Sleep deprivation and seizures:
A vicious cycle in epilepsy

By

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Abstract

Epilepsy is a neurological syndrome that is estimated to affect 1 in 25 people at some point in their lives. Epilepsy is a disease characterized by spontaneous recurrent seizures. Epilepsy occurs in many forms, with varying site(s) of seizure genesis (foci) and extent of synchronous activity. Regardless of how epilepsy manifests, underlying the condition is a state of neural hyperexcitability, which may be due to genetic predisposition or acquired from the incidence of seizures with other causes. Acquired epilepsy usually begins with a seizure arising from things such as encephalitis, autoimmunity, or traumatic brain injuries. Following the initial insult, the regions of the brain affected by the seizure may begin to alter local circuit structure and intrinsic properties that elevate the susceptibility for subsequent seizures. These anatomical and physiological processes are collectively termed epileptogenesis, which likely persists after the onset of epileptic seizures. Among people with epilepsy, poor sleep is a common symptom. Reciprocally, it has been reported that sleep loss is among the most potent triggers for epileptic seizures, even if seizures are otherwise well controlled. So, it appears that sleep loss and seizures participate in a vicious cycle in epilepsy. As the mechanisms of epileptogenesis and disease progression are continually elucidated, physiological ramifications of sleep dysregulation are emerging as a potential nexus of control over some broader pathologies associated with epilepsy. Investigation of mechanisms that drive disease progression would benefit current medical practices and direct treatments toward a cure. Conventional therapies are often ineffective in controlling seizures, especially in one of the most frequently diagnosed and resistant forms of epilepsy, temporal lobe epilepsy (TLE). In TLE, a common site of seizure generation and propagation is the hippocampus. My thesis work attempts to disentangle the vicious cycle in TLE by independently investigating 1) the effects of acute sleep loss on the hippocampus in seizure-naive mice, 2) investigating sleep and hippocampal function in early epileptogenesis, and 3) dysregulation of sleep and circadian rhythms in a model of TLE.

In the second chapter, I investigate the effect of 4-hour sleep deprivation (4SD) on GABAergic inhibition in the Dentate Gyrus (DG) and CA1 of the hippocampi of seizure-naïve mice. Using patch-clamp electrophysiology, I demonstrate that tonic GABAergic currents are significantly reduced in both regions studied. Additionally, I show that the function of two receptor subtypes that mediate hippocampal tonic inhibition is unaltered in the DG, while differentially regulated in CA1 neurons following 4SD. Finally, I demonstrate that the frequency and amplitude of miniature inhibitory postsynaptic currents (mIPSCs) are insensitive to 4SD treatment.

In the third chapter, I investigate the sleep characteristics and hippocampal neurotransmission in the early stages of epileptogenesis following induced status epilepticus (SE). First, I show that sleep efficiency (i.e., percent time spent awake, rapid eye movement (REM), and non-REM sleep) is not altered after SE. Second, I demonstrate that sleep restriction induced potentiation of NREM Delta power, a correlate to sleep pressure, is impaired after SE. Finally, I show that tonic inhibition in DG is

reduced at this acute point of epileptogenesis after SE, while mIPSC frequency and amplitudes are unaffected.

In the fourth chapter, I evaluate how circadian rhythms are dysregulated in a mouse model of TLE. I report that circadian rhythms of locomotor activity are significantly disrupted in TLE. I then demonstrate that transcription patterns of several core molecular controllers of the circadian Clock are altered in the central circadian pacemaker, the suprachiasmatic nucleus (SCN). Our data suggest that seizure activity can be communicated to and integrated into the SCN, thereby compromising accurate circadian timing of systems across the body and in the brain.

In summary, my thesis work demonstrates that circadian sleep regulation and the response to sleep deprivation, as well as tonic inhibition in the DG, are impaired at various points of epileptogenesis. Additional reduction of hippocampal tonic inhibition caused by transient sleep loss is a possible cause for the SD-induced increase in seizure susceptibility, especially in TLE.

Chapter 1 – Introduction & Background information

<u>What is Epilepsy?</u>

Epilepsy is a disease characterized by spontaneous recurrent seizures. Epilepsy occurs in many forms, with varying site(s) of seizure genesis (focus) and extent of synchronous activity. A common misconception about seizures is that they are all convulsive. Current classifications of seizure types published by the International League Against Epilepsy (ILAE) include several axes: method of onset, awareness, and motor involvement. During a seizure, the activity of a large group of neurons become synchronized. Based on how many regions of the brain are recruited to this synchronous activity at the onset, seizures can be classified as focal if it involves a single unilateral region, generalized if it involves multiple bilateral regions, or unknown if no seizure focus can be determined (Fisher et al., 2017). Interestingly, the severity of the motor component does not necessarily correlate to the extent of electrographic involvement at the onset. For example, generalized seizures usually have extreme motor components, whereas generalized nonmotor (absence) seizures cause loss of consciousness and appear as staring spells, with no convulsive components (Fisher et al., 2017).

An important distinction pertaining to epileptic seizures is that they are spontaneous, which is to say there is no causative injury known to have precipitated the seizure. The diagnostic criterion for seizures to be epileptic is for spontaneous seizures to become recurrent, with more than 24 hours separating episodes (Fisher et al., 2017). Underlying epilepsy is a hyperexcitable neural network. Neural hyperexcitability can be

due to genetic abnormalities that predispose an individual to epileptiform activity or acquired later in life. Acquisition of epilepsy is usually due to an injury that causes a seizure. Non-epileptic seizures are generally associated with injuries like high fever and encephalitis, traumatic brain injury, or autoimmunity (Chawla et al., 2002). Regardless of the cause, the initial seizure begins a series of structural and physiological changes that increase the susceptibility for subsequent seizures (Gowers, 1902). This process is called epileptogenesis and has had multiple definitions. One concept is that epileptogenesis is limited to the latent period between an initial seizure event and the incidence of the first spontaneous seizure. Recently, this notion has given way to the hypothesis that epileptogenesis is a continuous process that endures beyond the first epileptic seizure and contributes to progressively increasing the severity of epilepsy (Pitkänen et al., 2015). The development of chronic models of epilepsy has allowed the specific study of disease pathogenesis. Status epilepticus (SE) is sometimes associated with injuries and increases the risk of epileptogenesis beginning. SE is defined by a period of continuous or near-continuous seizures activity lasting anywhere from several minutes up to several hours. Induced SE models for acquired epilepsy include administration of chemoconvulsants (e.g., kainic acid, pilocarpine, bicuculline) or kindling with repeated electrical stimulation (Reddy & Kuruba, 2013). These research models generally exhibit neuropathologies that are similar to human TLE, including hippocampal sclerosis and focal or focal to bilateral tonic-clonic (secondary generalized) seizures (Bouilleret et al., 1999; Cavazos et al., 1994; Curia et al., 2008).

Temporal lobe epilepsy is one of the most common and difficult to treat of epilepsies. Multiple pharmaceutical and surgical methods exist as treatment, though

limited candidacy for surgical intervention and resistance to anti-seizure medications have outlined a need for alternative treatment (Téllez-Zenteno & Hernández-Ronquillo, 2012). Thus, interest in antiepileptogenic treatments has begun to grow, following our growing knowledge of the mechanisms of epileptogenesis to design treatments beyond seizure control to counter underlying conditions of epilepsy. Some target pathologies that have been identified for such treatment include circuit and synaptic reorganization, angiogenesis, intrinsic properties of neurons, inflammation, and gene expression. Throughout this introduction, I aim to outline how many of these mechanisms are influenced by vigilance state (e.g., awake, sleeping) or circadian rhythms and likely modify disease progression in epilepsy.

It has long been appreciated that basal sleep patterns are disrupted in people with most forms of epilepsy and that sleep loss is one of the most commonly reported triggers for seizures in people with otherwise well-controlled epilepsy (Foldvary-Schaefer & Grigg-Damberger, 2009). Recently, there has been growing evidence that they are closely interconnected processes and that disruptions in sleep and seizure susceptibility are locked in a vicious, deleterious cycle that is driving the development and/or progression of epilepsy. Studies investing the effect of promoting sleep has on epileptic seizures have revealed promising evidence for its therapeutic benefit (Fenoglio-Simeone et al., 2009; Roundtree et al., 2016). It is unknown which aspect of the cycle precedes the other, but regardless of the onset of sleep disruption, the effects of sleep dysregulation may be a nexus of control over some of the pathological changes to anatomy and physiology that are associated with epilepsy.

<u>Sleep</u>

Sleep is a conserved phenomenon from nematodes to humans. Much of our time is spent in this unconscious state when we are vulnerable to our environment, however the purpose and benefits of sleep far outweigh the prolonged susceptibility. Sleep creates the opportunity for your body and brain to complete tasks that it would otherwise be unable to complete. In the context of memory, sleep is critical for the conversion of fragile short-term memory into networks of neurons that can be called upon years later merely by seeing a familiar building or smelling a familiar fragrance. In the context of energy, sleep allows recuperation of energy stores spent during the waking period. But what makes you sleep? Numerous factors have been associated with the control of when you sleep and when you wake, but sleep homeostasis and circadian rhythms are the most influential.

Vigilance states and sleep scoring:

Sleep may seem to be simply an extended stint of unconsciousness; however, it is a time rife with physiological activity. Sleep itself is divided into multiple stages, each with a unique function. Individual sleep stages can be distinguished by electrical field activity at the skull, recorded as an electroencephalograph (EEG). In rodents commonly used to research sleep, sleep is divided into two vigilance states: non-rapid eye movement (NREM) and rapid eye movement (REM) states. Analyzing EEG of each vigilance state with a fast-Fourier transformation (FFT), the resulting discrete Fourier transform reveals each state contains a unique distribution of constituent frequency components, or power (Tukey & Cooley, 1965). EEG recordings during NREM sleep are dominated by relatively high amplitude, slow-wave activity as shown in Figure 1A, and FFT reveals relatively high

power of low (Delta, 1-4 Hz, Figure 1B) frequencies. The EEG waveforms during REM sleep (as seen in Figure 1C) are quite similar to waking EEG by appearance and are often associated with energy demands and neural activity akin to wakefulness. REM sleep EEG consists predominantly of low amplitude, rhythmic activity with peak power in the theta band, (5-9 Hz, Figure 1D). These general characteristics of EEG amplitude and frequency composition are used for determining vigilance states. Sleep scoring is often achieved by manual inspection of EEG recordings of frontal cortical activity coupled with muscle movement activity from electromyography (EMG) electrodes placed in the neck muscles. Several efforts have been made to circumvent this otherwise time-intensive process by creating an algorithm that can quickly and consistently determine vigilance states and support homogeneous scoring criteria that are often quite variable between labs. Current progress in clinical and basic science efforts can be reviewed in (Bastianini et al., 2015) and (Fiorillo et al., 2019).

Sleep scoring allows for subsequent analysis of sleep architecture. Discrete periods of wakefulness, NREM or REM sleep are termed bouts, and characterization of these bouts based on metrics of frequency, duration, and timing relative to environmental lighting condition is used to indicate the presence or absence of various sleep regulators. An example in mice is the consolidation of wakefulness to times of dark lighting conditions, a hallmark of a nocturnal species. This behavior is the manifestation of circadian regulators, which will be discussed in greater detail later in the introduction.
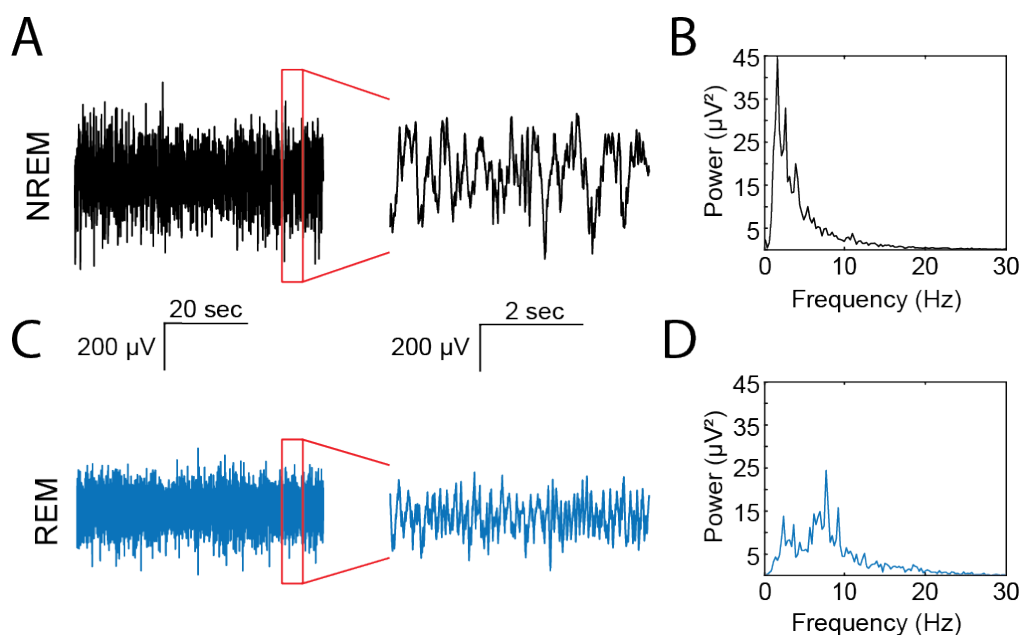
Figure 1: Power spectral analysis of EEG in sleep.

Sleep scoring is based on the unique cortical EEG patterns of each vigilance state. NREM sleep is determined by high amplitude, low-frequency activity (A), which can be appreciated in the power spectral density plot (B) which is the one-sided square magnitude of the Fourier transform of the EEG waveform across the frequency spectrum. High delta (1-4 Hz) power is indicative of slows-wave NREM sleep. REM EEG is significantly lower in overall amplitude (C), and the PSD shows peak power in the theta (5-9 Hz) frequency band.

Sleep and the autonomic nervous system:

The autonomic nervous system (ANS) is intimately associated with sleep and consists of three elements: the sympathetic (SNS), parasympathetic (PNS), and enteric (ENS) nervous systems (Zoccoli & Amici, 2020). The general physiologic response following activation of the SNS and PNS oppose each other and form the center of visceral homeostasis. Colloquially, SNS activity is referred to as the "fight or flight"

response because it favors arousal and increases such things as cardiorespiratory function, vasodilation, body temperature. Conversely, the PNS is known as the "rest and digest" response. At the transition from wake to NREM, the autonomic system shifts to favor PNS activity. PNS activation allows for homeostatic control of physiological functions: the heart rate slows, blood pressure decreases, respiration slows and becomes more regular, metabolism and metabolic demand decreases (Katayose et al., 2009), the immune system is suppressed (Kin, 2006), and body temperature decreases (Katayose et al., 2009). In addition to being responsive to sleep state, several of these systems may reciprocally affect sleep induction as well.

