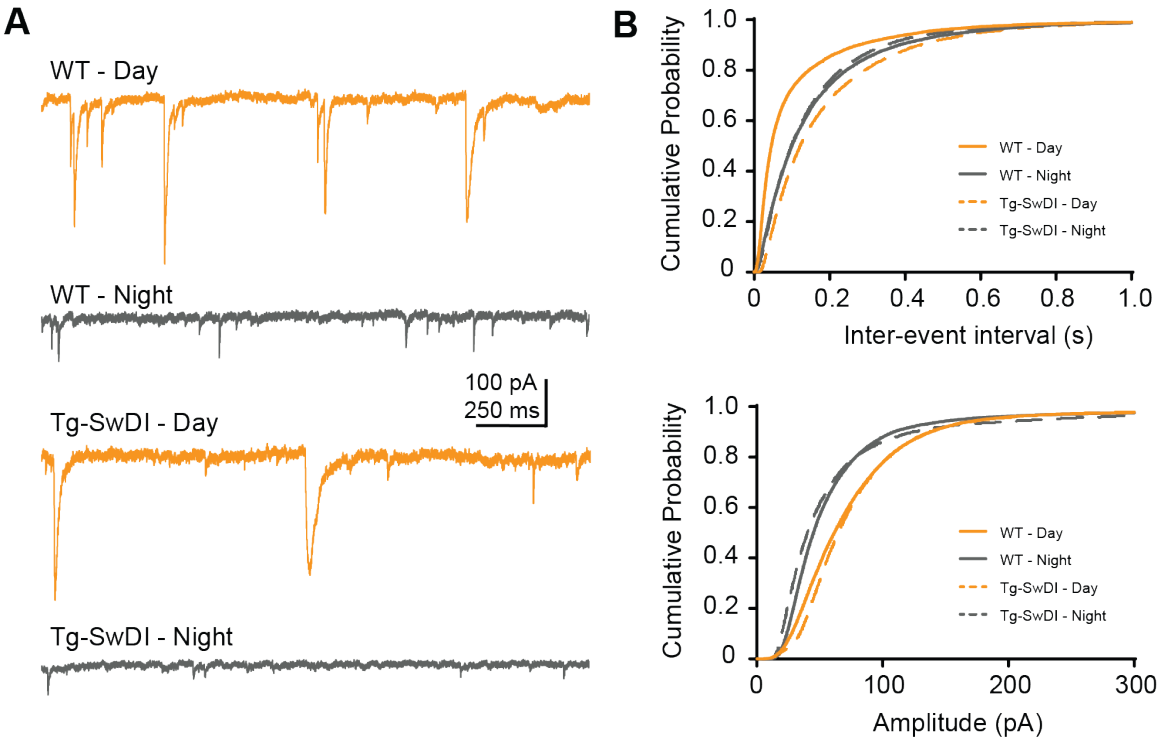


Figure 4. Increased daytime inhibitory input to CA1 pyramidal neurons impaired in Tg-SwDI mice. Whole cell patch clamp was used to measure amplitude and interevent interval (IEI) of sIPSCs in CA1 PCs after isolation with NBQX and CPP. Pipette recording solution contained CsCl and QX-314, and cells were held at -70mV. Representative traces (A) from WT (top) and Tg-SwDI (bottom) PCs at day (orange) and night (grey). Day/night differences in sIPSC inter-event interval (B, top) is reversed in Tg-SwDI mice. Day/night difference in amplitude (B, bottom) is preserved in Tg-SwDI mice (K-S test, Bonferroni-corrected alpha at $p < 0.0125$, $n = 7$-10 cells/group).

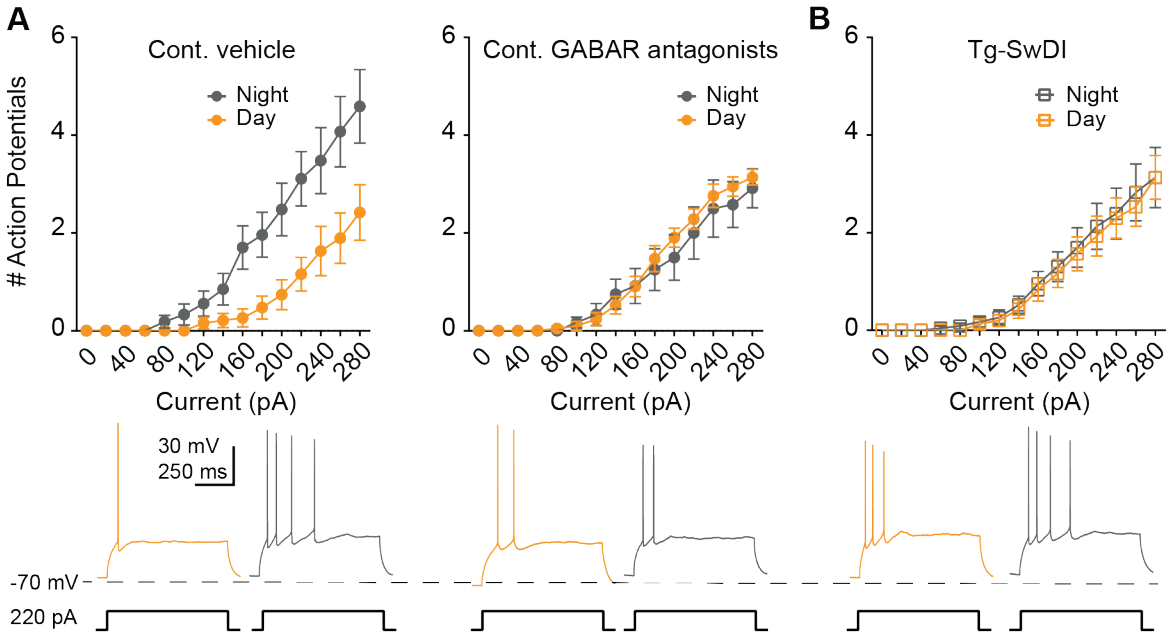


Figure 5. Loss of day/night differences in induced spike rate of hippocampal CA1 pyramidal cells in Tg-SwDI mice. Whole cell patch clamp was used to measure spikes per step with increasing current injections in CA1 PCs. Pipette recording solution contained KGlu, and cell voltage was not clamped. A. Whole-cell current clamp recordings of the number of spikes per step when recorded during the Day or Night in Per1::GFP mice in the absence (left; n = 19 cells (Day, orange), 27 cells (Night, grey)) or presence (right; n = 21 cells (Day), 12 cells (Night)) of GABA receptor antagonists; representative traces below (day: orange; night: grey). Three-way ANOVA indicated a significant Current Step X Treatment X Time of Day interaction ($p < 0.05$). In the vehicle- treated group, CA1 PCs produced more action potentials at Night compared to Day in response to increasing current injections (for Steps 160- to 280 pA, $p < 0.05$). In the presence of GABAR antagonists, the day/night difference in excitability was lost (simple main effects of Day vs Night at all current steps, $p > 0.601$). B. Pyramidal cell recordings from Tg-SwDI mice show loss of a day/night difference in induced spikes per step ($p = 0.742$; n = 30 cells (Day), 23 cells (Night)); representative traces below. See Supplementary Table 4 for statistical results.