The activity of the immune system requires precise regulation to mount an appropriate response to infection. Too little activation leads to an immunocompromised state and ineffective control of infections. Too much activation can lead to excessive collateral damage in the process of clearing infection and even autoimmunity. Elevated basal immune activation can be seen in chronic diseases such as rheumatoid arthritis, Crohn's disease (Breit et al., 2018), atherosclerosis, Alzheimer's disease, and epilepsy (Aronica & Crino, 2011a; Vezzani et al., 2016). Acetylcholinergic projections of the Vagus nerve are most associated with parasympathetic control of cardiac, respiratory, and gastrointestinal function. Stimulation of this nerve has been shown to decrease levels of pro-inflammatory cytokine Tumor Necrosis Factor (TNF) following an endotoxin challenge, and central neural activity induced by elevated inflammation acts as a fast-acting inflammatory reflex of the central nervous system (Kin, 2006; Kobrzycka et al., 2019). The output of this inflammatory reflex results in the activation of nicotinic acetylcholine receptors on macrophages which reduces the production of pro-

inflammatory molecules TNF, interleukin (IL)-1β, IL-6, and IL-18 while leaving the expression of the anti-inflammatory cytokine IL-10 unaffected (Borovikova et al., 2000). NREM sleep upregulation of parasympathetic activity would also serve to suppress immune activation by this very mechanism. Relative to sleep regulation, it has been shown that TNF signaling negatively regulates transcription of wake-promoting neurotransmitter orexin/hypocretin, whereas TNF receptor knock-out mouse models have elevated orexin transcripts and result in less spontaneous sleep and an impaired sleep response to infection-induced systemic inflammation (Kapás, 2008). Promotion of sleep by TNF and sleep-associated reduction in TNF expression suggests that anti-inflammatory effects of sleep may be a part of sleep regulation that can be co-opted by inflammatory conditions, including seizures.

Increasing evidence demonstrates that epilepsy also has a reciprocal relationship with inflammation. Surgically resected tissue from patients with multiple drug-resistant types of epilepsy has shown elevated activation of innate and adaptive immune systems (Aronica & Crino, 2011b; Vezzani et al., 2011). Conversely, elevated inflammatory states have been implicated in seizure susceptibility, as the administration of anti-inflammatory treatments (e.g., steroids and adrenocorticotropic hormone (ACTH)) was relatively effective in controlling seizures in some intractable pediatric epilepsies (Nasiri et al., 2017; Verhelst et al., 2005). While it is unknown to what extent sleep influences immune activity, it is conceivable that sleep dysregulation seen in epilepsy may be a factor contributing to elevated immune activation.

Patterns of EEG and CSF flow during sleep:

As we see with the interplay with the autonomic nervous system, sleep provides an opportunity for many beneficial cellular and system-level processes, and maintenance of the brain's environment is no small aspect of it. The central nervous system has a unique method of handling the exchange of nutrients, oxygen, and metabolic byproducts. Typically, access to the CNS is privileged and guarded by a blood-brain barrier (BBB). Within the CNS nutrients and oxygen are delivered via diffusion through interstitial fluid (ISF), and potentially toxic byproducts are cleared by ISF and the flow of cerebrospinal fluid (CSF). CSF is produced in the lateral ventricles of the brain by the choroid plexus and flows around the brain, ultimately drained through paths adjacent to several cranial and spinal nerves (e.g., the olfactory nerve through the cribriform plate and the optic nerve), exchanged into the venous sinus through the arachnoid villi, or through the ISF within perivascular spaces (i.e., the glymphatic system) (Cserr et al., 1992; Liddelow, 2015; Wardlaw et al., 2020). ISF and CSF are also the medium of endocrine signaling, and as such, tight regulation of its content is key. The flow of CSF is a dynamic feature and has been found to be upregulated during sleep. Cortical EEG and multi-unit activity recordings of cortical neurons have shown unique patterns of electrical activity within sleep states (Nelson et al., 2013). Non-rapid eye movement (NREM) sleep is associated with high amplitude cortical EEG that is predominated by slow-wave activity (delta, 0.5-4 Hz) (Nelson et al., 2013). Underlying the pattern seen in cortical EEG are on-off periods of individual neurons, where neurons tend to have rhythmic patterns of consolidated activity that define "up" states in EEG voltages. (Nir et al., 2011). Sleep states are also associated with an elevated flow of cerebrospinal fluid, which clears potentially harmful

waste metabolites from the brain (Cavus et al., 2005). The mechanisms behind this elevated flow may be the very rhythmic on-off states associated with NREM sleep creating pulses of filtration from the cerebral vasculature. Neuronal function relies heavily on oxygen and nutrients which are provided by a very finely tuned neurovascular unit rapidly responding to fluctuations of neuronal activity (Cavus et al., 2005). CSF flow and interstitial volume are influenced by the ebb and flow of the vascular filtration. In research published by Fultz et al. (2019), it was shown that slow-wave (delta) activity of surface EEG, fMRI bold signal of cerebral blood flow, and CSF flow through the fourth ventricle were correlated with each other and increased during NREM sleep. They found that cortical blood and CSF flow were closely coordinated, both being elevated during NREM sleep when compared to waking states and correlated to up-states of slow-wave (0.5-4 Hz) activity in cortical EEG. Clearing the brain of metabolites and ambient signaling molecules is likely an important factor in the rejuvenating effects of sleep.

Seizures are known to increase extracellular concentrations of neurotransmitter, as would follow the high level of neuronal activity and associated neurotransmitter release during widespread and synchronous activity inherent to seizures. Elevated CSF concentration of the excitatory neurotransmitter glutamate has been reported in seizure foci of patients with TLE, while concentrations of inhibitory GABA were unchanged (Cavus et al., 2005; Çavuş et al., 2016). A similar study conducted in rodents demonstrated that epileptic animals exhibited extracellular glutamate concentrations that oscillated with a circadian rhythm that peaked during the waking period, whereas controls did not (Sandhu et al., 2020). Interestingly, they showed no increase in mean glutamate concentrations between epileptic and control groups, lending the conclusion that impaired CSF turnover

could be the cause. The integrity of the neurovascular unit is emphasized when considering the etiology of epilepsy diagnosed in elderly patients, among whom neurovascular disease is the most common contributor to epileptiform activity (Ramsay et al., 2004). Conceptually, acute decreases in filtration would stagnate CSF flow and lead to abnormally high concentrations of neurotransmitters and toxic metabolites, while chronic effects could lead to damage of neural tissue. So, the possibility exists that deteriorating sleep patterns in epileptic patients affect CSF flow, neurotransmitter clearance, and exacerbate extrasynaptic aggregation of glutamate and associated seizure susceptibility. With that in mind, therapies that successfully improve sleep quality could be viable options for the treatment of neurological disease of hyperexcitability, particularly epilepsy.

Mechanisms of sleep regulation

We are beginning to appreciate the numerous roles that sleep has in maintaining healthy central and peripheral physiology. Just as sleep influences the physiology of the body, sleep itself is regulated by multiple elements, both internal and external, which work synergistically to coordinate behavior and combat the effects of extended wakefulness. Two primary elements of sleep regulation are circadian rhythms and sleep homeostasis, which provide a preference and pressure, respectively, for sleep timing (Borbély et al., 2016). In this section, I aim to provide an overview of the research into these two main sleep regulators.

Sleep Preference and the circadian pacemaker:

Perhaps chief among the factors regulating sleep preference is entrainment to external lighting conditions. For most vertebrates on earth, this entrainment drives a host of physiological processes in time with a circadian rhythm set by the sun. Circadian is derived from Latin and refers to any process that oscillates with a period lasting about (circa) a day (diem). Synchronizing internal biology with external cues has been observed in a wide range of species and levels of sentience and is an important feature of circadian controllers, however, it is not the defining characteristic. Circadian controllers can maintain the timing of internal processes even in the absence of external cues, or zeitgebers, which translated from German means 'time giver'. This section will introduce core concepts of the central circadian pacemaker, as well as discuss some relevant and potent modifiers of what is often referred to as the circadian Clock.

Core regulatory molecules of the circadian Clock:

At the center of circadian rhythms is a pacemaker mediated by a transcription-translation feedback loop (TTFL) of circadian Clock proteins (see schematic Figure 2). The first genes to be characterized and cloned were in the common fruit fly, *drosophila melanogaster*. There, a positive transcription factor, consisting of CLOCK and CYCLE proteins, promotes the transcription of the transcriptional repressors of the feedback loop: period (PER) and timeless (TIM). Upon translation and heterodimerization, the PER:TIM complex is translocated back into the nucleus where it represses transcription of Clock controlled genes (Dubowy & Sehgal, 2017). In mammalian cells, homologs of these

genes encoding circadian Clock proteins have been subsequently identified. Positive elements of the mammalian circadian Clock are the CLOCK and brain-muscle arnt like 1 (BMAL1) proteins, while the transcriptional repressors of the core pacemaker are period (PER1-3) and cryptochrome (CRY1/2) proteins. As shown in the schematic in Figure 2, transcription of *Per* and *Cry* is promoted by the heterodimeric basic-helix-loop-helix (bHLH) transcription factor CLOCK:BMAL1 binding the E-BOX enhancer and is followed by translation into proteins outside of the nucleus. Once translated and phosphorylated, dimers (PER:PER or PER:CRY) return to the nucleus where they mediate repression of circadian Clock-controlled genes. In the absence of zeitgebers, circadian rhythms enter a free-running state, where the cycle of transcription and translation would continue indefinitely, though period and amplitude of oscillations of expression may change without persistent entraining cues. In rodents, the free-running period length of these cycles is slightly shorter than 24 hours (Johnson, 1926), and in humans, they are slightly longer than 24 hours (Pagani et al., 2010). Unsurprisingly, systematic deletion of genes encoding each of these putative mammalian Clock proteins has highlighted the capacity of this feedback loop to endure single loss-of-function mutation in most of the genes, except for *Bmal1*. Periodicity and cryptochrome each have redundant genes and single deletions fail to significantly affect circadian rhythms or timing of locomotor activity. The activity of CLOCK can be substituted in part by NPAS2, which has even been shown the preferred binding partner for BMAL1 in some peripheral tissues (Landgraf et al., 2016). The activity of the circadian Clock is mediated by post-translational modifications on its constituent proteins, among which BMAL1 seems to be a nexus for the regulation of CLOCK:BMAL1 promotion of Clock-controlled genes.
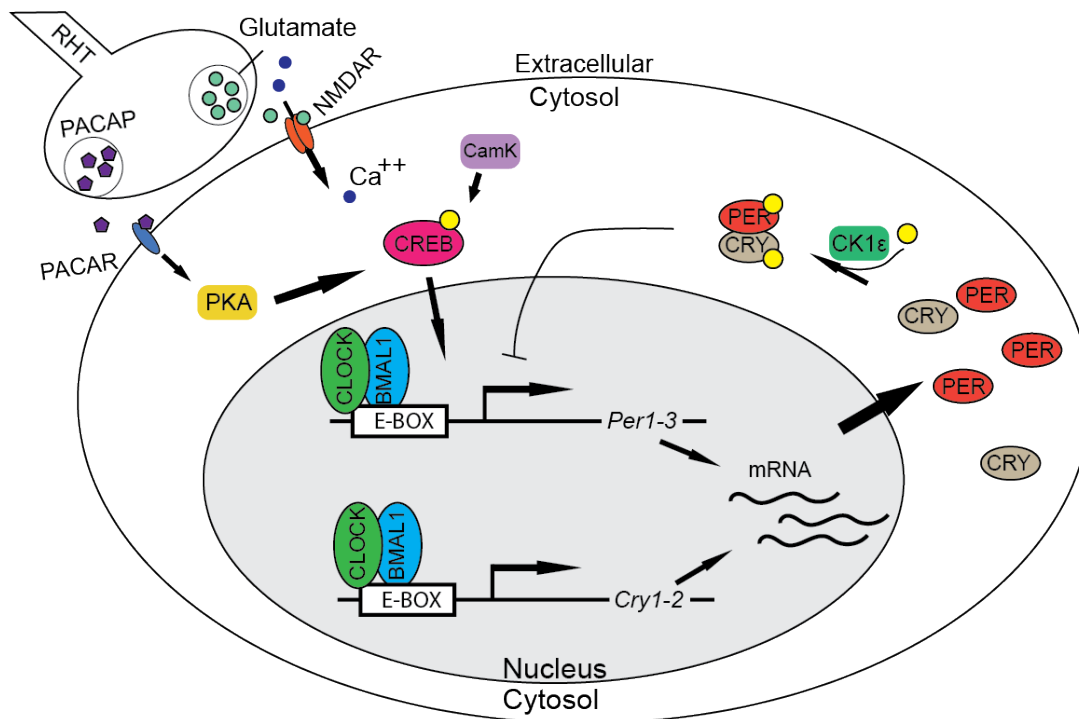
Figure 2: Schematic of the molecular mechanisms of the core circadian pacemaker.

The circadian Clock in the SCN is capable of autoregulation: CLOCK-BMAL1 heterodimers drive expression of PER1-3 and CRY1-2, which form repressive complexes after they are phosphorylated (yellow circles) by casein kinase1-epsilon (CK1ε). In light-entrained conditions, periodicity and cryptochrome expression peaks during the light-on period, reinforced by innervation from excitatory projections of the retinohypothalamic tract (RHT). Exposure to light causes the release of pituitary adenylate cyclase-activating peptide (PACAP) and glutamate from RHT terminals, activating CREB through AC/PKA and calcium/calmodulin-modulated kinase (CamKs) pathways, respectively. Phosphorylated CREB then enters the nucleus to repress the transcription of Clock-controlled genes.

BMAL1 – A hub of circadian modulation:

Circadian oscillations in the transcription of Clock-controlled genes rely heavily on

expression, post-translational modification, and protein-protein interactions of BMAL1.

Transcriptional efficiency and oscillation of Clock-controlled genes are at their greatest when BMAL1 binds with CLOCK, which is followed by the acetylation of BMAL1 at a highly conserved lysine 537 (Ac-K537), mediated by the acetyltransferase activity of its primary binding partner, CLOCK (Hirayama et al., 2007). While the ability of CLOCK:BMAL1 to bind DNA is maintained when acetylation of BMAL1 is blocked by site-directed mutation of lysine 537 to arginine (BMAL1$_{K537R}$), circadian rhythmicity of target genes is ablated. This suggests that the acetylation of BMAL1 is a key step in normal mechanisms controlling circadian transcription. Further study has demonstrated that BMAL1 also undergoes phosphorylation and SUMOylation events, each having the effect of decreasing protein half-life and expediting proteasome-mediated degradation (Cardone, 2005; Kondratov et al., 2003). The phosphorylation of BMAL1 has been shown to decrease its stability and depends on heterodimerizing with CLOCK (Cardone, 2005). Research into the phospho-accepting sites in BMAL1 identified GSK3β as the kinase responsible for the phosphorylation of serine-17 and threonine-21 residues (Sahar et al., 2010). Phosphorylation of BMAL1 S17/T21 upregulates ubiquitination and subsequent proteasome degradation. Like the ultimate ubiquitination following phosphorylation, the addition of a small ubiquitin-like modifier protein (SUMO) to lysine 259 decreases the lifespan of BMAL1 (Kondratov et al., 2003). Opposing the acetyltransferase activity of CLOCK, the histone deacetylase (HDAC) Sirtuin1 (SIRT1) catalyzes the deacetylation BMAL1-K537 (Nakahata et al., 2009). Sirt1 nuclear interaction with circadian regulatory machinery oscillates with a circadian rhythm that is antiphase to those of the acetylation of BMAL1 and histones at promoter regions of Clock-controlled genes (Asher et al., 2008; Nakahata et al., 2009). Co-immunoprecipitation assay of SIRT1 protein interactions

confirmed that direct association with CLOCK protein occurs, which follows that same circadian rhythm of nuclear presence. Sirt1 HDAC activity is dependent on NAD$^+$ (nicotinamide adenine dinucleotide), which offers a plausible link between the metabolic state of the cell and regulation of Clock-controlled genes (Bordone & Guarente, 2005).

Further evidence of the crosstalk between circadian controllers and cellular metabolism is the involvement of an accessory regulatory loop consisting of the heme sensing enzyme REV-ERBα. REV-ERBα transcription is promoted by the CLOCK:BMAL1 transcription factor. In turn, REV-ERBα negatively regulates BMAL1 expression, an activity that depends upon its coenzyme heme, which can inform the circadian clock of the oxidative metabolism in the cell (Yin et al., 2007). This mechanism is particularly evident in liver cells, where REV-ERBα and positive Clock proteins modulate PGC-1α expression, exerting negative and positive transcriptional control, respectively. PGC-1α is a transcriptional coactivator that is closely associated with lipid metabolism and gluconeogenesis (Yoon et al., 2001). Interestingly, PGC-1α seems to influence circadian rhythms of peripheral tissues *in vitro* where overexpression of PGC-1α leads to increased expression of CLOCK and BMAL1 (Liu et al., 2007). The effect of circadian Clock control in metabolism has been underscored by a study in which BMAL1 deletion led to a loss of glucose homeostasis (Rudic et al., 2004).

Indeed, BMAL1 is critical to circadian rhythms, and the importance of post-translational modifications is evident by reports that circadian rhythmicity persists regardless of BMAL1 expression pattern. Likely owing to the many mechanisms exerting control over BMAL1 activity as a transcription factor, circadian BMAL1 expression is not required for core Clock function, as it persists even when BMAL1 is constitutively

expressed (A. C. Liu et al., 2008). While there are numerous post-translational events that regulate the proteins of the core Clock, its effect on transcription is mediated by another system, chromatin modification.

Circadian regulation of chromatin structure:

The ultimate mechanism underlying transcriptional control is nucleosome modification, which is an intricate and complex system, the details of which are extensively reviewed by (McGinty & Tan, 2015) and (Bowman & Poirier, 2015). Briefly, nucleosomes are complexes of 8 basic proteins called histones (2 pairs of histones 2a and b, 3, and 4). Each histone subunit has a string of amino acids that extends out of the main octamer complex that is subject to post-translational modifications (e.g., acetylation, methylation, phosphorylation). Each modification works with varying effects to modify electrostatic interactions between adjacent nucleosomes and the chromatin (DNA) itself. A loose chromatin structure is referred to as euchromatin, and the loosened nucleosome-DNA interaction in euchromatin allows transcriptional machinery to access the DNA and is therefore associated with the activation of local genes. Conversely, sections of the genome that are tightly compacted are called heterochromatin, and transcription of genes isolated in this heterochromatic structure is repressed. Collectively, the dynamic post-translational modification of histones and associated changes in chromatin density constitute epigenetic regulation of gene expression. This is exactly what allows the body to make an immense variety of cell types, despite each one having the same DNA sequence (or hopefully very nearly the same sequence!). Within epigenetics, classes of enzymes that modify histone tails are called 'writers', whereas 'reader' proteins selectively

bind these post-translational modifications and recruit proteins or protein complexes with varying end functions.

In the context of circadian rhythms, the CLOCK:BMAL1 transcription factor mediates modification of histone tails. Modifications mediated by the circadian Clock are acetylation of Lysine 9 and 27 (H3K9-Ac and H3K27-Ac) of histone 3 (Koike et al., 2012). In addition to CLOCK-mediated acetylation of BMAL1, CLOCK has been shown to possesses limited histone acetyltransferase capabilities. In a study published by (Doi et al., 2006), an acetyl-CoA binding site, a putative indicator of histone acetyltransferases, was identified in CLOCK. They reported that the purified mCLOCK was indeed capable of acetylating histones. Transcriptional activation by CLOCK:BMAL1 alone has been reported before when mCLOCK and mArnt3 (a.k.a. BMAL1) were co-expressed in HEK-293 cells and measured with a *mPer1*:luciferase assay, though that activity was only marginally above background noise (Takahata et al., 2000). Takahata et al. and others since have shown that the transcriptional co-activators CBP and P300 are much more effective activators of circadian transcription (J. Etchegaray et al., 2003; Takahata et al., 2000).

Transcriptional repression is mediated by members of the negative limb of the circadian Clock: period and cryptochrome, as well as REV-ERBα. Once translated period and cryptochrome proteins are phosphorylated by casein kinase 1 epsilon and delta (CK1-ε and δ) (Meng et al., 2008), which stabilizes the protein. As phosphorylated PER and CRY accumulate, they colocalize with the CLOCK:BMAL1 transcription factor and recruit transcriptional co-repressors. In research published in 2011 by Duong et al, protein complexes containing period proteins were evaluated to determine affiliated co-

repressors capable of mediating the negative feedback of the TTFL. Using protein co-immunoprecipitation with antibodies specific for PER1 and PER2, they demonstrated that Sin3a and HDAC1 were in complex with PER1 and PER2, which was dependent on the presence of a chaperone protein PSF (polypyrimidine trace-binding protein-associated splicing factor) (Duong et al., 2011). They went on to test the occupancy of this complex at the *mPer1* promoter region and found that PER2, PSF, SIN3a, and HDAC1 had synchronous occupancy patterns across the 24-hour light-dark cycle, demonstrating that they were indeed co-localizing. In systems where PSF protein expression was inhibited with shRNA, repressor complex occupancy near the *Per1* E-box enhancer region was significantly reduced while histone acetylation (H3K9 and H3K27) levels were increased, results that mirrored DNA binding and histone acetylation patterns in *Per1/2* double knock-out mice. Destabilization of PERx/PSF/SIN3a/HDAC1 has the effect of truncating the period of the circadian Clock, which indicates that, while this complex is involved with circadian histone deacetylation, it is not the only mechanism at play in circadian transcriptional repressors.

Another HDAC studied for its effect on circadian histone acetylation in association with the circadian Clock is Sirtuin1 (SIRT1). SIRT1 is a class-III histone deacetylase whose activity is dependent on NAD^+. SIRT1 complexes with PER/CRY and mediates deacetylation of histone 3, as well as of BMAL1. The role that SIRT1 plays in transcriptional repression of Clock-controlled genes is perhaps more important than SIN3a/HDAC1, as demonstrated by Nakahata *et al*. In their 2008 study, deletion of *Sirt1* significantly ablates circadian oscillations as shown in luciferase reporter assays driven by the mPer1 promoter sequence (Nakahata et al., 2008). The relative influence of SIRT1

on circadian transcriptional control coupled with its sensitivity to metabolic activity through its NAD$^+$-dependence suggests that SIRT1 mediates the integration of the metabolic state into sleep patterns (Bordone & Guarente, 2005). Additionally, Sirt1 activity has been shown to decline with aging and is a likely cause of deteriorating in the strength of circadian sleep patterns (Braidy et al., 2011).

In addition to histone acetyltransferases, repression of circadian transcription includes a methyltransferase. Enhancer of Zeste homolog 2 (EZH2) is the catalytic element of Polycomb Repressor Complex 2 (PRC2). EZH2-mediated trimethylation of histone 3 at Lysine 27 (H3Kme3) acts to promote heterochromatin formation and, in concert with PRC1, repression of local genes (Erhardt et al., 2003). The methylation must be preceded by deacetylation, likely mediated by one of the above HDAC-containing complexes. Some evidence exists to indicate that polycomb-group (PcG) protein complexes may contain HDAC1 or HDAC2, in which case they may be self-sufficient to mediate the H3K27Ac-H3K27me3 transition. Characterization of the enzymatic components of polycomb-group (PcG) complexes was reported by van der Vlag & Otte (1999), where embryonic ectoderm development (EED) protein co-immunoprecipitation studies demonstrated that HDAC1 and HDAC2 participated in a subset of EED/EZH2-containing complexes (van der Vlag & Otte, 1999). PRC2 is most associated with coordinated expression of genes during early development and stability of the epigenome during cell replication (Laugesen & Helin, 2014), but it has been reported that that PRC2 also interacts with cryptochrome, suggesting that PRC2 also plays a role in transcriptional repression of the circadian Clock. In a study published by J. P. Etchegaray et al. (2006), EZH2-mediated repression of Clock-controlled genes was tested with an

mPer1:Luciferase reporter assay. Etchegaray demonstrated that EZH2-mediated repression of Luciferase activity required the presence of CRY1 or CRY2, and responded in a CRY-concentration dependent manner.

The integration of the members and co-factors of the circadian Clock can be seen in every organ and results in the circadian rhythm of up to 15% of actively expressed genes (Zhang et al., 2014). While these rhythms can be sustained independently in each peripheral system, their activity must be synchronized for effective function as a system. This function requires a central circadian Clock capable of surveilling and integrating cues from the internal and external environments. In vertebrates, this responsibility falls on the Suprachiasmatic nucleus.

The Suprachiasmatic nucleus – The master circadian pacemaker:

Every organ has the capability to drive an internal circadian pacemaker, but without synchrony, this ability would mean very little for the physiological stability of the system. The maintenance of synchronous circadian rhythms among all tissues is mediated by the suprachiasmatic nucleus (SCN). Neurons within SCN are largely GABAergic and further anatomical subdivisions can be differentiated by expression of neuropeptides including vasoactive intestinal peptide (VIP), gastrin-releasing peptide (GRP), and vasopressin peptide (VP), which generally define ventrolateral (core), central, and dorsomedial (shell) subregions of the SCN, respectively (Morin, 2013). RHT projections into the SCN primarily target VIPergic neurons of the SCN core, but also sparsely innervate other regions (Diego Carlos Fernandez et al., 2016; Morin, 2013). Local signaling increases the

synchrony of neuronal activity within the SCN and is mediated by the paracrine release of neuropeptides and GABA (Morin, 2013). Axon tracing experiments have shown multiple targets of SCN within the central nervous system (CNS), of which several are known regulators of sleep (i.e., ventrolateral preoptic nucleus (VLPO) and lateral hypothalamus (LH)). The SCN also communicates with peripheral systems through endocrine signaling pathways as demonstrated by the ability of parabiosis to coordinate peripheral circadian Clocks in SCN lesioned animals (Guo et al., 2005).

The electrical code of circadian rhythms:

Neuronal activity is mediated by electrical transduction. Each neuron receives both excitatory and inhibitory signals onto its dendrites and cell body. These signals mediate fast and slow mechanisms to ultimately control the flow of ions into (influx) or out of (efflux) the cell. These currents have the effect of changing the voltage across the cell surface (V_m, membrane voltage), which is constantly maintained actively with ion pumps and passively with leak channels. When the membrane voltage is elevated (depolarized) enough, it reaches an action potential (AP) threshold and begins a cascade effect on ion channels that are sensitive to V_m. The AP itself is primarily initiated in the cell body or soma of the neuron by a high concentration of voltage-gated Sodium channels at the axon initial segment, which allow positively charged sodium cations (Na^+) to rapidly enter the neuron and further depolarize the cell. As the membrane depolarizes, voltage-gated potassium channels are activated which results in the efflux of positively charged ions (K+), which rapidly lower the membrane potential and terminate the AP. This is referred to as an All-or-Nothing response and once initiated, is propagated down the axon until it

activates voltage-gated calcium channels at synapses which mediate the release of neurotransmitter.

Much of the control over APs is mediated by the V_m responsiveness of the cell to the flow of ions across its membrane. Following Ohm's law (voltage=current*resistance, V=IR), changes in membrane resistance alter the voltage change of the cell membrane to any given current. In electrically active cells, membrane resistance is influenced by the number and properties of ion channels in the membrane, more channels lead to lower R_m and less V_m change per unit of current.

The activity of a neuron can be measured indirectly by recording the fast-changing ion concentrations in the exterior of the cell, which relate to the interior voltage change and are called local field potentials (LFPs). With this technique, it is possible to measure how frequently groups of neurons generate (or "fire") APs or "spikes". In light entrained mice, LFP recordings have demonstrated that the spiking frequency of SCN neurons is at its highest when lights were on, and lowest during periods of darkness (Nakamura et al., 2008). This oscillation in spike frequency is truly circadian, as it persists even in the absence of external lighting cues *in vivo* (Jones et al., 2015). The fluctuations in firing rates are mediated by circadian regulation of ion channel function and expression in the membrane and are well summarized by (Kuhlman & McMahon, 2006). Briefly, they describe active roles for currents mediated by L-type voltage-gated calcium channels (VGCCs), Fast-delayed rectifier K^+ channels, and calcium (Ca^{2+}) activated big K^+ (BK) channels. Periods of high-frequency spiking are associated with increased L-type VGGC conductance, which increases the probability of firing an action potential, and elevated expression of FDR channels, which terminate AP by rapidly repolarizing the neuron,

priming it for subsequent APs. During periods of low spike frequency, BK channels are expressed in the membrane at higher levels, replacing the role of FDR currents to terminate the AP. BK channels are active longer than FDR channels and increase the amount of time necessary for the neuron to depolarize to AP threshold, thereby slowing the frequency of AP generation. When ion channels are activated, the currents they mediate can be determined with the equation $I = G(V_m - V_{eq})$, where I is the current, G is the conductance of the channel, and $(V_m - V_{eq})$ is the driving force, which is the difference between V_m and the equilibrium potential (V_{eq}) of the ion to which the channel is permeable. In the case of potassium, the equilibrium potential of K^+ $(V_{eq,K})$ in mammalian neurons is -95 to -90 mV (Xu, 2013) and currents through any K^+ channel will drive the V_m toward $V_{eq,K}$ and the size of that current depends on the difference between V_m and $V_{eq,K}$. In the context of circadian rhythms in SCN neurons, high-frequency spiking periods are associated with low basal K^+ currents, which depolarize the cell away from $V_{eq,K}$, and is likely associated with lower expression of K^+ channels in the membrane, which would increase R_m and the V_m responsiveness of the cell. Conversely, increased basal K^+ currents hyperpolarize the V_m and decrease R_m, contributing to lower frequency firing rates.

While this pattern is circadian and capable of self-regulation via Clock-controlled expression of ion channels, its pattern of spiking is supported and enhanced by the light-activated release of the excitatory neurotransmitter glutamate by the RHT. The role of RHT activity in the entrainment of circadian rhythms was confirmed in a study by Jones et al. (2015), in which they mimicked the activity of the RHT briefly during periods of darkness by optogenetically stimulating the SCN of rodents held in constant darkness.

The onset of behavioral activity was successfully re-entrained to coincide with the timing of the optogenetic stimulation (1-hour duration, 8 Hz stim). In *in vitro* experiments, they showed that the entrainment of SCN firing patterns was dependent on action potentials and VIPergic signaling, further outlining the role of VIP in local network signaling and SCN synchrony (Jones et al., 2015).

Normally, the circadian molecular Clock is regulated by calcium-dependent signaling (O'Neill et al., 2008), as shown in the basic schematic in Figure 2. The RHT primarily releases glutamate onto SCN neurons, which supports light-specific increases in electrical activity and associated intracellular calcium concentrations. In both light-entrained and free-running conditions, the rhythmic influx of calcium increases the activity of Ca^{2+}/calmodulin-dependent protein kinase (CAMK) which also upregulates the phosphorylation of CREB (O'Neill et al., 2008). The phosphorylated CREB then binds to promoter regions of *Per1* and *Per2*, enhancing their transcription and entraining the phase of the rhythm of the circadian Clock. Less frequently, RHT terminal release pituitary adenylate cyclase-activating protein (PACAP) onto SCN neurons, which contributes to CREB promotion by increasing protein kinase (PK)A and PKC enzymatic activity and enhancing calcium-permeable ion channels, including NMDA glutamate receptors and L-type VGGCs. In sum, much of the circadian Clock involves calcium homeostasis as mediated by spiking frequency and Ca^{2+}-permeable ion channels, so any factor that affects cell firing rate in the SCN would extend to influence circadian rhythms. Rhythms of SCN activity relative to lighting conditions are conserved across species (Shuboni et al., 2015). However, the integration of SCN output within connected nuclei is divergent between diurnal and nocturnal animals, enabled by unique responses to RHT activity.