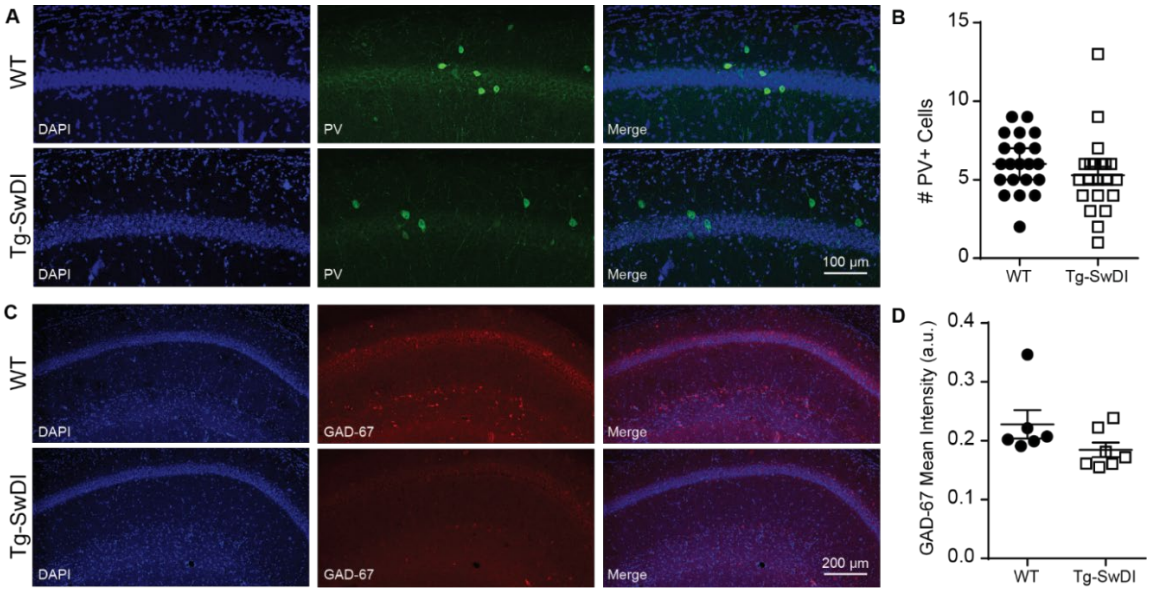


Figure 6. No change in interneuron markers in area CA1 of Tg-SwDI mice. Immunohistochemistry for GAD-67 and Parvalbumin from ZT 3-6 in CA1 of Tg-SwDI and WT mice. A. DAPI (left) and GAD-67 (middle) fluorescence in a coronal hippocampal slice from WT (top) and Tg-SwDI (bottom) mice and merged (right). Scale bar = 200 µm. B. Quantification of mean intensity, $p > 0.05$, n = 21 sections/group. C. DAPI (right) and parvalbumin (middle) fluorescence in a coronal slice from WT (top) and Tg-SwDI (bottom) mice merged (right). Scale bar = 100 µm. D. Quantification of PV^+ cell count, $p > 0.05$, (n = 21 sections/genotype).

	Genotype	Time of Day	Interaction
4-month females	χ^2 (1) = 3.117, p = 0.077	χ^2 (1) = 8.857, p = 0.003	χ^2 (1) = 12.076, p = 0.001
4-month males	χ^2 (1) = 0.193, p = 0.660	χ^2 (1) = 6.003, p = 0.014	χ^2 (1) = 1.633, p = 0.201
9-month females	χ^2 (1) = 0.663, p = 0.415	χ^2 (1) = 1.030, p = 0.310	χ^2 (1) = 6.852, p = 0.009
9-month males	χ^2 (1) = 0.268, p = 0.605	χ^2 (1) = 3.136, p = 0.077	χ^2 (1) = 8.526, p = 0.004

Supplementary Table 1. Statistical analysis of spontaneous alternation.

	Genotype	Time of Day	Interaction
Bmal1	$F_{1, 104}$ = 12.522, p = 0.001	$F_{3, 104}$ = 1.695, p = 0.173	$F_{3, 104}$ = 1.071, p = 0.365
Bmal2	$F_{1, 104}$ = 16.301, p < 0.001	$F_{3, 104}$ = 0.781, p = 0.507	$F_{3, 104}$ = 0.478, p = 0.698
Cry1	$F_{1, 104}$ = 24.517, p < 0.001	$F_{3, 104}$ = 2.231, p = 0.089	$F_{3, 104}$ = 0.165, p = 0.919
Cry2 females	$F_{1, 45}$ = 6.844, p = 0.012	$F_{3, 45}$ = 0.583, p = 0.629	$F_{3, 45}$ = 1.107, p = 0.356
Cry2 males	$F_{1, 49}$ = 1.641, p = 0.206	$F_{3, 49}$ = 0.657, p = 0.582	$F_{3, 49}$ = 1.957, p = 0.133
Per1	$F_{1, 104}$ = 19.791, p < 0.001	$F_{3, 104}$ = 0.460, p = 0.711	$F_{3, 104}$ = 0.650, p = 0.585
Per2 females	$F_{1, 45}$ = 19.938, p < 0.001	$F_{3, 45}$ = 8.189, p < 0.001	$F_{3, 45}$ = 0.974, p = 0.414
Per2 males	$F_{1, 51}$ = 0.368, p = 0.547	$F_{3, 51}$ = 4.670, p = 0.006	$F_{3, 51}$ = 2.209, p = 0.098
Rev-erb alpha females	$F_{1, 45}$ = 21.708, p < 0.001	$F_{3, 45}$ = 0.537, p = 0.659	$F_{3, 45}$ = 0.305, p = 0.822
Rev-erb alpha males	$F_{1, 51}$ = 7.104, p = 0.010	$F_{3, 51}$ = 0.383, p = 0.766	$F_{3, 51}$ = 1.067, p = 0.371
18S females	$F_{1, 45}$ = 6.568, p = 0.327	$F_{3, 45}$ = 0.779, p = 0.512	$F_{3, 45}$ = 0.395, p = 0.757
18S males	$F_{1, 51}$ = 0.978, p = 0.327	$F_{3, 51}$ = 0.057, p = 0.982	$F_{3, 51}$ = 0.748, p = 0.529

Supplementary Table 2. Statistical analysis of circadian clock mRNA expression.

4 months	Genotype	Time of Day	Interaction
BMAL1	$F_{1, 100}$ = 0.0065, p = 0.940	$F_{3, 100}$ = 18.602, p < 0.001	$F_{3, 100}$ = 5.214, p = 0.002
PER2	$F_{1, 98}$ = 9.357, p = 0.003	$F_{3, 98}$ = 3.913, p = 0.011	$F_{3, 98}$ = 0.871, p = 0.459
pGSK3-β norm to tGSK3-β	$F_{1, 89}$ = 2.242, p = 0.138	$F_{3, 89}$ = 11.584, p < 0.001	$F_{3, 89}$ = 1.160, p = 0.330
9 months			
BMAL1	$F_{1, 95}$ = 6.553, p = 0.012	$F_{3, 95}$ = 2.249, p = 0.088	$F_{3, 95}$ = 0.754, p = 0.523
PER2	$F_{1, 95}$ = 9.764, p = 0.002	$F_{3, 95}$ = 2.331, p = 0.079	$F_{3, 95}$ = 0.073, p = 0.974
pGSK3-β females	$F_{1, 44}$ = 25.568, p < 0.001	$F_{3, 44}$ = 0.994, p = 0.404	$F_{3, 44}$ = 0.274, p = 0.844
pGSK3-β males	$F_{1, 44}$ = 0.191, p = 0.664	$F_{3, 44}$ = 1.646, p = 0.193	$F_{3, 44}$ = 0.125, p = 0.945

Supplementary Table 3. Statistical analyses of circadian clock protein expression.

	Control Vehicle vs GABA blockers
Current Step	$F_{1.7, 128.6} = 71.179, p < 0.001$
Current Step X Treatment	$F_{1.7, 128.6} = 0.296, p = 0.710$
Current Step X Time of Day	$F_{1.7, 128.6} = 1.813, p = 0.173$
Current Step X Treatment X Time of Day	$F_{1.7, 128.6} = 3.487, p = 0.04$
Treatment	$F_{1, 75} = 0.019, p = 0.891$
Time of Day	$F_{1, 75} = 3.011, p = 0.087$
Treatment X Time of Day	$F_{1, 75} = 4.470, p = 0.038$
	Tg-SwDI
Current Step	$F_{1.6, 83.5} = 44.488, p < 0.001$
Current Step X Time of Day	$F_{1.6, 83.5} = 0.062, p = 0.909$
Time of Day	$F_{1, 51} = 0.11, p = 0.742$

Supplementary Table 4. Statistical analysis of action potential generation in response to depolarizing current injections.