The SCN output and divergent chronotypes:

There are several patterns of behavioral activity (chronotypes) that occur despite the consistent activity patterns of the SCN relative to environmental lighting. Neurons in the SCN are connected to multiple regions with multiple, bidirectional tracts, forming the extended circadian neural network. While the SCN is important for keeping light-entrained circadian rhythms, it has limited direct influence in controlling circadian rhythms. Using so-called 'masking' experiments, the response to brief exposure to light during otherwise dark periods has helped to identify multiple regions of the brain that also receive output from the SCN, including the intergeniculate nucleus (IGL), ventral sub-paraventricular zone (vSPZ), olivary pretectal nucleus (OPT), and lateral hypothalamus (LH) (Diego C Fernandez et al., 2016; Morin, 2013). Generation of chronotype (i.e. diurnal or nocturnal rhythms) is mediated by differential response to RHT innervation within these retino-recipient neurons in the extended circadian neural network. Studies in a species of diurnal rodent show that specific lesioning of LGH or the OPT cause a conversion from diurnal to nocturnal behavioral rest/wake pattern while lesioning the SCN alone stripped the circadian rhythms of locomotor behavior (Gall et al., 2013, 2016). These divergent chronotypes likely stem from differential RHT-mediated light responses as measured by the expression of the immediate early gene, cFos. In diurnal animals, RHT activation leads to increased cFos expression in IGL, vSPZ, and OPT. Conversely, the same light stimulus led to no change or reductions in cFos expression in the same regions of nocturnal brains. Despite the differential effects on behavior and cFos expression patterns in these other retino-recipient regions in diurnal and nocturnal systems, the response of SCN neurons was the same between diurnal and nocturnal rodents. (Gall et al., 2016;

Shuboni et al., 2015). These studies demonstrate a neural component that underlies the modification of SCN output in response to external lighting cues that contribute to unique chronotypes.

Hypothalamus-Pituitary-Adrenal axis and circadian rhythm synchrony:

Just as RHT activity can modify SCN output by modulating the extended circadian neural network, variable responses to SCN endocrine signaling influence synchrony of peripheral circadian rhythms. Using the same chronotype models, it has been demonstrated that endocrine signaling is differentially regulated in diurnal and nocturnal animals. One endocrine component of synchronization between SCN and peripheral Clocks is glucocorticoid signaling (Balsalobre et al., 2000). Adrenal corticosterone release is the result of a sequential release of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH), by neurons in the hypothalamus and pituitary, respectively, which form the hypothalamus-pituitary-adrenal (HPA) axis (Smith & Vale, 2006). The paraventricular nucleus (PVN) is the hypothalamic component of the HPA and receives SCN output via VP signaling and exhibits differential responses in diurnal and nocturnal animals, resulting in HPA activity that is promoted or inhibited, respectively (Kalsbeek et al., 2008). Inhibition of hypothalamic glucocorticoid receptor signaling leads to increased HPA axis activity, which demonstrates an autoregulatory capacity of glucocorticoid expression (Laryea et al., 2013). Glucocorticoid receptors are widely expressed and mediate a host of physiological responses including lipid and liver metabolism, immune system suppression, and modulation of neuronal function (Coutinho & Chapman, 2011; Garabedian et al., 2017). The implications of glucocorticoid levels on

peripheral circadian transcription rhythms are mediated by glucocorticoid response elements (GRE) regulating the expression of period genes and synchronizing peripheral Clocks in a manner also specific to chronotype (Dickmeis, 2009; Yamamoto et al., 2005).

Transforming growth factor-2 – A direct role of the SCN in rest-activity regulation:

Transforming growth factor-2 is the only peptide produced in the SCN that is known to affect locomotor activity directly. In a study published in 2001, Kramer et al. identified 32 neuropeptides that were secreted by isolated SCN neurons (Kramer et al., 2001). Of those peptides, TGF2 was the only one that had a significant effect on activity patterns when perfused into the third ventricle of mice. TGF2 is a ligand for the endothelial growth factor receptor (EGFR). Kramer et al. demonstrated the specificity of EGFR activation with complementary experiments in which either TGF2 or EGF were perfused for three weeks in animals held in constant darkness. Both EGFR ligands elicited a profound and suppressive effect on locomotor activity. They also reported that this effect was reversible and did not seem to significantly alter the circadian rhythms of the SCN. After cessation of EGFR activation, mice showed a resurgence of locomotor activity akin to the timing and levels that are seen in control mice perfused for the same amount of time with artificial cerebrospinal fluid and housed in constant darkness.

Subsequent study has revealed the role of glucocorticoids on EGFR signaling (Lauriola et al., 2014). This subjects TGF2 signaling to the activity of the HPA, which is differentially regulated in diurnal and nocturnal systems. This HPA-TGF2 signaling interaction outlines another method of circadian dysregulation in epilepsy in which

seizure-induced increases in HPA activity perturbs sleep-wake patterns and increases seizure susceptibility (O'Toole et al., 2014).

Sleep Homeostasis – A role of adenosine in sleep:

As mentioned, circadian control of sleep can be considered to exert a preference for sleep timing. In a study by Liu *et al.* published in 2012, the consolidation of sleep during lights-on periods was ablated in SCN-lesioned rats, while the proportion of time spent asleep (i.e., sleep efficiency) is unaltered (X. G. Liu et al., 2012). In these SCN-ablated animals, sleep regulation falls back on sleep homeostasis. Conceptually, sleep homeostasis is the response to sleep debt accrued during wakefulness and serves a restorative function for the neurons and glia in the CNS. EEG delta (1-4 Hz) power of slow-wave NREM sleep is high immediately following extended periods of wakefulness and decreases with time spent in NREM sleep. This feature of NREM EEG is regarded as a biomarker for sleep pressure, an idea that has been strengthened by sleep deprivation experiments in rodent models that show recovery NREM sleep following sleep deprivation is much elevated relative to the levels seen after normally occurring bouts of sleep (Nelson et al., 2013).

Underlying the characteristic EEG patterns of slow-wave sleep are periods of high spiking frequencies (Up-states) and periods of quiescence (Down-states) that oscillate at 1-4 Hz. Underlying these slow oscillations is the coordinated activity of interconnected networks in the thalamus and cortex. Coordinated slow thalamocortical network oscillations have been successfully reproduced in slice experiments by modulating

equilibrium potentials and activity of ion channels, the specifics of which extend beyond the scope of this introduction and are comprehensively reviewed by (Neske, 2016). However, one component that is thought to affect network oscillations is adenosine, which has been shown to regulate potassium conductances and truncate Up-state duration (Phillis et al., 1975). The effect of adenosine signaling on sleep is mediated by modifying the activity within sleep and wake-promoting regions in the mesopontine tegmentum, hypothalamus, and basal forebrain (Rainnie et al., 1994; Strecker et al., 2000; Toossi et al., 2016).

Adenosine is produced by the rapid breakdown of ATP that is released in synaptic vesicles in an activity-dependent manner. Prolonged states of elevated synaptic activity during wakefulness lead to the accrual of adenosine in the extracellular space (Dunwiddie et al., 1997). A microdialysis study examined extracellular adenosine in sleep-wake modulating regions of the brain and demonstrated that adenosine concentrations increase during periods of wakefulness and decrease during NREM sleep in the basal forebrain, though it was postulated that the relatively small size of other regions tested prevented detection of similar patterns (Porkka-Heiskanen et al., 2000). Adenosine is a ligand for a class of G-protein coupled receptors that consist of A_1, A_{2A}, $A_{2B,}$ and A_3 receptors. In the CNS, adenosine A_{2A} and A_1 are preferentially expressed and localize near synapses (Ballesteros-Yáñez et al., 2018). The effects of adenosine signaling on slow-wave sleep can be appreciated by the systemic modulation of adenosine receptors. One of the most familiar agents is caffeine, a nonselective antagonist of adenosine receptors. The acute effect of caffeine consumption is known to stave off fatigue and increase wakefulness, though caffeine also affects subsequent slow-wave sleep.

Specifically, caffeine causes a reduction in slow-wave sleep amplitude (Landolt et al., 1995). Conversely, potentiating adenosine A_1 signaling has been shown to increase the amount of SWS and increase NREM delta power, mimicking the increases observed following sleep restriction (Benington et al., 1995; Radulovacki et al., 1984). Additionally, in animals with targeted deletion of the gene encoding adenosine A_1 receptors (*Adora1*) in the brain exhibit impaired wake-dependent modulation of NREM delta power following sleep restriction, though time spent in NREM sleep is not significantly affected (Bjorness et al., 2009). In these *Adora1*-KO mice, working, but not episodic, memory performance was also impaired following sleep deprivation relative to *adora1*-WT mice. Therefore, the homeostatic response of NREM delta power following extended wakefulness is important for cognitive function and is mediated by adenosine A_1 receptor signaling. These studies underscore the role of adenosine in mediating compensatory modulations of slow-wave activity/NREM delta power in response to the amount of waking behavior that preceded it, highlighting its importance in sleep homeostasis, especially signaling mediated by adenosine A_1 receptors in mediating slow-wave sleep.

As introduced, circadian rhythms and sleep homeostasis are heavily influenced by electrical activity. As with the study of optogenetic re-entrainment of circadian behavioral rhythms, untimely electrical activity associated with epileptic discharges can upset normal circadian rhythms of the extended neural network and potentially mimic the activity of the RHT. Given the extensive interconnectivity of the SCN and relay nuclei (Morin, 2013), it is plausible that seizures can directly influence SCN entrainment. Circadian rhythmicity of seizures has long been described in most epilepsies (Gowers, 1902), an observation that hints at an intimate relationship between the circadian Clock and rhythmic seizure

susceptibility. Assessment of locomotor activity patterns following induced status epilepticus has demonstrated a progressive deterioration consolidation activity in mice (Matos et al., 2018). Additionally, kainate treatment caused a transient dysregulation in the transcription of several core Clock genes, including transient increases of *Cry1* and *Per1* in what would be considered the latent phase (~30 days post-SE), and a progressive decline in the transcription of *Cry2*, *Bmal1,* and *Clock* (Matos et al., 2018). This would suggest that chronic hyperexcitability following kainate treatment is effectively integrating into the circadian molecular Clock by progressively increasing activity/CREB-mediated expression of circadian transcriptional repressors. By all accounts, adenosine signaling is neuroprotective. Adenosine signaling induced by seizure activity and mediated by adenosine A_1Rs is generally thought to be an internal mechanism by which seizures are terminated (Masino et al., 2014), and likely contribute to depressed cortical EEG following seizures. These reports indicate that seizure mediated disruption of sleep is more likely due to circadian rhythm dysregulation than adversely affecting mechanisms of sleep homeostasis.

The Hippocampal Formation

The role of the hippocampus gained notoriety in 1957 with a report concerning the practice of bilateral temporal lobotomy for the treatment of intractable epilepsy, a procedure that included resection of the hippocampus (Figure 3A). Patient H.M. was one case included in the report and, in addition to significantly reducing seizure occurrence, patient H.M. developed anterograde amnesia, the inability to form new episodic memories (Scoville & Milner, 2000). Since then, the roles that the hippocampus has in memory and epilepsy have been researched extensively.

The hippocampus contains anatomically discrete populations of morphologically and functionally unique neurons connected by highly organized axon tracts that mediate the flow of information through a trisynaptic circuit, as shown in Figure 3B (Moser, 2011). The major anatomic structures of the hippocampus are the dentate gyrus (DG) and the cornu amonis (CA), in which the principal excitatory neurons are granule cells and pyramidal neurons, respectively. The CA structure is further subdivided into three regions: CA1, CA2, and CA3. DG granule cells (DGGCs) receive information from the entorhinal cortex via a tract of axons called the perforant path. DGGCs then project to the neurons in the CA3 via mossy fibers, which in turn project to CA1 through an axon tract called the Schafer collaterals. CA1 neurons then project out of the hippocampus and back to the entorhinal cortex (Amaral et al., 2007). This trisynaptic circuit mediates the main flow of information through the hippocampus, though multiple elements influence how information is processed. Axon tracts of the trisynaptic circuit also synapse on inhibitory interneurons, which modify the communication between principal excitatory neurons and

facilitate hippocampal processing (Buzsáki, 1984; Freundl & Buzsáki, 1996). Compared with CA3 and CA1, the dentate gyrus is known to have a significantly greater density of inhibitory interneurons that mediate feed-forward inhibition. This strong inhibition contributes to the concept of the dentate gate, which heavily regulates activation of DGGCs and thereby controls which signals are allowed to enter the trisynaptic circuit (Krook-Magnuson et al., 2015). The effect of the dentate gate is evident in how it filters incoming activity, acting as a low-pass filter and selectively repressing higher (>7 Hz) frequency activity (Scullin & Partridge, 2012). DG repression differs from the selective potentiation of signals received by CA synapses, which combine with DG to affect overall hippocampal filtering (Stepan et al., 2015). This hippocampal filter is a manifestation of short-term plasticity, which is supported by intrinsic excitability of and interconnectivity between excitatory and inhibitory neurons.

Memory is enabled by long-term plasticity, which is the ability of synapses to modulate their strength. Long-term potentiation (LTP) is considered a cellular correlate for memory formation. Kinase signaling pathways have been shown to mediate local protein translation and *de novo* transcription of synaptic proteins required for the development of long-term plasticity, notably the ERK/MAPK pathway (Peng et al., 2010). It has been shown that ERK/MAPK activity exhibits circadian rhythmicity in the hippocampus, which explains the differential efficacy of hippocampus-dependent memory acquisition between training that occurred during the lights-on period versus lights-off. This is a difficult effect to decipher, considering that sleep propensity and possibly confounding effects of sleep deprivation are also variable across the 24-hour lighting cycle. Some evidence to support the effect of the circadian Clock system on memory is

provided by reports that VIP, a major signaling molecule released by the SCN, activates ERK/MAPK signaling and potentiates NMDA-mediated synaptic transmission (Hamnett et al., 2019; Yang et al., 2009). Assuming seizures are capable of transiently driving subjective day-like activity in VIPergic SCN neurons, night-time seizures may reinforce their effects on the pathologic synaptic interconnectivity between neurons active during the seizure. Outside of circadian rhythms of signaling, associated circadian rhythms of sleep itself are also influential on normal and pathologic synaptic plasticity.
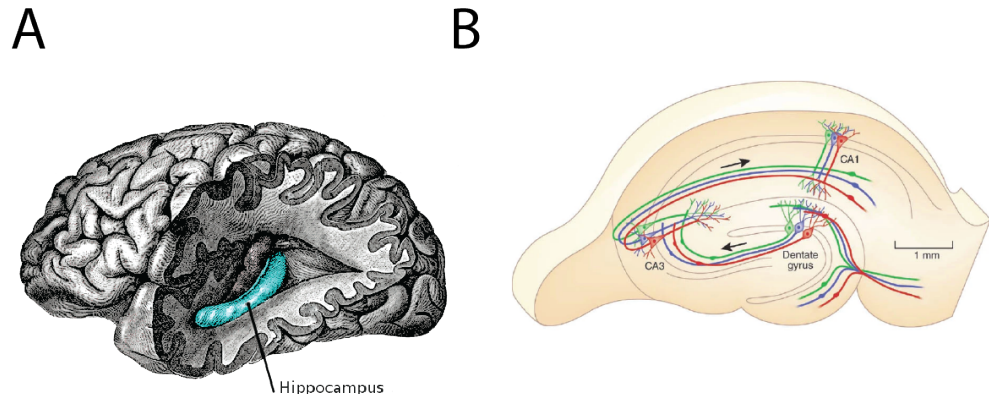

Figure 3: Anatomy of the hippocampus

The hippocampus is a structure situated in the temporal lobe in humans (A). Within the hippocampus is a highly organized, laminar circuit of axon tracts shown in the cross-section schematic in panel B. The trisynaptic circuit is a series of projections starting in the entorhinal cortex (EC) and sequentially innervating the dentate gyrus (DG), and cornu amonis (CA)3 and CA1 that mediates the primary flow of information through the hippocampus. Credit: Panel A is sourced from Gray's Anatomy plate 739 (Clinkingbeard, 1974) and panel B is adapted from (Moser, 2011).