ABSENCE OF DIURNAL REGULATION OF SPATIAL MEMORY AND INHIBITORY TRANSMISSION IN THE HIPPOCAMPUS OF THE J20 MODEL OF ALZHEIMER'S DISEASE

by

ALLISON R. FUSILIER, CHANGBYEONG CHANG, LACY K. GOODE, STEFANI D. YATES, JAMES BLACK, ERIK D. ROBERSON, AND KAREN L. GAMBLE

ABSTRACT

Circadian rhythms are endogenous oscillations that occur over 24 hours in a multitude of biological processes, including neurophysiology and brain function. Circadian rhythms are disrupted in disease, including in Alzheimer's disease (AD), where patients experience disruptions in their sleep wake cycle and time-of-day specific exacerbation in epileptiform activity. We previously showed that in a mouse model of amyloidosis, decreased inhibition during the day in the hippocampus is coincident with cognitive impairment, but little has been done to elucidate the role of loss of day-night differences in hippocampal inhibition in AD mice. In this study we use electrophysiological and behavioral assays to probe day-night differences in neurophysiology and cognition in the J20 mouse model of AD. While inhibition onto CA1 pyramidal cells of the hippocampus is reduced overall in J20 mice, we failed to statistically detect previously established diurnal variations in inhibitory transmission in non-transgenic littermate controls. Studies of day-night differences in physiology and disruptions of that physiology may provide insight into possible therapeutic targets as well as the timing of those interventions (e.g. morning or evening) to most effectively ameliorate Alzheimer's disease symptoms or delay pathological onset.

INTRODUCTION

Circadian rhythms are endogenous oscillations occurring over a 24-hour period that modulate physiology and behavior[1]. These rhythms are driven by a cellular transcription-translation feedback loop known as the molecular clock[1], which drives expression of 43% of all protein encoding genes in at least one tissue, including ~600 in the hippocampus alone[2,3]. Circadian regulation of hippocampal activity extends to function: in fact, there is a well-established relationship between circadian rhythms and cognition. In humans, performance in hippocampal-dependent cognitive tasks improves rapidly after waking and remains high until habitual bedtime. Both environmental and genetic disruptions in circadian rhythms result in cognitive impairment in humans and rodents[4-8], underscoring the importance of circadian rhythms in learning and memory.

Alzheimer's disease is the most common cause of dementia worldwide[9], and the most prevalent neurodegenerative disease[10]. It is characterized by pathogenic amyloid beta (Aβ) plaque accumulation, tau tangles, and cognitive impairment[10]. Subclinical epileptiform activity or seizures, which are indicative of network hyperexcitability, are also present in early Alzheimer's disease[11]. Interestingly, epileptiform activity is greater and seizure thresholds are lower during the inactive phase (night in humans) in general and in Alzheimer's disease[12,13]. This may be exacerbated in Alzheimer's disease patients because of documented disruptions in their sleep wake cycle, which is driven by the circadian clock[14]. Still, the contribution of circadian disruption to Alzheimer's disease pathogenesis is severely understudied. Multiple mouse models of AD display impairments in cognitive

function, including learning and memory assays[15-21]. However, most of these studies conducted in nocturnal mice only examined cognition at one time of day, usually during the day. Because of well documented day-night, or diurnal differences in cognitive performance[22], examining hippocampal function in mice only during the day could preclude insight into circadian regulation of hippocampal physiology and pathophysiology. In a recent study, we showed that Tg-SwDI mice–a mouse model of amyloidosis–fail to show day/night differences in a hippocampus-dependent spatial working memory task. Whether this phenotype is specific to the Tg-SwDI model or is conserved across more specific mouse models of AD, remains to be studied.

In addition to epileptiform activity[23] in patients with AD, studies of post-mortem tissue show reductions in interneuron populations including parvalbumin-expressing interneurons[24,25] that drive gamma oscillatory activity[26,27]crucial for memory formation[28-30]. These oscillations are impaired in AD, making it crucial to study interneuron output across time of day in AD. This work aimed to address the question of whether day-night differences in hippocampal inhibitory signaling and spatial memory are impaired in the J20 mouse model of AD.Synaptic integration and spike initiation in neurons is controlled by synaptic inhibition, which strongly influences neuronal output and information processing (Farrant and Nusser, 2005). Importantly, the balance of excitation to inhibition (E/I) is crucial to the proper functioning of circuits, and E/I imbalances have been implicated in a number of neurodevelopmental disorders and neurodegenerative diseases including schizophrenia, autism spectrum disorders, and Alzheimer's disease (Fernandez et al., 2007; Kehrer et al., 2008; Gogolla et al., 2009; Roberson et al., 2011). Thus, understanding the

mechanisms that modulate the strength of inhibitory transmission is fundamental to unraveling how neuronal circuits function in normal and disease states.

METHODS

Animals All animal procedures followed the Guide for the Care and Use of Laboratory Animals, U.S. Public Health Service, and were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Mice of both sexes were maintained on a 12:12 light/dark cycle with ad libitum access to food (LabDiet Rodent 5001 by Purina) and water. Experiments were performed in male and female mice of the J20 line, which expresses an hAPP minigene with the Swedish (K670M/N671L) and Indiana (V717F) familial Alzheimer's disease (AD) mutations under control of the PDGF promoter[31].

Spatial Working Memory The spatial working memory task (adapted from [32-34]) was conducted during the day at Zeitgeber time (ZT) 3 and night ZT 15 with a T-maze. ZT 0 is when lights turn on, and ZT 12 is when lights turn off. During night testing, dim red light (< 10.0 lux) was used for visualization. Groups were counterbalanced to ensure a control for possible testing order differences. Animals were entrained to a 12:12 light:dark cycle. Mice were allowed to acclimate to the behavior room for 30 minutes each day immediately prior to five-minute researcher handling or testing. Mice were handled for 4 consecutive days prior to testing to reduce anxiety. Mice were tested at 3-4 months of age. Male and female mice were tested separately, and NT and J20 mice interleaved during trials. Mice were placed at the base of the T-maze and allowed to freely choose an arm of the T. Upon

entry to an arm, a gate was closed, trapping the mouse in the arm for 30 seconds. The mouse was removed, the gate opened, and the mouse returned to the base of the maze after a 10-second delay. An alternation was recorded if the mouse entered the opposite arm as the last entry. Each mouse performed 5 trials, and an alternation percentage was calculated as (# alternations / # of trials). Each trial was completed in under two minutes. Animals were only excluded from analysis if there was a notable directional bias in arm choices (i.e. animals only used the left arm in all 10 entries).

Electrophysiology At 3-6 months of age, mice were euthanized with cervical dislocation and rapid decapitation, brains were removed, and 350 μm coronal slices were prepared using a VT1200S vibratome (Leica Biosystems). Slices were cut in ice cold cutting solution containing the following (in mM): 110 choline chloride, 25 glucose, 7 MgCl2, 2.5 KCl, 1.25 Na2PO4, 0.5 CaCl2, 1.3 Na-ascorbate, 3 Na-pyruvate, and 25 NaHCO3, bubbled with 95% O2/5% CO2. Slices were rested at room temperature for at least one hour in recovery solution containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 Na2PO4, 2 CaCl2, 1 MgCl2, 25 NaHCO3, 25 glucose and 2 kynurenic acid bubbled with 95% O2/5% CO2. Recordings were made in standard artificial cerebral spinal fluid (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 Na2PO4, 2 CaCl2, 1 MgCl2, 25 NaHCO3, and 25 glucose bubbled with 95% O2/5% CO2. Patch pipette solution contained (in mM): 140.0 CsCl, 10.0 EGTA, 5.0 MgCl2, 2.0 Na-ATP, 0.3 Na-GTP, 10.0 HEPES, and 0.2% biocytin (pH 7.3, 290 mOsm), and QX-314 (sodium channel antagonist, 5 mM) added at time of use. Examples of biocytin filled pyramidal neurons from both NT and J20 mice are shown in Figure 2B. Patch pipettes (BF150-086; Sutter Instruments, Novato, CA) were

pulled on a Sutter P-97 horizontal puller (Sutter Instruments, Novato, CA) to a resistance between 3-5 MΩ. Recordings were performed perfusion of oxygenated ACSF at 2.5 mL/min. The blind patch technique was used to acquire whole-cell recordings from CA1 pyramidal neurons. Neuronal activity was recorded using an Axopatch 700B amplifier and pClamp11 acquisition software (Molecular Devices, San Jose, CA). Signals were filtered at 5 kHz and digitized at 4 kHz (Digidata 1550B). Spontaneous inhibitory post-synaptic currents (sIPSCs) were recorded between ZT 2-6.5 (day) or ZT 12.5-17 (night) and pharmacologically isolated with bath perfusion of NBQX (AMPAR antagonist, 10 μM, Hello Bio) and CPP (NDMAR antagonist, 5 μM, Hello Bio). Miniature IPSCs (mIPSCs) were recorded as above with the addition 0.5 μM TTX (voltage-gated sodium channel inhibitor, Tocris). All cells were dialyzed for 5 min prior to experimental recordings and held at -70 mV. Cells used for analysis had access resistance less than 30 MOhms that did not change by more than 20% for the duration of recording.

Statistical Analysis Results were calculated using SPSS software (versions 25 and 27).

Spontaneous Alternation Spontaneous alternation was analyzed using ordinal regression analysis (Logit link function) with the day/night/genotype group as a fixed factor and alternation as an ordinal outcome (four categories: 0%-20%, 40%, 60%, 80%-100% alternations). Post hoc planned comparisons were used to assess day/night differences within genotype with a Holm's corrected α for multiple comparisons. Data for males and females were combined since there were no apparent sex differences in alternation score distribution ($p = 0.28$ and 0.96 for NT and J20, respectively.

Electrophysiology Inhibitory postsynaptic currents were automatically detected using pClamp's template search analysis then manually inspected for false event detection. The amplitudes and interevent interval (IEI) were analyzed using a generalized estimating equation (GEE) with an unstructured working correlation matrix structure, a subject effect of cell, and a within-subject effect of postsynaptic events to account for multiple events from each cell. To account for the non-normal distribution of the data, upper (10%) and lower (10%) outliers were trimmed, then amplitude values were transformed using a log transformation, and IEI values were transformed by adding 1 then performing a log transformation. These transformations allowed for normal distribution of the data prior to analysis.

RESULTS

J20 mice lack typical diurnal spatial working memory

We first tested if diurnal variation in hippocampus-dependent spatial working memory is compromised in J20 mice using spontaneous alternation in a T-maze. This task has demonstrated day-night differences in mice[17,33] and other rodents[35,36]. To account for diurnal differences in locomotor activity that could affect the number of arm entries, this assay employs trials rather than a time limit as other versions of assaying spatial memory in a T- or Y-maze[37]. Additionally, this method of testing spatial working memory does not require multiple days of training that could disrupt sleep and circadian rhythms. Male and female NT and J20 mice were tested during both day and night at 3-4 months of age. As expected, NT mice had more successful alternations at night compared to day (**Figure 1**, $p = 0.025$). In J20 mice, however, performance was overall decreased compared to NT controls at both day ($p < 0.001$) and night ($p < 0.001$, Figure 1). This suggests a loss in diurnal variation in spatial working memory in the J20 mice.

Night-time inhibitory transmission is reduced in J20 mice

A recent study from our lab showed that inhibition onto the primary output cell of the hippocampus, CA1 pyramidal cells (CA1 PCs) is greater during the day compared to night in WT control mice, but that increased day-time inhibition is absent in Tg-SwDI mouse model of amyloidosis[17]. To determine if diurnal differences in inhibition exist in J20 mice, spontaneous inhibitory post-synaptic currents (sIPSCs) were recorded from CA1

PCs during the day and night in 3–6-month male and female J20 and non-transgenic (NT) littermate control mice (**Figure 2, Supplementary Table 1**).

Amplitude of sIPSCs onto CA1 PCs was significantly greater in NT mice compared to J20 mice (main effect, $p = 0.036$; **Figure 2C, top**), although it is a difference of ~2pA and is likely not biologically relevant (NT: 34.91 +/- 1.02; J20: 33.04 pA +/- 1.02). Amplitude did not appear to vary with time of day (main effect, $p = 0.38$; interaction, $p = 0.28$). Regarding event frequency, the effect of Genotype on interevent interval (IEI) was dependent upon time of day (interaction, $p = 0.049$; **Figure 2C, bottom**). Specifically, IEIs recorded at night were shorter in NT mice than J20 mice ($p = 0.034$), indicating a higher frequency of events at night in the NT mice compared to J20 mice. In addition, while it did not reach significance ($p = 0.068$), IEIs of NT mice were slower during the day than night. This trend to higher night-time sIPSC frequency in the NT mice contrasts with our previous findings in two studies using C57/Bl6 mice[17] (Chapter 2 of this dissertation). In these studies, inhibition was increased during the day compared to night. Inhibitory event sIEIs in J20 mice were nearly the same when recorded during the day and night ($p > 0.05$). Miniature inhibitory post-synaptic currents (mIPSCs) were recorded in parallel in the presence of the voltage-gated sodium channel blocker tetrodotoxin, to assess whether the increased night-time inhibition onto CA1 PCs in NT mice is action potential-dependent (Figure 3). There were no significant time of day or genotype effects on mIPSC amplitude (Figure 3B, top). However, the main effect of time of day on mIPSC IEI was trending toward significance (Figure 3B, bottom; $p = 0.063$) suggesting that IEIs may be shorter at Night compared to Day. Finally, J20 mice had longer event IEIs than NT controls (main

effect, $p = 0.012$; Figure 3B, bottom). Overall, these results suggest that spontaneous vesicle fusion in the J20s is reduced compared to NT mice irrespective of time of day.

DISCUSSION

Patients with AD exhibit circadian disruption as evidenced by altered rest-activity patterns[14,38-48]. Sleep deprivation and genetic disruption of the molecular clock accelerate amyloid beta pathology and plaque formation in multiple mouse models of AD[49-51]. While spatial memory deficits and synaptic dysfunction have been reported in the J20 mouse model[15,16,52-56] , there are no examinations of circadian dysfunction. Given the evidence of circadian dysfunction in AD patients as an early symptom, it is important to examine altered physiology and behavior of disease models at different times of day as it may guide future design of potential therapies. This study provides evidence that normal diurnal differences in spatial memory are absent from this AD mouse model by age 3-4 months, around the time of synaptic loss and changes in neural plasticity, but before amyloid plaque detection.

Molecular clock function is linked to spatial memory performance[57]. Several core clock components (mRNA and protein) are reduced in multiple AD models including cell culture and mouse models. Two other AD mouse models (APP/PS1 and 5XFAD) show reduced *Per2* mRNA and/or protein levels in the SCN or hypothalamus, compared to age-matched controls[58,59]. In the hippocampus, intracranial injection of Aβ31-35 in WT mice reduced the nocturnal peak in PER2 protein levels[60]. In young APP/PS1 mice, hippocampal clock gene expression was not different from WT controls, with both genotypes showing a time-of-day difference[61]. However, by 12-15 months, APP/PS1 mice showed reduced

diurnal rhythms in hippocampal clock gene mRNA expression[62]. The Tg-SwDI model has reductions in gene expression of *Bmal1*, *Bmal2*, *Cry1*, *Per1*, and *Reverbα*, across both males and females, and reduced PER2 expression specific to female mice at 4 months of age[17]. A similar investigation into molecular clock expression in J20s may reveal further insights into hippocampal circadian dysfunction in the J20 model. Further, it is still unknown if changes in molecular clock expression and neurophysiology in the hippocampus occur in parallel, if one precedes the other, or if one is causal to the other. Careful and thorough work will be required to answer these questions, and we hope our study lays a foundation for future studies.