Sleep and homeostasis of neural excitability

Modification of memories and synapses is intimately associated with sleep. Both NREM and REM sleep have been investigated in processes of memory consolidation,

modification, and extinction (Vyazovskiy et al., 2008). The effectiveness of memory formation is coupled with processes of synaptic and intrinsic homeostasis, whereby synaptic drive and firing rates can be sustained over time. Several related hypotheses exist for the mechanism by which these properties are maintained during sleep, including the synaptic homeostasis hypothesis (SHY) (Tononi & Cirelli, 2014) and synaptic replay (Abel et al., 2013; Lewis & Durrant, 2011; Weber et al., 2014).

Most long-term plasticity follows the Hebbian principle that neurons that "fire together, wire together", meaning that a synapse between two neurons where the presynaptic and postsynaptic neurons are activity-dependent. Hebbian LTP is mediated by the insertion of additional fast-acting AMPA glutamate receptors on the postsynaptic density. If left unchecked, continual synaptic strengthening would inevitably saturate, leading to potentially catastrophic hyperexcitability and an inability to store new information. The synaptic homeostasis hypothesis suggests that synapses formed or strengthened during wakefulness and associated learning are compensated for by down-scaling synapses during NREM sleep, which may not be isolated to those synapses that were active during wakefulness. Indeed, in vivo functional assay of evoked cortical synaptic response demonstrates progressive potentiation during waking and depression during sleep, likely in response to varying synaptic localization of glutamatergic AMPA receptors (Barnes, 1996).

Complementary to the notion of synaptic scaling, research into how cortical neurons can integrate strengthened synapses and maintain responsiveness to weaker ones revealed that neurons can scale their intrinsic excitability, allowing for stable firing rates in the presence of varying synaptic drive (Hengen et al., 2016). The study showed that

changes in intrinsic excitability following diminished synaptic input induced by monocular sensory deprivation can occur over a much longer time window on the order of days. Sensory deprivation results in a rapid decrease of relative firing rates in the contralateral visual cortex, which returns to baseline after several days (Hengen et al., 2016). Interestingly, these adjustments to firing rate were found only to occur during periods of wakefulness. It may be that these two homeostatic responses interact in a call-and-response manner, in which neurons that undergo gross changes to synaptic drive (e.g., lost in sensory deprivation, gained during memory acquisition, or pathologically accumulated in sleep deprivation or epilepsy) actively maintain a set level of activity by adjusting firing rates to accommodate the new level of synaptic input.

<u>GABAergic neurotransmission and modulation</u>

Up to this point, much of the focus in epilepsy research has been geared toward excitatory/glutamatergic neurotransmission, an understandable direction for conditions of hyperexcitability. As I have alluded to previously, individual neurons integrate both excitatory *and* inhibitory signals, relying on a balance between them for normal function. Intuitively, a gain of excitatory or loss of inhibitory signaling would have a similar net effect toward hyperexcitability. Inhibition in the CNS is primarily mediated by γ-aminobutyric acid (GABA). GABA mainly activates two classes of receptors, GABA-A and -B receptors, mediating fast ion conductance or slow signal transduction, respectively. GABA-A receptors (GABA$_A$Rs) are pentameric channels composed of varying combinations from a pool of 19 subunits (α1-6, β1-4, γ1-3, δ, ε, θ, π, ρ1-3) and in the hippocampus commonly consist of 2 alpha, 2 beta, and either a gamma-2 or delta subunit (Sieghart et al., 1999). Specific GABA$_A$R composition influences sub-cellular trafficking,

responsiveness to ligand, and dynamics of desensitization (Lüscher & Keller, 2004). Under normal physiological conditions in adults, the opening of GABA$_A$ receptors allows the movement of chloride into neurons. Chloride currents are affected by membrane voltage and chloride concentration equilibrium set by competing actions of potassium-chloride cotransporter type-2 (KCC2) expelling chloride and influx through sodium-potassium-chloride cotransporter type-1 (NKCC1) (Liu et al., 2020; Orlov et al., 2015; Watanabe et al., 2019).

Currents mediated by ionotropic GABA$_A$Rs are divided into several categories: GABA$_{A,Fast}$, GABA$_{A,Slow}$ and GABA$_{A,tonic}$. These represent inhibitory currents mediated by diffusion of neurotransmitter following release from synaptic vesicles. GABA$_{A,fast}$ currents are inhibitory post-synaptic currents (IPSCs) mediated by GABA$_A$Rs within the synapse in immediate response to high concentrations (mM) of GABA. Diffusion of GABA out of the synapse leads to activation of perisynaptic GABA$_A$Rs, located adjacent to the synapse, giving rise to GABA$_{A,slow}$ currents (Zarnowska et al., 2009). Membrane-bound transporters within and surrounding the synapse mediate sequestration of GABA into pre-synaptic terminals or adjacent astrocytes, that allow recycling or degradation of GABA, respectively (Soudijn & van Wijngaarden, 2000). This cellular internalization of neurotransmitter is collectively referred to as reuptake. Neurotransmitter that escapes reuptake contributes to an ambient GABA concentration that can activate extrasynaptic receptors that contribute to persistent GABA$_{A,tonic}$ conductances (Goodkin et al., 2007). The schematic in Figure 4 summarizes the movement and activity of GABA. In addition to conductances mediated by ligand-bound GABA$_A$Rs, another tonic inhibitory conductance is mediated by the spontaneous opening of GABA$_A$Rs (O'Neill & Sylantyev,

2018). In the hippocampus, the GABA$_A$Rs responsible for ligand-mediated tonic currents are those that contain an alpha5 or delta subunits (Glykys et al., 2008). GABAergic inhibition is vital for precise coordination of neuronal function within a circuit (O'Neill & Sylantyev, 2018; Vida et al., 2006), as mediated by inhibitory interneurons (Buzsáki, 1984). Overall intrinsic excitability can be greatly affected by the tonic inhibitory conductance, a persistent controller influencing neuronal integration of excitatory and inhibitory synaptic drive.
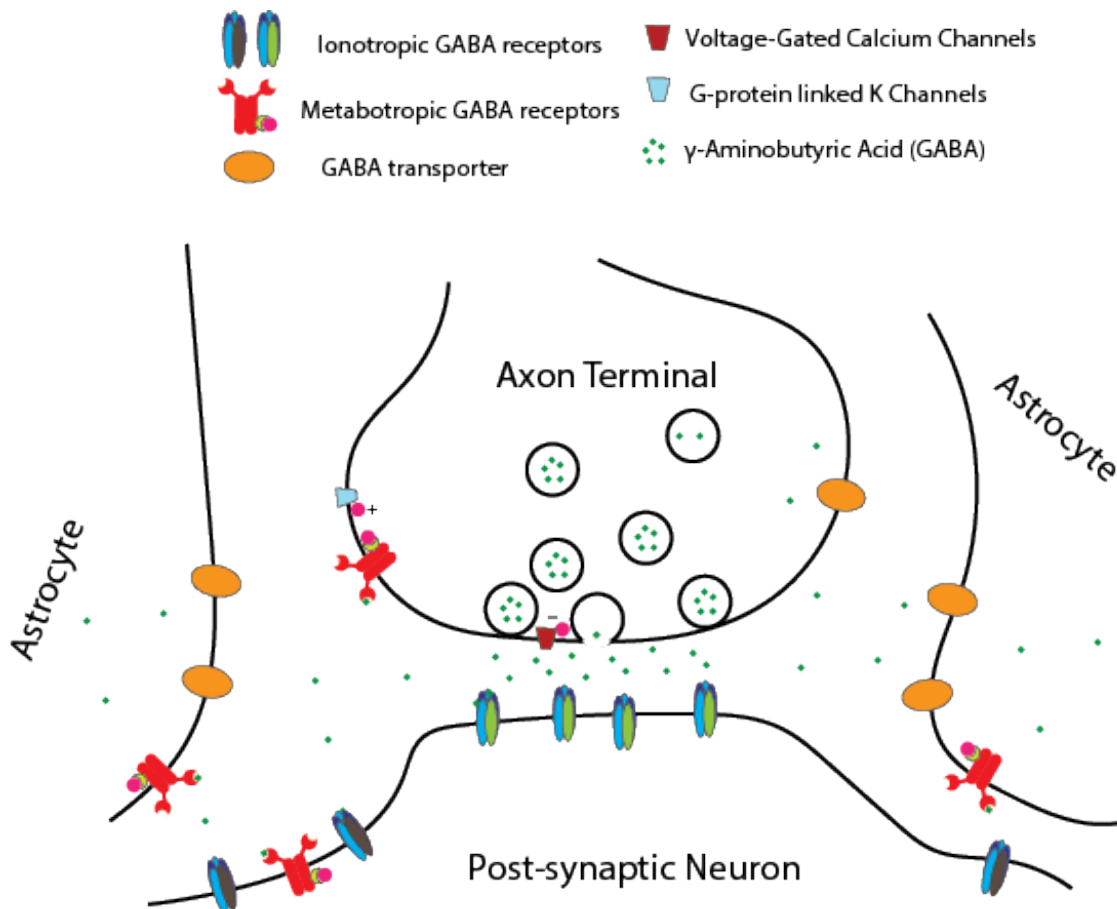

Figure 4: Elements of GABAergic neurotransmission.

GABAergic neurotransmission starts with the release of GABA from synaptic vesicles onto synaptic GABA$_A$ receptors. It then diffuses out of the synaptic cleft and is subject to reuptake by membrane-bound GABA transporters. GABA that remains in the extracellular space leads to activation of extrasynaptic GABA$_A$ and GABA$_B$ receptors, modulating tonic currents and intracellular signaling cascades.

One mechanism in homeostatic control of excitability is an activity-dependent down-regulation of $GABA_A$ receptor signaling. This homeostatic downregulation of $GABA_A$Rs has been demonstrated following both orthosteric and allosteric enhancement that varies based on duration. The process of activity-dependent downregulation of $GABA_A$Rs begins with post-translational modification mediated downregulation of receptor function, followed by elevated internalization, channel degradation, and ultimately repression of subunit expression (Dave et al., 1990). Assuming healthy physiology, cessation of $GABA_A$R potentiation results in withdrawal, the severity of which is directly correlated with level and duration of said potentiation. In the case of acute sleep deprivation, the sustained and elevated neuronal activity would also be accompanied by interneuron-mediated feedforward GABAergic signaling, which potentially leads to desensitization and internalization of $GABA_A$Rs. Upon termination, there would be a transient depression of inhibition while neurons readjust to the new level of synaptic neurotransmission. If this sleep deprivation were to occur in an otherwise pathologically hyperexcitable (e.g., epileptic) brain, it follows that a transient reduction of inhibition would increase seizure susceptibility. In severe cases of ethanol (i.e., potentiator of $GABA_A$Rs) withdrawal, it has been reported that seizures occur even in non-epileptic individuals (Goodkin et al., 2007). Prolonged (~45 minutes) periods of seizures themselves can increase receptor internalization, which is thought to be a focused effect on benzodiazepine-sensitive $GABA_A$Rs (Goodkin et al., 2007). Given that GABAergic signaling is sensitive to acutely elevated synaptic signaling, it may be an underlying factor of how acute sleep loss can elevate neuronal excitability and seizure susceptibility.

<u>Research aims and thesis outline</u>

Our understanding of the relationship between epilepsy and sleep is continually expanding, though much is left to be discovered about the vicious cycle that exists between sleep loss and seizures. So, too, are the mechanisms underpinning epileptogenesis continually being identified and refined. As many current therapies are targeted at controlling seizures, there is a dire need for the development of therapies to inhibit the epileptogenesis and potentially reverse pathology. These advancements in medicine begin with basic science. Our knowledge of the physiological effects of sleep, circadian rhythms, and seizures provides indirect evidence that these systems are capable of interacting with each other. But is targeting one sufficient to beneficially influence the others?

Work in this thesis attempts to disentangle the constituents of what I call the vicious cycle in epilepsy. The following chapters will present research aimed at characterizing sleep disruptions before and after the onset of epilepsy, as well as evaluating the acute effects of sleep deprivation in a seizure-naive brain.

In the second chapter, I describe research that examines the effect of acute sleep deprivation on tonic GABAergic inhibition in excitatory neurons of the hippocampus of seizure-naive mice. In the third chapter, I evaluate the longitudinal dynamics of sleep in response to acute sleep deprivation following induced status epilepticus, as well as the chronic effects of status epilepticus on tonic inhibition in the hippocampus. In the fourth chapter, I characterize circadian rhythms of locomotor activity and molecular Clock in a

genetic model of temporal lobe epilepsy. In the final chapter, I will integrate the results of my thesis work into the current body of literature and outline questions that were generated along the way and posit potential research that would expound upon work presented in this thesis.

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Chapter 2 – Acute sleep loss disrupts tonic GABAergic inhibition

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Abstract

Societal pressures that restrict healthy sleep hygiene are ubiquitous, affecting cognitive performance and endangering the future health of people around the globe. Sleep disruptions have been associated with many neurologic disorders. In the present study, we were interested in how transient sleep loss is so potent in triggering seizures in epilepsy, which already has persistent sleep disruption and hyperexcitability. Some elements of GABAergic inhibition have been shown to be susceptible to sleep loss, however, little is known of what physiologic effect that sleep disruptions may have on GABAergic neurotransmission in the hippocampus. Here, we use whole cell patch-clamp electrophysiology techniques to investigate the effect of 4-hour sleep deprivation (4SD) on miniature synaptic and tonic GABAergic currents in principal cells of the dentate gyrus (DG) and CA1 in the hippocampal formation. We report that the amplitude and frequency of miniature inhibitory postsynaptic currents (mIPSCs) are not affected by 4SD, whereas tonic GABAergic currents are decreased in both regions. In the DG, 4SD did not alter currents activated by 1 µM THIP, a competitive $GABA_A$ receptor ($GABA_AR$) agonist with high-selectivity for receptors containing delta subunits ($\delta GABA_AR$) and blocked by L-655,708, a negative allosteric modulator with high-selectivity of GABAARs containing an

alpha5 subunit (α5GABA$_A$Rs). However, in the CA1, 4SD increased THIP activated currents and decreased L-655,708 sensitive currents. Loss of tonic inhibitory currents would suggest that neurons in the hippocampus are becoming hyperexcitable during sleep deprivation. If this loss of inhibition were to occur in an individual with an underlying condition of hyperexcitability, such as epilepsy, it could explain why

Introduction

Sleep deprivation:

Sleep problems have become a serious issue around the world over the last several decades, forcing the Centers for Disease Control to declare 'insufficient sleep syndrome' an epidemic and the World Health Organization to classify hypersomnolence (i.e., excessive fatigue) as a non-communicable disease (Chattu et al., 2018). Increasing work demands and rapid increase of smartphone use have not only decreased the amount of time asleep by pushing active periods later but have also increased periods of light exposure, which confuses the natural circadian regulation of sleep (Pieters et al., 2014; Shockey & Wheaton, 2017; Touitou et al., 2016). Deterioration of sleep hygiene may underlie reports that over 40 percent of adolescents and 30 percent of adults have insufficient sleep, a figure that is on the rise (Liu et al., 2013; Pieters et al., 2014). Poor sleep has been associated with the incidence of obesity, cardiovascular disease, anxiety, and ADHD, and bodes ill for future health and cognitive performance of those sleep-deprived individuals (Liu et al., 2016; Shi et al., 2018).