In the Tg-SwDI mouse model of amyloidosis, there is a lack of diurnal variation in inhibition onto CA1 PCs of the hippocampus and in action potential firing in SCN neurons[63]. This led us to examine diurnal variation in inhibitory transmission in the J20 model. Unexpectedly, we detected greater inhibition onto CA1 PCs in NT mice at night, in contrast to what we have seen previously in C57/Bl6 mice (increased inhibition during the day)[17] (Chapter 2 of this dissertation). While the unexpected results from the NT mice make any genotype differences in inhibitory transmission harder to interpret, the finding that inhibition is reduced overall in J20 mice is in line with the hypothesis of hyperexcitability in AD. However, a direct comparison to the literature is tricky since other studies were conducted at one time of day, and in a different region of the hippocamps (dentate gyrus)[56], or the cortex[55]. Another caveat in the interpretation of these results is that we assessed both neuronal physiology and behavior in NT and J20 mice at only two times of day and thus cannot differentiate a shift in phase from an absence of rhythm.

ACKNOWLEDGEMENTS

This work was supported by NIH grants R01NS082413 (KLG), R56AG061785 (EDR and KLG), RF1AG059405 (EDR), T32NS061788 (ARF), and F31AG066385 (ARF).

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FIGURES

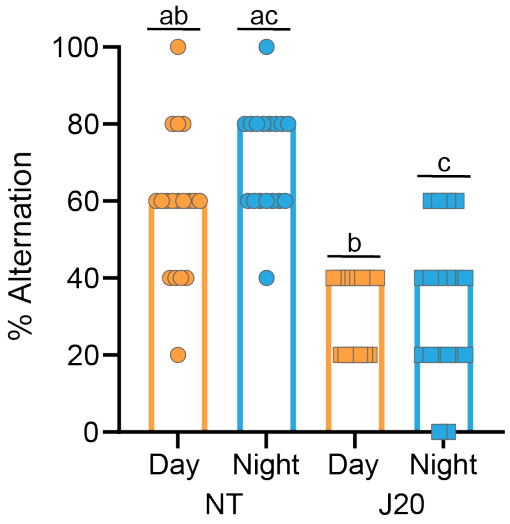

Figure 1. Absence of diurnal variation in spatial working memory in J20 mice at age 3-4 months. Percent alternation at ZT 3 vs ZT 15 in NT (circles) and J20 (squares) mice tested during the day (orange) and at night (blue). NT mice had a significant day-night difference in alternation with more successful alternations at night (comparison a, $p = 0.025$). J20 mice lacked diurnal variation in alternations ($p = 0.596$). Performance during the day (comparison b, $p < 0.001$) and the night (comparison b, $p < 0.001$) was better in NT mice compared to J20.

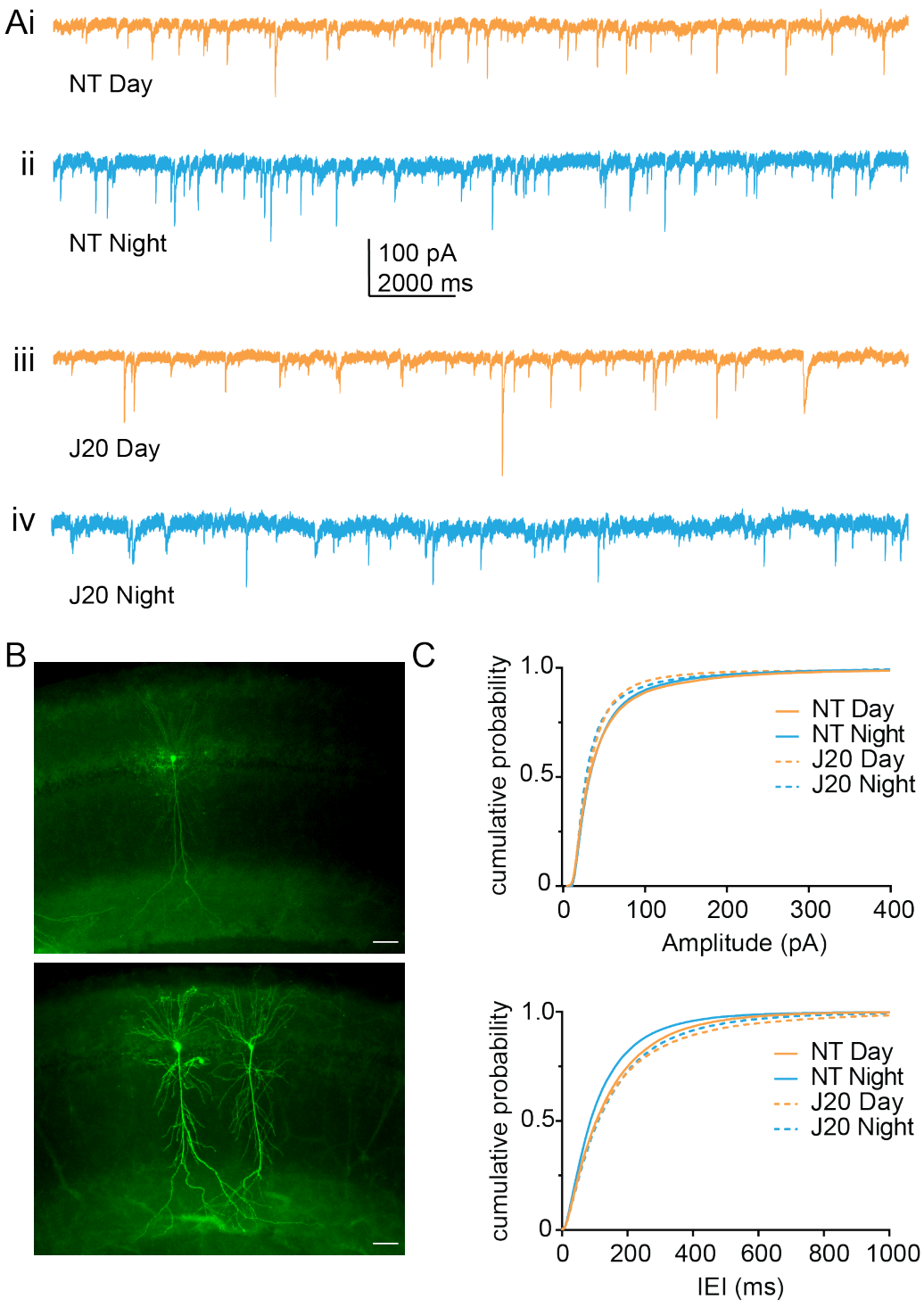

Figure 2. Night-time inhibitory transmission is reduced in J20 mice at 3-6 months of age. A. Representative sIPSC traces from CA1 PCs from NT mice during the day (i) and night (ii) and J20 mice during the day (iii) and night (iv). B. Biocytin filled CA1 **PCs** from NT (top) and J20 (bottom) mice, scale bar is 200 microns. C. Reduced event amplitude (top) at both times of day in J20 mice (dashed) compared to NT mice (solid, p

= 0.036). Bottom; NT mice (solid) have a shorter IEI at night (blue) compared to J20 mice (dashed, p = 0.034).

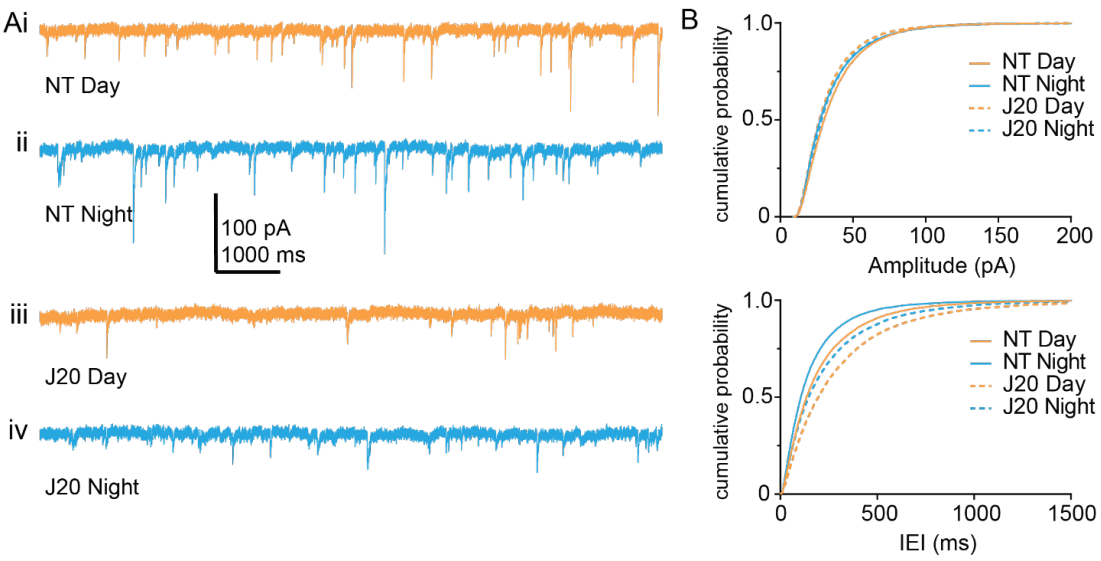

Figure 3. Reduced mIPSC IEIs in J20 mice. A. Representative mIPSC traces from CA1 PCs from NT mice during the day (i) and night (ii) and J20 mice during the day (iii) and night (iv). B. No diurnal or genotype difference in mIPSC amplitude (top). mIPSC IEI (bottom) was trending to significance ($p = 0.063$) with longer IEIs at Night compared to Day in NT and J20 mice. There was also a main effect of genotype ($p = 0.012$), with longer event IEIs in J20 mice compared to NT controls.

Experiment	Measure	Transformation	Genotype	Time of Day	Interaction
sIPSC	Amp	Log10(amp)	$\chi 2$ (1) = 4.384, p = 0.036	$\chi 2$ (1) = 0.769, p = 0.381	$\chi 2$ (1) = 1.172, p = 0.279
	IEI	Log10(IEI+1)	$\chi 2$ (1) = 2.290, p = 0.130	$\chi 2$ (1) = 3.892, p = 0.085	$\chi 2$ (1) = 3.892, p = 0.049
mIPSC	Amp	Log10(amp)	$\chi 2$ (1) = 1.406, p = 0.236	$\chi 2$ (1) = 2.316, p = 0.128	$\chi 2$ (1) = 1.384, p = 0.239
	IEI	Log10(IEI+1)	$\chi 2$ (1) = 6.383, p = 0.012	$\chi 2$ (1) = 3.468, p = 0.063	$\chi 2$ (1) = 0.471, p = 0.492

Supplementary Table 1. Statistical analyses of IPSCs.

GENERAL DISCUSSION

Circadian rhythms are intrinsically generated oscillations in physiology and behavior that occur over a 24-hour period. While circadian rhythms in the hippocampus have been detected at the molecular and behavioral (i.e., cognition) level, a detailed examination of time-of-day variation in synaptic physiology is lacking. Unraveling daily rhythms in neurophysiology can offer insight into how our bodies regulate neural signaling to generate time-of-day appropriate behaviors or to adapt to changes in the environment. Finally, day-night differences in physiology and disruptions of that physiology may provide insight into possible therapeutic targets, and also when interventions should be administered to most effectively ameliorate Alzheimer's disease symptoms or delay pathological onset.

Summary of Findings

This dissertation set out to better understand the day-night influence on hippocampal CA1 neurophysiology and how hippocampal circadian regulation is disrupted in disease. I addressed the following questions:

- Cognitive function that relies – at least in part – on the hippocampus varies as a function of time of day, but the underlying time of day variations in hippocampal neural circuit function have not been examined in detail. While changes in

hippocampal LTP have been shown across time of day, an understanding of other measures of electrical activity at the level of the single neuron is lacking. Do synaptic inputs, intrinsic properties, and resulting excitability of CA1 pyramidal cells vary diurnally?

  - In Chapter 2, I provide evidence that inhibition and excitation onto CA1 pyramidal cells vary across time of day in a complimentary manner. While CA1 pyramidal cell excitability and sag are also influenced by time of day, the magnitude and directionality depend on several factors, including synaptic transmission, sex, and position in the hippocampus.

- One of the most notable symptoms in patients with AD is cognitive dysfunction. Along with disruption of sleep-wake cycles, worsening of cognitive dysfunction in humans with AD occurs in the evenings, pointing to impaired circadian function in the hippocampus. Is diurnal regulation of hippocampal-dependent cognitive function and neuronal physiology impaired in Alzheimer's disease?

  - In Chapters 3 and 4, I provide evidence that diurnal regulation of spatial memory is impaired in Alzheimer's disease. I also provide evidence that diurnal regulation of CA1 pyramidal cell neurophysiology is altered in AD, along with molecular clock expression.

Diurnal regulation of hippocampal neurophysiology

Synaptic inputs onto CA1 pyramidal cells

We first examined whether synaptic inhibition and excitation were regulated by time of day. Our sIPSC and mIPSC data suggest that that action potential-dependent inhibition onto CA1 pyramidal cells is greater during the day compared to night (Chapter 2, **Figure 1**). Given that these inhibitory currents were pharmacologically isolated with glutamate receptor antagonists, it is likely that increased day-time interneuron activity is spontaneously generated. Indeed, reports in the literature suggest that some interneurons in area CA1 are spontaneously active[246-250]; however, definitive evidence of time-of-day variation in spontaneous interneuron firing in the hippocampus is lacking. Unpublished data from our lab suggest that parvalbumin-positive (PV+) inhibitory interneurons express the components of an intrinsic molecular clock and exhibit the potential for rhythmic changes in excitability, with resting membrane potential (RMP) being significantly more hyperpolarized at night compared to day in hippocampal slices.

Second, we found that excitatory input onto CA1 pyramidal cells is numerically greater during the night compared to day, and that in the absence of action-potential dependent neurotransmitter release, this difference persists in males, but is absent in females. This suggests that the increased excitation at night in females is likely action-potential driven. Major excitatory input onto CA1 pyramidal cells arrives from the axons of principal neurons of the downstream area CA3 (Schaffer collaterals; CA3 pyramidal cells) or from the entorhinal cortex (Temperoammonic pathway). CA3 pyramidal cells also exhibit circadian rhythms in excitability, with larger calcium current, decreased afterhyperpolarization, and reduced spike frequency adaptation at night compared to

day[251]. This increased night time CA3 pyramidal cell excitability could translate to increased sEPSC onto CA1 pyramidal cells at night compared to day. Additionally, microdialysis studies have detected a higher concentration of glutamate at night compared to day in the striatum, nucleus accumbens[252,253] and SCN to name a few. This is in line with our data in Chapter 2 suggesting that spontaneous excitatory drive onto CA1 pyramidal cells is enhanced at night compared to day (Chapter 2, **Figure 2**), and with pyramidal cell excitability, which is greater at night (Chapter 2, **Figure 3**).

That excitation and inhibition onto CA1 pyramidal cells peak at opposite times of day is in line with previously published work[254,255], however, those reports find increased excitation during the day and increased inhibition at night. Multiple factors can account for the discrepancies in these results. While both studies used male and female mice, sex differences were not accounted for, and while both studies looked at day-night differences, they were not done in the same range of the light and dark phase as ours.

CA1 pyramidal cell excitability and sag ratio

Because of the diurnal variation in excitatory and inhibitory input onto CA1 pyramidal cells, we next wanted to determine whether the net outcome of CA1 pyramidal cell excitability also varied across time of day. We found that there were no day-night differences in spikes per step in pyramidal cells recorded from anterior slices. In posterior slices from male mice, cells fired more spikes per step at night compared to day, and overall compared to cells from female mice. These results suggest that CA1 pyramidal cells from male mice are more excitable than those from female mice. We then wanted to

determine if the day-night differences in excitability were present in the absence of synaptic transmission and are an intrinsic property of these neurons. When synaptic transmission was blocked, in cells from posterior slices, the increased excitability in male mice at night compared to day and compared to those from female mice at night persisted. Interestingly, in this condition, cells recorded from anterior slices were more excitable in males compared to females (Chapter 2, **Figure 3**).