Aside from societally driven effects on our sleeping patterns, neurological conditions, such as epilepsy (Foldvary-Schaefer & Grigg-Damberger, 2009), Alzheimer's disease (Peter-Derex et al., 2015), Parkinson's disease (Dhawan et al., 2006), and Autism Spectrum Disorder (Konofal et al., 2010) frequently have comorbid sleep problems, and preexisting hyperexcitability could be exacerbated additional, acute sleep perturbations caused by a night of particularly poor sleep. Thus, investigating the independent effects that acute sleep deprivation has on neural excitability of seizure naive

individuals is of great importance, informing standard of care for these neurological maladies and the public of the ramifications of poor sleep habits. In the case of epilepsy, there is long-standing clinical precedence of sleep disruption leading to elevated seizure susceptibility (Foldvary-Schaefer & Grigg-Damberger, 2009; Haut et al., 2007), which in turn contributes to further perturbation of sleep patterns. Understanding how sleep deprivation may increase one's susceptibility to seizures and, conversely, how seizures affect sleep patterns may serve to inform how epilepsy develops as a disease and provide therapeutic methods to combat the progression toward neural hyperexcitability.

GABAergic neurotransmission:

In the hippocampus, GABA$_A$Rs are commonly composed of 2 alpha, 2 beta, and either a gamma-2 or delta subunit. Specific receptor composition influences sub-cellular trafficking, responsiveness to ligand, and dynamics of desensitization (Lüscher & Keller, 2004). GABA$_A$R mediated currents come in at least two varieties: phasic and tonic. The former is mediated by receptors at or near synapses, termed inhibitory postsynaptic currents (IPSCs), which are transient and high in amplitude and constitute point-to-point communication. Phasic GABAergic inhibition is vital for precise coordination of neuronal function within a circuit (O'Neill & Sylantyev, 2018; Vida et al., 2006), and is mediated by synaptic drive from inhibitory interneurons (Buzsáki, 1984). Tonic currents are a more global response mediated by receptors with diffuse distribution and a high affinity for binding GABA, enabling them to respond to low, ambient concentrations of GABA (Soudijn & van Wijngaarden, 2000). Persistent inhibitory currents can greatly affect overall intrinsic excitability (Nusser & Mody, 2002). GABA$_A$Rs associated with tonic

currents in the hippocampus α5GABA$_A$Rs or δGABA$_A$Rs (Glykys et al., 2008). In addition to currents mediated by ligand-bound GABA$_A$Rs, a portion of tonic inhibitory currents can be mediated by spontaneous gating of GABA$_A$Rs (O'Neill & Sylantyev, 2018).

Here, we investigate the effects that 4 hours of total sleep deprivation have on inhibitory neurotransmission in seizure naive mice. Specifically, we report a reduction in GABAergic tonic inhibitory currents in the dentate gyrus (DG) and CA1 in the hippocampus following 4SD, a structure that is implicated in seizure generation in some epilepsies (e.g., temporal lobe epilepsy, TLE) (Franck et al., 1995; Klinzing et al., 2019; Scoville & Milner, 2000).

Methods

Animals:

For all experiments, C57bl/6 male mice from Envigo (Madison, WI) were used. All animals were allowed >3 days to acclimate to the environmental conditions of the vivarium before any experiments. We used male mice due to low and stable serum progesterone-derived neurosteroid concentrations, which have direct effects on $GABA_ARs$ that mediate tonic currents and fluctuate during the estrous cycle. All procedures were approved by UW-Madison Institutional Animal Care and Use Committee and adhered to NIH standards of care (NRC, 2011).

Sleep deprivation:

All animals were housed individually, and locomotor activity was monitored via passive infrared motion sensors for the 12-hour dark period before the experiment. Sleep deprivation began at the start of the light period and was achieved via a persistent novel environment lasting 4 hours (4SD) (Colavito et al., 2013). Briefly, animal activity was monitored for behaviors indicative of sleep onset (e.g. nesting, extended behavioral arrest). When these behaviors were seen, novel objects were introduced into the cage, thereby encouraging exploration and persistent vigilance. Control animals were allowed to rest undisturbed for the first 4 hours of the light phase.

Solution preparation:

Solutions were prepared including high-sucrose cutting solution (CS, containing in mM: 80 NaCl, 24 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 25 D-glucose, 0.5 $CaCl_2$, 4 $MgCl_2$, 75 sucrose, 1 Na-L-Ascorbate, and 3 NaPyruvate) and artificial cerebrospinal fluid (aCSF, containing in mM: 125 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 25 d-glucose, 2 $CaCl_2$, 1 $MgCl_2$ and adjusted to 325 ± 2 mOsmol with sucrose). High-chloride (KCl) intracellular solution was made in a batch to ensure constancy of the intracellular milieu across data sets and contained in mM: 140 KCl, 10 EGTA, 10 HEPES, 2 Mg_2ATP, 0.3 NaGTP, and 20 phosphocreatine and was adjusted to pH 7.3 with 5 N KOH and 310 mOsmol. All solutions were aerated with a 95% O_2/5%CO_2 gas mixture.

Slice preparation:

Mice were anesthetized with isoflurane before decapitation and brain isolation. Horizontal hippocampal slices (400 µm) were prepared as described in Bischofberger et al. (2006) in ice-cold CS with a vibratome (VT1000s Leica, Nußloch, Germany). Slices were then transferred to an aerated holding chamber filled with a warm (37°C) 1:1 mixture of CS:aCSF for 1 hour, after which the temperature was allowed to equilibrate to room temperature (22°C).

Whole cell patch-clamp recordings:

After transfer to a submersion recording chamber (22°C), slices were equilibrated for 5 minutes. Cells were visualized with an upright microscope (Axioskop, Zeiss, Germany) equipped with infra-red differential interference contrast (IR-DIC) optics, CCD camera and controller (Hamamatsu, Japan). DGGCs and CA1 pyramidal cells were identified visually based on morphology and anatomical location. We acquired whole-cell patch recordings in the voltage-clamp configuration (-70 mV) with a Multiclamp 700B amplifier (Molecular Devices, San Jose, CA) filtered with a 2 kHz low-pass, 4-pole Bessel filter, digitized at 20 kHz with a Digidata 1333 (Molecular Devices, San Jose, CA) and acquired with Axograph X on a Macintosh G4 computer. Borosilicate patch pipettes were filled with KCl intracellular solution and had a resistance of 2-5 MΩ when submerged in aCSF. GABAergic currents were isolated with a base solution containing 100 nM tetrodotoxin (TTX, Tocris, cat: 1069), 25 µM D-(-)-amino-5-phosphonovaleric acid (AP5, Tocris, cat: 0106), and 5 µM 6,7-dinitroquinoxaline-2,3-dione (DNQX, Tocris, cat: 2312). Cell stability was assessed with a 5-minute baseline voltage-clamp recording prior to the initiation of tonic current assays. Cellular parameters were calculated from current responses to a -5 mV step command (i.e., membrane capacitance (C_m), series resistance (R_s), input resistance (R_{in}), and resting membrane potential (V_m)) recorded before and after any drug application. Recordings were included in analyses if R_s was less than 10 MΩ and drifted less than 2 MΩs throughout the recording. To measure tonic inhibitory currents, 6-minute recordings were acquired, in which we perfused base solution for 1-2 minutes, followed by perfusion of the base solution with 100 µM Bicuculline Methiodide (BMI, Tocris, cat: 2503). To measure currents mediated by $GABA_A$Rs containing delta subunits, we used

THIP (4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride), a GABA$_A$R agonist with a high affinity for delta subunit-containing GABA$_A$Rs. To measure THIP activated currents, 10-minute recordings were acquired with serial perfusion of three solutions: base, base + 1 μM THIP (Tocris, cat: 0807), and base + 100 μM BMI. To measure of currents mediated by α5GABA$_A$Rs, we utilized L655,708 (Tocris, cat: 1327), a GABA$_A$R benzodiazepine-site inverse agonist with high selectivity for α5GABA$_A$Rs. L-655,708 blockade of tonic currents was measured with 15-minute recordings with serial perfusion of four solutions: base, base+5 μM GABA (Tocris, cat: 0344), base+GABA+30 μM L655,708, and base+GABA+100 μM BMI. Miniature IPSCs were measured during the baseline period prior to any solution changes.

Analysis:

Tonic currents were calculated from 60-second segments of steady-state voltage-clamp traces, converted to all-point histograms, to which a Gaussian function was fit to the outward half of the distribution, yielding a mean current and standard deviation. The current density was calculated by normalizing the difference between steady-state holding currents to the membrane capacitance of the cell. Statistical differences between tonic currents of control and 4SD recordings were evaluated with unpaired, two-tailed t-tests.

Miniature IPSCs (mIPSCs) were detected using Axograph X with a variable amplitude template calculated by fitting a double exponential to a consensus IPSC waveform (rise: 0.48 ms, decay: 16 ms) and amplitude threshold 4 times the RMS noise.

Median values were determined for each cell before being combined into lists based on hippocampal subfield and SD treatment. Unpaired, two-tailed t-tests were used to compare median inter-event interval (IEI, latency previous mIPSC except for the first event, which is calculated as the latency from start) and amplitude values from each recording. Using the bootstrapping method, we created 1,000 probability distribution functions (PDFs) from lists drawn randomly and consisting of half the total number of detected mIPSCs for each grouped variable. From each set of PDFs, an estimated distribution was generated with an average and 95% confidence interval for each metric and hippocampal region.

All t-tests were considered significant with p-values less than 0.05.

<u>Results</u>

Tonic inhibitory currents were reduced following 4-hour sleep deprivation:

Transient insults to sleep bear deleterious effects in seizure naive and epileptic individuals, though the underlying mechanisms are not completely understood, especially in the context of hippocampal GABAergic neurotransmission. Therefore, we tested the effect of 4SD on GABAergic tonic inhibition in principal excitatory cells of the dentate gyrus and CA1 in the hippocampus of seizure naive mice. We observed that at 4SD significantly reduced tonic inhibitory current density in DGGCs (Figure 1B, Control: 0.44±0.05 pA/pF, n=16; 4SD: 0.18±0.03 pA/pF, n=18, p<0.05). Additionally, we saw a significant reduction of tonic inhibitory current density in CA1 pyramidal neurons following 4SD (Figure 1C, Control: 0.25±0.02 pA/pF, n=14; 4SD: 0.13±0.03 pA/pF, n=11, p<0.05). These significant reductions in tonic inhibition are consistent with an increase in neuronal excitability in seizure naive mice.
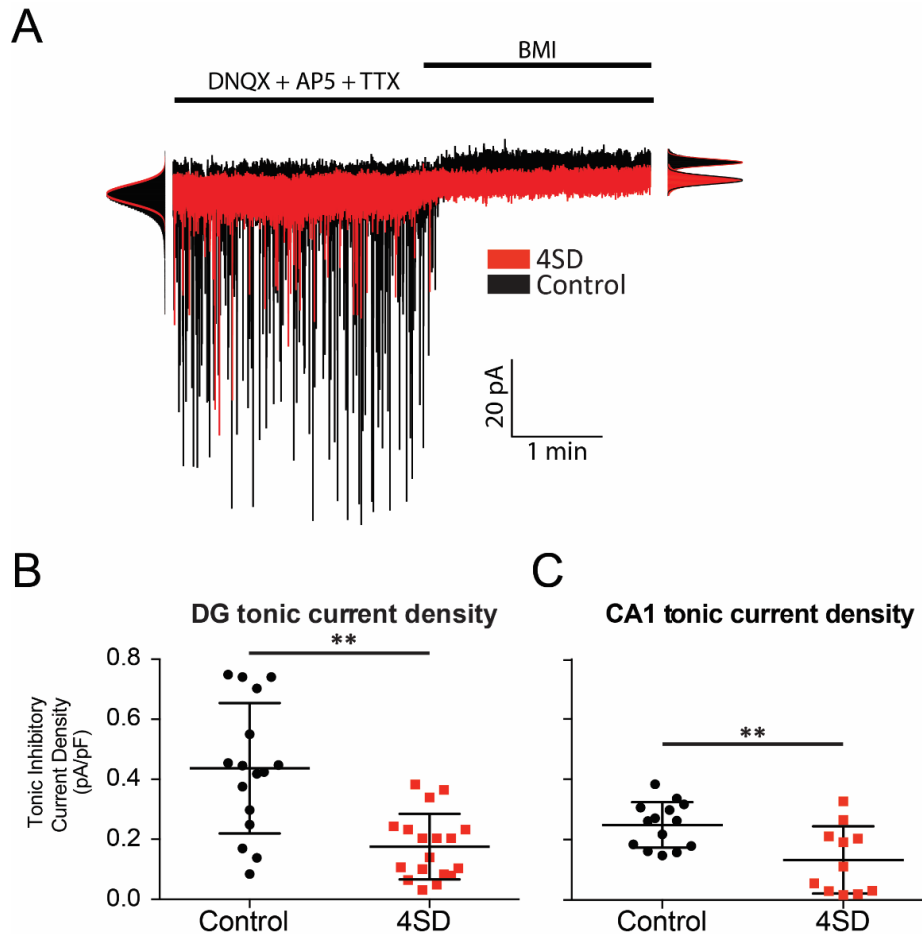
Figure 1: Analysis of 4SD-induced changes to tonic GABAergic currents.

GABAergic inhibition was assessed in principal excitatory cells in the dentate gyrus and CA1 regions of the hippocampal formation in acute hippocampal preparations from animals treated with 4-hours of total sleep deprivation and age-matched controls. GABAergic currents were isolated pharmacologically with glutamate blockers AP5 and DNQX, and action potentials were inhibited with tetrodotoxin. Voltage-clamp recordings were acquired from neurons in the whole-cell patch configuration held at -70 mV. Representative VC charts are shown for control (black) and 4SD (red). All point histograms generated from 60-second segments prior to and following application of bicuculline (BMI), and gaussian functions were fit to the inward going half of the distribution, opposite the mIPSC shown here as downward deflections at baseline. Tonic current magnitude was calculated as the difference between means of these fit functions before and after BMI application. Each current magnitude was normalized to the membrane capacitance (Cm, pF) to yield current density. Following 4SD, mean tonic current density were significantly reduced in dentate gyrus granules cells (B, Control=0.437±0.054 pA/PF, n=16; 4SD=0.176±0.026 pA/pF, n=18, p<0.001) and CA1 pyramidal cells (C, 0.249±0.02 pA/pF, n=14; 4SD=0.133±0.034 pA/pF, n=11, p<0.05).