What determines neuronal excitability and is it subject to time-of-day modulation? The most obvious candidate influencing excitability is fast excitatory transmission through glutamate receptors (AMPARs and NMDARs). Broadly, excitatory glutamate input onto neurons provides depolarizing conductances (excitatory post synaptic potentials, EPSPs) that summate to produce action potentials. While subthreshold EPSPs can spatially and temporally summate to generate APs, the efficacy of EPSP to spike coupling is thought to depend on several factors including the magnitude and timing of GABA-mediated inhibitory postsynaptic potentials (IPSPs)[256], as well as passive and active properties of the post-synaptic neuron[257].

In addition to directly hyperpolarizing membrane potential through generation of IPSPs, fast inhibitory transmission is typically mediated by $GABA_A$ receptors ($GABA_ARs$), which often have a reversal potential close to the resting potential. At resting potential, even though little current is generated by these synapses due to the small driving force, the resulting increase in membrane conductance that they introduce effectively short-circuits excitatory synaptic currents by locally reducing the input resistance. For a given excitatory input, the size and duration of the EPSP will be reduced, and the temporal and spatial window over which signal integration can occur

will be narrowed, making it less likely that an action potential will be generated. This shunting inhibitory conductance can therefore reduce neuronal excitability. As previously stated, unpublished data from our lab suggest that PV+ inhibitory interneurons express molecular clock components and exhibit the potential for rhythmic changes in excitability, with RMP more hyperpolarized at night compared to day. PV+ interneurons, among others, may therefore govern time of day dependence of neuronal excitability by providing a hyperpolarizing conductance or by creating a shunt[258,259]. Further, extrasynaptic $GABA_AR$s, due to their higher affinity, can be activated by low concentrations of GABA in the extracellular space, inducing what is known as tonic inhibition[259]. This tonic GABA in the hippocampus is dependent on neuronal activity – blocking action potential release through the addition of the voltage gated sodium channel blocker tetrodotoxin (TTX) reduces the concentration of tonic GABA and might be a mechanism through which synaptic activity modulates excitability. Indeed, it has been hypothesized that tonic GABA modulates excitability levels in the SCN in a circadian fashion via their action on extrasynaptic $GABA_AR$s[260]. Unfortunately, empirical evidence is currently lacking.

Different neurons are equipped with a unique complement of voltage-gated ion channels that contribute to active conductances specific to that neuron. Further passive membrane properties such as membrane resistance and membrane capacitance that depend on neuronal morphology contribute to passive membrane properties of a neuron. Together, these active and passive conductances also determine the intrinsic excitability of the cell. Most of our understanding about how circadian rhythms influence excitability comes from work in the neurons of the SCN, which exhibit strong intrinsic 24-hour

rhythmicity in firing rate, input resistance, resting membrane potential[261,262], and a variety of ionic conductances[263-266]. More than 600 genes show circadian expression in hippocampus[267], including voltage-gated channels (for a subset see **Table 3**). Though a full discussion of how each of these channels affects excitability is out of the scope of this dissertation, future studies linking rhythmic expression of one or several of these channels to rhythms in excitability is crucial.

Gene	Function	AP	PP
Hcn1	Hyperpolarization activated cyclic nucleotide gated K^+ channel 1	X	
Kcnab1	Small conductance Ca^{2+}-activated K^+ channel	X	
Kcnab2	Small conductance Ca^{2+}-activated K^+ channel	X	
Kcnab3	Small conductance Ca^{2+}-activated K^+ channel	X	
Kcnc1	Voltage-gated, fast, delayed rectifier, K^+ channel	X	
Kcnc3	Voltage-gated, fast, delayed rectifier, K^+ channel Shaw family	X	
Kcnip1	Voltage-gated, K^+ channel interacting protein 1; A-type K^+ current	X	
Kcnip2	Voltage-gated, K^+ channel interacting protein 2; A-type K^+ current	X	
Kcnip4	Voltage-gated, K^+ channel interacting protein 4; A-type K^+ current	X	

Kcna1	Voltage-gated, delayed rectifier, K^+ channel; Shaker	X	
Scn8a	Voltage-gated, resurgent and persistent Na^+ channel	X	
Cacnb2	Voltage-gated Ca2+ channel	X	
Cacng3	Voltage-gated Ca2+ channel	X	
Cacna1a	Voltage-gated Ca2+ channel (P/Q)	X	
Kcnj3	G-protein coupled, inwardly rectifying K^+ channel (GIRK1)		X
Kcnk3	Voltage-insensitive, two-pore K^+ channel; K^+ leak current		X
Gabra1	$GABA_AR$, Alpha1	Synaptic	
Gabra4	$GABA_AR$, Alpha 4		
Gabrb2	$GABA_AR$, Beta 2		
Gabrg3	$GABA_AR$, Gamma 3		

Table 3. **Putative clock-dependent genes coding for ion channels**. Key: AP, active properties; PP, passive properties.

When examining sag ratio across time of day, we again found no differences in cells recorded from anterior slices. In cells recorded from posterior slices, there was

overall greater sag in male mice compared to female mice, and sag was significantly lower in female mice at night compared to day. In the presence of synaptic antagonists, the differences observed in sag in cells recorded from posterior slices were eliminated, while differences in anterior slices were uncovered. In male mice, sag was greater during the day compared to night, and there was no difference in female mice. During the day, sag is greater in male mice than females, but this is reversed at night, where females have greater sag than males. To our knowledge, this is the first studying examining a measure of HCN channel function (sag) between day and night in the hippocampus. Given that in the SCN, HCN channel function varies with time of day[268], and in the hippocampus, HCN channel expression varies along the dorsoventral axis[269], it is not surprising that we found that diurnal regulation of sag depends on both these factors in addition to sex. Intrinsic properties of CA1 pyramidal cells vary along the dorsoventral axis, at least partially due to differences in receptor composition. Most reports examining intrinsic properties, including sag, do so in the presence of synaptic antagonists, therefore, a suitable comparison of our results in the in-tact circuit is difficult. Overall, we found that diurnal regulation of sag in anterior cells seems to be a product of intrinsic excitability, while in posterior cells, it seems to be driven by consequence of synaptic transmission (Chapter 2, **Figure 4**).

When taken together, the measures of excitability and sag suggest that diurnal variation is intrinsic to anterior, dorsal-like CA1 pyramidal cells, but dependent on synaptic transmission for ventral-like pyramidal cells. Measurements of inhibitory and excitatory synaptic transmission also vary along the dorsal ventral axis, which could account for the differences seen with the circuit in-tact. Expression of ion channels that

may contribute to intrinsic dorsal-ventral differences include A-type potassium channels[270], M-type potassium channels[271], and GIRK channels[272,273], therefore investigation into their function across time of day is warranted.

Basal synaptic transmission and LTP

As diurnal variation depends on sex, we next sought to determine whether increased night-time LTP depends on sex. When assessing basal synaptic transmission across time of day, we found no difference between males and females, and no day-night difference, indicating that evoked basal synaptic transmission does not vary across time of day. We also found that LTP magnitude is greater at night compared to day regardless of sex.