4SD alters THIP activated currents in CA1 but not DG:

One mechanism by which 4SD may affect tonic inhibitory current density is the population size and post-translational modifications of two $GABA_AR$ isoforms that are associated with tonic inhibition and expressed at the cell membrane. The first subtype is the $\delta GABA_AR$. Using THIP, a competitive agonist with high selectivity for $\delta GABA_ARs$, we found that tonic inhibitory current density mediated by $\delta GABA_ARs$ in DGGCs was unaffected by 4SD, (Figure 2B: Control: 0.539 ± 0.061 pA/pF, n=24; 4SD: 0.506 ± 0.034 pA/pF, n=14, p>0.05). Counterintuitively, we found tonic currents activated by THIP perfusion increased in CA1 pyramidal cells following 4SD (Figure 2C: Control:0.358 ± 0.043 pA/pF, n=17; 4SD: 0.789 ± 0.068 pA/pF, n=6, p<0.05). These data suggest that $\delta GABA_ARs$ are not the mechanism for reduced tonic inhibition following 4SD in the DG, and, surprisingly, results from CA1 pyramidal cells indicate an effect opposing the initial observation of reduced tonic inhibition: an increased function of $\delta GABA_ARs$.
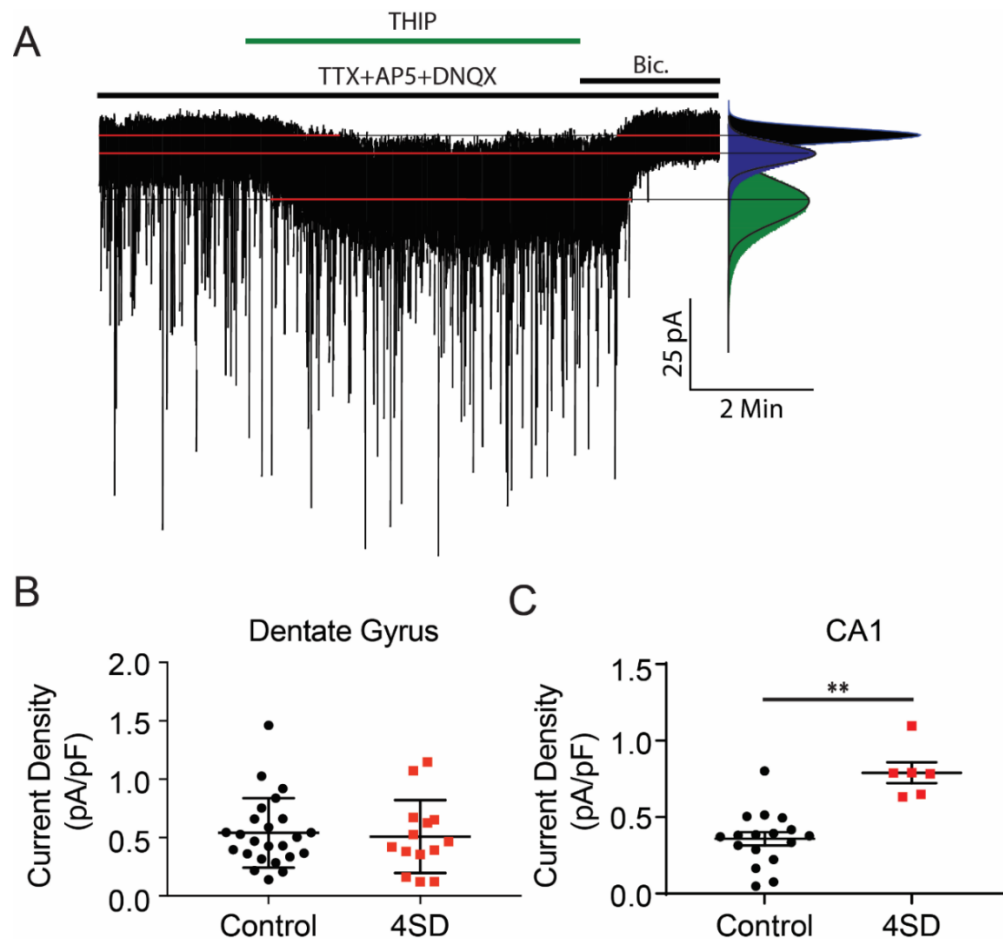
Figure 2: Analysis of 4SD-induced changes to THIP-sensitive tonic currents.

Currents mediated by GABA$_A$Rs containing delta subunits were determined pharmacologically with the highly selective 1 µM THIP. GABAergic currents were isolated pharmacologically with glutamate blockers AP5 and DNQX, and action potentials were inhibited with tetrodotoxin. Voltage-clamp recordings were acquired from neurons in the whole-cell patch configuration held at -70 mV. A representative VC chart is shown in panel A. All point histograms generated from 60-second segments of steady-state recording at baseline and in the presence of 1 µM THIP. Bicuculline was applied to ensure that recordings included GABAergic currents only. Gaussian functions were fit to the inward going half of each distribution, opposite the mIPSC shown here as downward deflections at baseline. The magnitude of tonic currents activated by THIP was calculated as the difference in mean current at baseline and with THIP and was normalized to membrane capacitance (Cm, pF). Following 4SD, mean THIP activated current density were unaffected in dentate gyrus granules cells (B, Control: 0.539±0.061 pA/pF, n=24; 4SD: 0.506±0.034 pA/pF, n=14, p>0.05), while mean current density was significantly increased in CA1 pyramidal cells (C, Control=0.358±0.043 pA/pF, n=17, black; 4SD: 0.789±0.068 pA/pF, n=6, red, p<0.05).

4SD alters L-655,708 sensitive currents CA1 but not DG:

The second subtype of $GABA_ARs$ that is closely associated with tonic currents is the $\alpha5GABA_AR$. We investigated the effect of 4SD on currents mediated by $\alpha5GABA_ARs$ by quantifying 30 µM L-655,708-mediated block of tonic currents while increasing the signal to noise with 5 µM GABA. We perfused L-655,708 in the presence of 5 µM GABA, followed by total blockade of $GABA_ARs$ with 100 µM BMI, and calculated the percentage of overall tonic currents blocked by L-655,708 perfusion. We found that the percentage of tonic currents blocked by 30 µM L655,708 in DGGCs was not affected by 4SD (Figure 3B: Control: 30.72±4.43%, n=11; 4SD: 26.12±5.284%, n=8, p>0.05). In CA1 pyramidal cells, we found that 30 µM L-655,708 blocked a smaller percentage of enhanced tonic currents after 4SD (Figure 3C: Control: 40.72±5.34%, n=17; 4SD: 24.09±2.98%, n=12, p<0.05). Taken together, the significant alterations to L-655,708 sensitive currents may outweigh the paradoxical increase in currents sensitive to THIP following 4SD and explain the overall loss of currents in CA1 pyramidal cells. However, the lack of 4SD-mediated effects on THIP and L-655,708 sensitive currents in DGGCs argues against the notion that changes in $GABA_AR$ function and expression are the mechanism of decreased overall tonic inhibitory currents in DG. In which case, mechanisms regulating the extracellular GABA concentration may be causing the loss of tonic inhibitory currents in the DG in 4SD, including reuptake and synaptic release.
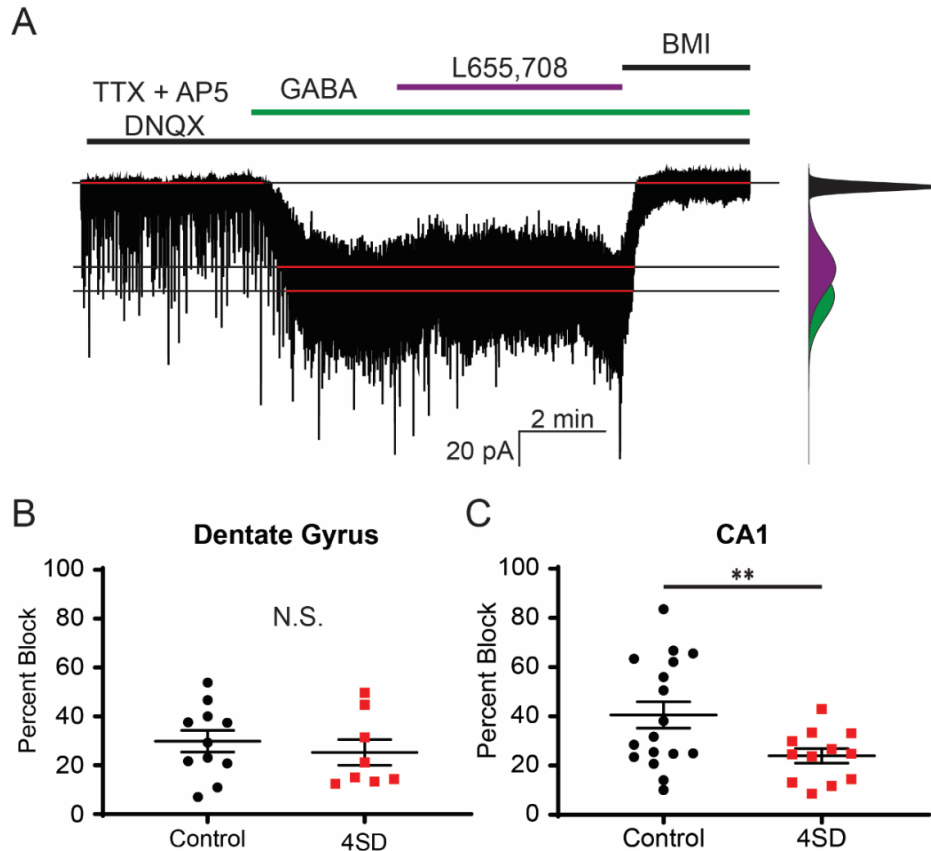
Figure 3: Analysis of 4SD-induced changes to L-655,708-sensitive tonic currents.

Contribution of alpha5 subunit-containing GABA$_A$Rs (α5GABA$_A$Rs) were determined pharmacologically with L655,708, a GAB$_A$R negative allosteric modulator with activity at the benzodiazepine binding site and high selectivity for α5GABA$_A$Rs. GABAergic currents were isolated pharmacologically with glutamate blockers AP5 and DNQX, and action potentials were inhibited with tetrodotoxin. Voltage-clamp recordings were acquired from neurons in the whole-cell patch configuration held at -70 mV. A representative VC chart is shown in panel A. All point histograms generated from 60-second segments of steady-state recording at steady state in the presence of 5 µM GABA, 30 µM L-655,708, and 100 µM Bicuculline. Gaussian functions were fit to the inward going half of each distribution, opposite the mIPSC shown here as downward deflections at baseline. The magnitude of tonic currents mediated by α5GABA$_A$Rs was calculated as the amount of current blocked by L-655,708 (I_{GABA}-$I_{L-655,708}$) as a percentage of overall currents (I_{GABA}-$I_{Bicuculline}$). Percent block of tonic currents with L-655,708 was not affected by 4SD in dentate gyrus granule cells (B, Control: 30.72±4.43%, n=11; 4SD: 26.12±5.284%, n=8, p>0.05), however L-655,708 block was significantly decreased in CA1 pyramidal cells (C, Control=40.72±5.34%, n=17, black; 4SD=24.09±2.98%, n=12, red, p<0.05).

Miniature IPSC amplitude and inter-event interval are unaffected by 4SD:

Ligand-gated tonic inhibitory currents are usually maintained by a low ambient concentration of extracellular GABA. Ambient GABA is largely generated from spillover from synaptic release (Glykys & Mody, 2007). We detected miniature inhibitory postsynaptic currents (mIPSCs) from baseline voltage-clamp recordings in CA1 and DG neurons. For each mIPSC, amplitude (pA) and inter-event interval (IEI, ms) were calculated. Median values were determined for each cell and grouped for subsequent analyses based on the hippocampal region and 4SD-treatment. Analyses of mIPSC amplitude suggested no significant effect of 4SD in either the DGGCs (Control: 6.4±0.16 pA, n=97; 4SD: 6.143±0.137 pA, n=65: p>0.05, figure 4A) or the CA1 pyramidal cells (Control: 7.51±0.23 pA, n=80; 4SD: 7.72±0.25 pA, n=53: p>0.05, Figure 4D). Analysis of median IEI revealed no effect of 4SD on mIPSC IEI in DGGCs (Control: 0.656±0.027 sec, n=97; 4SD: 0.775±0.066 sec, n=65: p>0.05, Figure 4G), or CA1 (Control: 0.361±0.017 sec, n=80; 4SD: 0.368±0.019 sec, n=53: p>0.05, Figure 4K). Like the median analysis, bootstrap analysis of IEI and amplitude metrics suggested subtle differences in probability distribution functions and cumulative distribution functions. These data indicate that mIPSC frequency and amplitude are insensitive to acute sleep loss and are unlikely the cause for a reduction in tonic inhibition observed following 4SD.
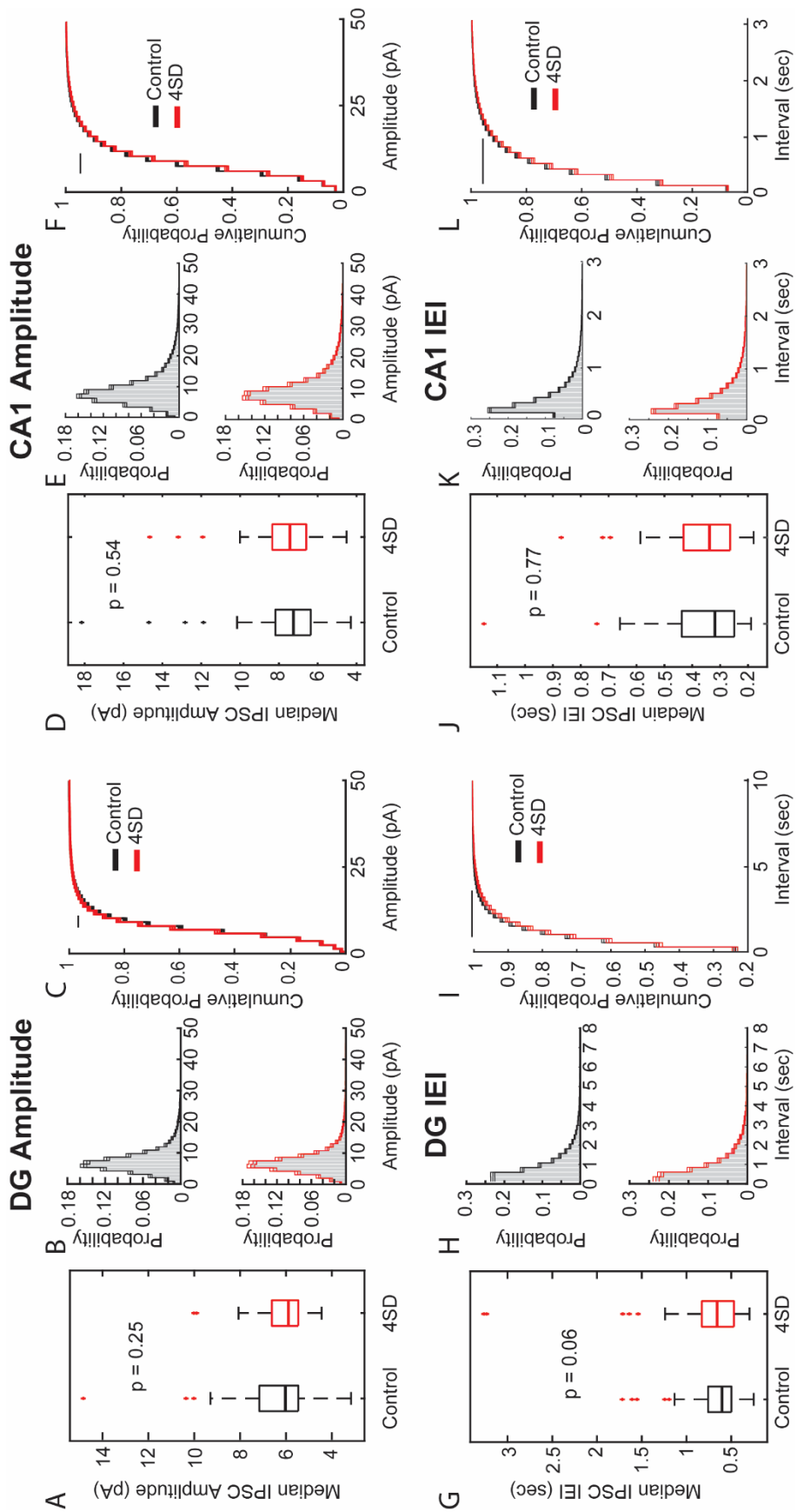
Figure 4: Analysis of 4SD-induced changes to miniature IPSC amplitude and IEI.