The lack of day-night difference in basal synaptic transmission is in line with work from the Colwell lab, who also failed to detect a day-night difference in basal synaptic transmission at the CA3-CA1 synapse in two different strains of mice[104]. However, they did detect a day-night difference in population spike amplitude recorded in the cell body layer of CA1 in response to Schaffer collateral stimulation of increasing magnitude, suggesting overall neuronal excitability is greater during the night compared to day, as we detected in **Figure 2** of Chapter 2. Our LTP results (greater magnitude during night compared to day) are also in line with previous work from our lab and others[103-106]. However, we show for the first time that despite differences in circadian regulation of synaptic transmission and excitability, the sex difference does not extend to day-night differences in LTP (Chapter 2, **Figure 5**). An important note for this

experiment is that we were underpowered to account for differences along the anterior-posterior axis. The lack of segregation of these data according to slice position along the anterior-posterior axis may have occluded detection of a sex difference in circadian regulation of LTP; this possibility cannot be entirely ruled out. Other factors that might have influenced our results is the use of a "less naturalistic" high frequency stimulation (HFS; 100 Hz for 0.5s, 2X). We used HFS with the intent to replicate previous findings from our lab and others, however, using a more natural stimulation patterns such as theta-burst stimulation might reveal latent sex differences.

Altered hippocampal diurnal regulation in AD

Spatial working memory

Patients with AD exhibit circadian disruption as evidenced by altered rest-activity patterns[188,274-281]. Sleep deprivation and genetic disruption of the molecular clock accelerate amyloid beta pathology and plaque formation in multiple mouse models of AD[201-203] and likely accelerate AD pathology in humans[282]. Given the evidence of circadian dysfunction in AD patients, it is important to examine it in models of disease, especially when trying to design and test potential therapies. Here, we showed that that normal diurnal differences in spatial memory are absent from both the J20 and Tg-Swdi AD mouse models by age 3-4 months (Chapter 3, **Figure 1**; Chapter 4, **Figure 1**) around the time of synaptic loss and changes in neural plasticity in J20 mice, and before amyloid plaque detection in both models.

Clock gene expression

There is evidence that molecular clock expression is linked to day-night differences in spatial working memory[70,76,283]. With the finding of altered spatial memory performance in Tg-SwDI mice, we next examined clock gene expression. We found reductions in expression of several clock components at both the mRNA and protein levels (Chapter 4, **Figures 2 and 3**), consistent with findings in other AD models[205-209]. Evidence suggests that reduced clock function may lead to several issues including amyloid beta accumulation, synaptic dysfunction, oxidative stress, altered proteostasis, immune dysfunction, and neuroinflammation [284]. Importantly, many ion channel-related genes examined in CircaDB database show significant 24-hour rhythms in expression in the SCN[285]. Therefore, it is possible that deficits in the molecular clock alter neuronal membrane physiology by dysregulation of these ion channels.

Inhibitory transmission onto and excitability of CA1 pyramidal cells

We found altered diurnal regulation of inhibitory transmission onto CA1 pyramidal cells. In experiments in Tg-SwDI mice with WT controls, we found that inhibition in WT mice was greater at night compared to day, with larger event amplitudes and shorter IEIs during the day (Chapter 3, **Figure 4**). In Tg-SwDI mice, event amplitudes were larger during the day, but IEIs were longer during the day compared to night, indicating a higher frequency of events at night compared to day (Chapter 3, **Figure 4**). Overall, diurnal inhibition was reversed in the Tg-SwDI mice compared to controls. When this experiment was performed in J20 mice and NT littermate controls,

we detected greater inhibition onto CA1 PCs in NT mice at night (Chapter 4, **Figure 2**), in contrast to what found in WT mice (increased inhibition during the day)[286] (Chapter 2, **Figure 1**). While the unexpected results from the NT mice make any genotype differences in inhibitory transmission harder to interpret, the observation that inhibition is reduced overall in J20 mice is in line with previous findings[287-289]. Additionally, differences in sex affect diurnal regulation of synaptic transmission (shown in Chapter 2) and position along the dorsoventral axis affects synaptic transmission[269,270,290,291]. Neither of those factors were accounted for in these studies.

In Chapter 2 of this dissertation, we found that diurnal regulation of pyramidal cell excitability and sag ratio depend on both position along the axis and sex. Additionally, in our study of the Tg-SwDI mice, we were not powered enough to look at differences between male and female mice. When measuring diurnal variation of CA1 PC excitability, we found that the increased night-time excitation seen in controls was absent in Tg-SwDI mice (Chapter 3, **Figure 5**), similar to what we observed when pharmacologically inhibiting GABAergic transmission onto CA1 PCs (Chapter 3, **Figure 5**). The lack of day/night differences in CA1 PC excitability and sIPSCs in TgSWDI mice support the hypothesis of hyperexcitability in AD[173,196,292].

The findings of these studies emphasize the impact of diurnal regulation in cognitive function and neurophysiology. Uncovering how day-night regulation of physiological processes change with the progression of disease will be important in our ability to target and treat different disease symptoms, and even to accurately detect potential biomarkers.

Future Directions

The focus of this dissertation was on examining day-night differences in hippocampal neurophysiology and how those are altered in disease. In Chapter 2 of this dissertation, we uncovered that synaptic transmission, position along the dorsoventral axis, and sex affect the diurnal variation in synaptic physiology and intrinsic properties of neurons, emphasizing the complexity of diurnal regulation on neuronal physiology. In the future, experiments designed to examine diurnal variation should be designed with those factors in mind.

While we saw differences in diurnal variation based on position in the hippocampus in our studies of neuronal excitability, we were unable to account for those differences in our studies of inhibitory and synaptic transmission. A more thorough examination on how sex and spatial differences affect synaptic transmission is needed. In addition to determining these differences in WT mice, another important question is how that is altered in disease. The factors of sex and location could have played a role in the results seen in both the J20 and Tg-SwDI mice, so a more thorough examination of diurnal changes in inhibition in those models is warranted.

Determining whether day-night differences in excitability exist in interneurons will be important in order to better understand diurnal regulation of hippocampal function, especially in regard to the complimentary nature of inhibition and excitation over the course of the day. This will also aid in our understanding of circadian dysfunction and its role in hyperexcitability in AD.

Because of the radically differing results in diurnal variation of sag ratio, it would be interesting to examine how HCN channel function varies across time of day and how disrupting the function of HCN channels alters CA1 PC excitability between day and night.

Now that we have uncovered differential diurnal regulation based on sex, an important question to answer is whether diurnal differences in performance of hippocampal-dependent tasks vary between males and females. This could be especially impactful in treatment of diseases that affect one sex more than another, such as in AD.

The field has long hypothesized that diurnal regulation of cognitive function is produced by underlying diurnal regulation of neurophysiology, but we lack empirical evidence connecting these two measures. If pyramidal cell excitation is higher at night and performance in hippocampal dependent tasks is greater at night, it would be interesting to see if chemogenetically reducing excitability at night disrupts night time enhancement of cognitive performance.

Another important question is whether molecular clock function directly impacts hippocampal neurophysiology. A potential experiment to address this would be to disrupt circadian rhythms by knocking out *bmal1* and assessing if rhythms in hippocampal neurophysiology persist.

Concluding Remarks

This dissertation set forth to determine the influence of diurnal variation on hippocampal function and how that influence is altered in AD. The results presented in

this dissertation advance our understanding of time of day regulation of synaptic transmission onto and excitability of CA1 pyramidal cells. Time of day influences depend largely on sex and position in the hippocampus. Understanding how diurnal regulation of hippocampal function is altered by these factors is critical to understanding circadian dysfunction in AD and other neurological diseases.

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 24-Mar-2020

TO: Gamble, Karen Lynnette

FRC

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 24-Mar-2020.

Protocol PI:	Gamble, Karen Lynnette
Title:	Circadian Dysfunction and Neurodegenerative Disease
Sponsor:	National Institute of Neurological Disorders andStroke/NIH/DHHS
Animal Project Number(APN):	IACUC-09708

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 22-Mar-2023.

Institutional Animal Care and Use Committee (IACUC)

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 22-Jun-2021

TO: Roberson, Erik

FRC

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 22-Jun-2021.

Protocol PI:	Roberson, Erik
Title:	Circadian changes in network excitability and Alzheimer diseasepathogenesis
Sponsor:	National Institute on Aging/NIH/DHHS
Animal Project Number(APN):	IACUC-21847

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 08-Sep-2022.

Institutional Animal Care and Use Committee (IACUC)

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