Miniature inhibitory post-synaptic currents (mIPSCs) were detected in voltage-clamp patch-clamp recordings in DG and CA1 principal neurons in the presence of AP5, DNQX, and TTX. Amplitude and inter-event interval were measure for each mIPSC detected from DG control (n=38,708 mIPSCs, from 97 recordings) and 4SD (n=23,422 mIPSCs, from 65 recordings) and CA1 control (n=65,973 mIPSCs, from 80 recordings) and 4SD (n=45,648 mIPSCs, from 43 recordings). Median values of each metric and each recording were determined before pooling the data based on cell and treatment type. We found no significant difference between control and 4SD for amplitude in DG (A, Control=6.4±0.16 pA, n=97; 4SD=6.143±0.137 pA, n=65, red, p>0.05) or CA1 (D, Control=7.51±0.23 pA, n=80, black; 4SD=7.72±0.25 pA, n=53, red, p=0.54). Comparing median inter-mIPSC interval we found no significant effect of 4SD in DG granules cells (G, Control=0.656±0.027 sec, n=97; 4SD=0.775±0.066 sec, n=65, red, p=0.06) or CA1 pyramidal cells (J, Control=0.361±0.017 sec, n=80, black; 4SD=0.368±0.019 sec, n=53, red, p=0.77). Using combined lists, we assessed the overall distribution of amplitude and IEI of detected mIPSCs. We determined 95% confidence intervals using the bootstrap method with 1,000 iterations. We graphically represented these bootstrap analyses in probability distribution functions of amplitudes in DG (panel B) and CA1 (panel E), and for inter-event intervals in DG (panel H) and CA1 (panel K) in each panel gray = original data with overlaid lines from bootstrap and 95CI, (top = control, black, bottom = 4SD, red) and compared the distributions of 4SD by plotting mean and 95CI in cumulative distribution functions. Bins with non-overlapping 95CIs suggests statistical significance and are indicated with black bars in CDFs for amplitudes in DG (panel C), CA1 (panel F), and inter-event intervals in DG (panel I) and CA1 (panel L).

<u>Discussion</u>

In the present study, we tested the hypothesis that GABAergic currents within the hippocampus are affected by the acute loss of sleep, providing a potential mechanism for the observation that transient exacerbation of sleep loss in epilepsy increases excitability. Our findings demonstrate a significant reduction of tonic inhibitory currents in principal excitatory cells of the dentate gyrus and CA1. A subsequent investigation into the function of GABA$_A$Rs known to contribute to tonic inhibition in the hippocampus (Glykys et al., 2008), primarily α5GABA$_A$Rs and δGABA$_A$Rs, demonstrated altered function in CA1, but not DG, following 4SD. The function of α5GABA$_A$Rs and δGABA$_A$Rs in DGGCs were not affected by acute sleep loss, however CA1 pyramidal neurons exhibited decreased function of α5GABA$_A$Rs subunits while the function of δGABA$_A$Rs increased. Our analyses of mIPSCs did not find any substantial changes in frequency or amplitude, suggesting that synaptic supply of extracellular GABA is not affected by 4SD.

Ramifications of poor sleep are well known anecdotally and empirically, as acute and chronic sleep deprivation incurs detrimental effects in memory and long-term health. Sleep deprivation is especially potent in people suffering from epilepsy, where its effects are often linked to elevated seizure susceptibility (Cobabe et al., 2015; Foldvary-Schaefer & Grigg-Damberger, 2009). The hippocampus is known as a site of seizure generation in temporal lobe epilepsy (Scoville & Milner, 2000). Overlaying these two observations highlights a potential mechanism behind elevated seizure susceptibility following acute sleep deprivation: hyperexcitability in the hippocampus.

Many studies have endeavored to elucidate the effects of sleep deprivation in the central nervous system. In cortical neurons, 4SD has been shown to induce hyperexcitability (Yan et al., 2011). Counterintuitively, similarly brief sleep deprivation (3-4 hours) caused an increase in protein expression of $GABA_AR$ subunits beta2/3 (del Cid-Pellitero et al., 2017; Modirrousta et al., 2007). In the present study, we observed a pyramidal neuron-specific changes in THIP and L-655,708 sensitivity following 4SD, which may underscore a fundamental difference between the plasticity of $GABA_AR$ function and expression between the two cell types in the context of acute sleep deprivation. Paradoxical (rapid eye movement) sleep deprivation lasting 72-hours decreases hippocampal neuron excitability and impairs LTP formation and maintenance at Schaffer collateral-CA1 and performant path-DG synapses (McDermott et al., 2003). Our previous investigation into 72-hour sleep fragmentation produced similar impairment of Schaffer collateral-CA1 LTP maintenance and hippocampal-dependent performance in the Morris Water maze behavioral task (Wallace et al., 2015). These studies had a greater effect on REM sleep, which are unlike studies, such as the present one, which involve total deprivation of both NREM and REM sleep. One study reported that 12-hour total SD upregulated the metabotropic $GABA_B$ receptor expression in CA1 pyramidal cells, which appear to be a homeostatic change critical for LTD maintenance (Tadavarty et al., 2011). We can see that type and duration of sleep disruption can have differential effects neuronal function through changes in intrinsic excitability and impairments to synaptic plasticity. It may be that the effects of acute sleep deprivation may be compensated for within several days. Despite the advancements in our understanding of SD-induced impairment of memory and neuronal function in the hippocampus, relatively

little is known about the effects of acute SD on ionotropic GABAergic neurotransmission. To our knowledge, this study is the first to directly assess the effects of acute sleep deprivation on tonic GABAergic inhibition in the hippocampus with slice electrophysiology.

Ligand mediated tonic currents are chiefly influenced by three elements, synaptic release (Glykys & Mody, 2007), the responsiveness of the post-synaptic cell to GABA (e.g. receptor population size, affinity for binding GABA, ion gradient, etc.), and reuptake by surrounding neurons and astrocytes (Song et al., 2013). We demonstrate that tonic currents are indeed altered after 4SD, though the mechanisms remain unclear. We evaluated two candidates we considered most likely to affect tonic GABAergic currents. Firstly, we tested the function of $\alpha 5 GABA_A Rs$ and $\delta GABA_A Rs$ that are responsible for mediating most tonic inhibitory currents in the hippocampus (Glykys et al., 2008). Secondly, we characterized miniature inhibitory postsynaptic currents, the primary source of ambient, extracellular GABA (Glykys & Mody, 2007). Despite DGGC tonic currents being reduced following 4SD, we see no effect in the currents that are sensitive to THIP and L-655,708. Considering that mIPSC frequency and amplitudes are also resistant to 4SD, we are left with a few plausible causes for the reduced tonic inhibition. The most obvious is the function of reuptake machinery. GAT-1 is the membrane-bound GABA transporter commonly found in hippocampal neurons. Its presence at the membrane is increased by BDNF signaling and inhibited by PKC activity (Law et al., 2000). It has been reported that BDNF levels are stable or even down-regulated, and PKC-activity increases, following sleep deprivation (Abrial et al., 2015; Zhang et al., 2013). Both findings suggest that SD-sensitive regulators of GAT-1 surface occupancy support GABA-transporter internalization and reduced capacity for GABA reuptake following sleep deprivation. This

would more likely increase extracellular [GABA] and enhance tonic inhibition than account for the loss of tonic inhibitory currents. As an alternative mechanism, some studies have shown that GABA-independent, spontaneous, currents can contribute to overall tonic inhibition in DGGCs (O'Neill & Sylantyev, 2018; Wlodarczyk et al., 2013). These studies show that the activity of the inverse agonist bicuculline can be reversed by coincubation with SR-95531 (gabazine), after which some tonic current returns while IPSCs remain blocked, likely mediated by displacement of bicuculline at the GABA-binding sites, which allows spontaneous gating of $GABA_A$Rs. Our initial tonic inhibition experiments used 100 µM bicuculline to block currents mediated by both ligand-gated and spontaneous $GABA_A$R activity. Our functional experiments of $\alpha5GABA_A$Rs and $\delta GABA_A$Rs in DGGCs indicate no difference in the function of each subtype, though leaving the question of the relative contribution of spontaneous gating to the measured tonic currents, as well as any susceptibility to 4SD, unresolved.

Unlike the receptor function in DGGCs, CA1 pyramidal cells did exhibit altered function of $\delta GABA_A$Rs and $\alpha5GABA_A$Rs, though they changed in opposing directions. Specifically, 4SD caused a decrease in L-655,708-sensitive currents and an increase in THIP-sensitive currents. In the CA1, alpha5 subunits are expressed in greater levels than delta subunits and $\alpha5GABA_A$Rs contribute a greater proportion of BMI sensitive currents (Glykys et al., 2008). Measuring $\alpha5GABA_A$Rs with L-655,708 has been reported to have moderate efficacy and requires a very low concentration to maintain selectivity to alpha5 subunits (Kd~2.4 nM) (Quirk et al., 1996). Though we were using concentrations far higher and potentially activating $GABA_A$Rs containing other α-subunits, our results agree with previous reports of L-655,708 block using lower concentrations (50 nM), which

demonstrated ~29% block in DGGCs and ~70% block in CA1 pyramidal cells, with residual tonic currents on par with those measured in tissue from in α5 subunit knockout animals (Glykys et al., 2008). A possible explanation for divergent 4SD-responses in the CA1 could be that δGABA$_A$R function is increasing to compensate for a loss of α5GABA$_A$R-mediated currents, but that it is inadequate to counter the loss of function, resulting in a net decrease in tonic currents.

Conclusion:

Tonic inhibitory currents are an integration of many discrete mechanisms converging on GABAergic neurotransmission. In the present study, we provide evidence which demonstrates that BMI-sensitive tonic inhibitory currents are indeed reduced in principal neurons of the DG and CA1 in the hippocampus following 4SD. Such changes are not likely due to fluctuations in synaptic communication, as determined by mIPSC analyses, making the presynaptic release, synaptic receptor mobilization or internalization unlikely.

In the DG, the function of δGABA$_A$Rs and α5GABA$_A$Rs, assessed with THIP activation and L-655,708 block of tonic currents, respectively, are not affected by 4SD, despite the loss of tonic currents observed overall. Future studies of reuptake and spontaneous GABAergic currents may provide insight into the 4SD-sensitivity of tonic inhibitory currents in DGGCs.

In the CA1, we observed an increase in currents activated by THIP and a decrease in L655,708-sensitive currents following 4SD, suggesting that increased δGABA$_A$R and decreased α5GABA$_A$R function. It may be that the increase in δGABA$_A$R-mediated currents is a compensatory response to a loss of α5GABA$_A$R-mediated currents but is inadequate to maintain tonic currents. Future studies of tonic inhibition and its susceptibility to acute sleep deprivation would be expanded by studies designed to investigate spontaneously gated tonic inhibition, the activity of membrane GABA transporters, and subunit expression, localization, and post-translational modification state of GABA$_A$Rs, especially α5GABA$_A$R and δGABA$_A$Rs.

In the context of epilepsy, the baseline state of tonic GABAergic inhibition and its relative susceptibility to 4SD remain to be determined. Many FDA approved anti-seizure drugs impart enhancement of GABAergic inhibition, and several target tonic inhibition (Greenfield, 2013). If 4SD-induced loss of tonic inhibition that we observe in seizure naive mice also occurs in epileptic animals, temporary treatment with tonic inhibition enhancing drugs may be a beneficial *ad hoc* therapeutic intervention when faced with acute sleep deprivation, especially for suppression of SD-induce seizure susceptibility in people with epilepsy.

Author Contributions

The author(s) declare their contributions as follows: Conceived and designed experiments: MJ, RM, EW. Performed experiments: EW, JE. Analyzed Data: EW. Contributed materials/reagents/ analysis tools: RM, MJ. Wrote the paper: EW. All authors reviewed the manuscript.

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Chapter 3 – Sleep dynamics and tonic inhibition in early Kainate-induced epileptogenesis

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Abstract

The development of epilepsy, or epileptogenesis, is a much-debated topic. It remains unclear which processes involved in structural and physiological changes give rise to spontaneous seizures after an initial seizure. Epileptogenesis is a term which encompasses these processes; it consists of a latent period between the first seizure(s) and the onset of epileptic seizures, and a chronic period driving disease progression. The relationship between sleep and seizures in epilepsy is an accepted clinical dogma, owing to ubiquitous sleep problems among people with epilepsy and the potency of sleep loss in triggering seizures. While recent evidence has begun to define the nature and mechanisms of this vicious cycle, much remains to be determined about its origins and contributions to driving seizure susceptibility. Here we sought to determine if sleep is affected in early epileptogenesis following kainate-induced status epilepticus (SE) by evaluating the dynamics of sleep architecture and EEG power spectra. Kainate-induced SE is a frequently used model of temporal lobe epilepsy (TLE) and leads to TLE-like pathologies in the hippocampus including cell death and circuit rearrangement. In Chapter 2, we determined that tonic inhibition in neurons of the hippocampus is sensitive to acute (4-hour) sleep deprivation. Expression of delta subunit-containing GABA receptors, which contribute to tonic inhibitory currents in the hippocampus, has been shown to be down-regulated within 4 days of pilocarpine-induced SE, which leads to hyperexcitability. The level of excitability of hippocampal neurons is known to affect characteristic activity during

slow-wave sleep. Here, we evaluate the electrophysiological effects of kainate-induced SE on inhibitory neurotransmission in the dentate gyrus (DG).

<u>Introduction</u>

Epileptogenesis:

Epilepsy is a neurological disease characterized by recurrent seizures and the manner with which seizures manifest varies greatly among individuals. The International League Against Epilepsy (ILAE) report a differential diagnostic schema of seizures that vary by type of onset (e.g., focal or general), the effect on awareness, and involvement of motor function (Fisher et al., 2017). The ILAE also defines epilepsy as the occurrence of 2 or more seizures separated by >24 hours. There are multiple known routes to the development of epilepsy, however the disease is known to have underlying neurological pathologies that lead to hyperexcitability that gives rise to elevated seizure susceptibility (Pitkänen et al., 2015). This state of hyperexcitability is founded in either genetic predisposition (e.g., channelopathy) or acquired from injury (e.g., high fever, encephalitis, dysplasia in the CNS, and traumatic brain injury) triggering acute and therefore non-epileptic seizures. It is thought that these initial seizures lead to anatomical and physiological changes within the network of neurons that were affected by the initial seizure. Collectively, the development of these pathologies leading to the development of epilepsy is termed epileptogenesis. Canonically, epileptogenesis was considered to be restricted to a 'latent' period between the time of initial injury and the development of spontaneous seizures, however the tendency of seizures to increase in frequency and severity after epilepsy has been diagnosed lends to the conclusion that the processes of epileptogenesis extend beyond the first epileptic seizure. It is unknown if the mechanisms contributing to latent and extended epileptogenesis are the same or arise from distinct processes.

Sleep problems in epilepsy:

Epilepsy causes disruptions in sleep (Foldvary-Schaefer & Grigg-Damberger, 2009). Conversely, sleep loss is a known and potent trigger for seizures, regardless of how well controlled the underlying disease is. This reciprocal interactivity of sleep loss and seizures forms a vicious cycle in epilepsy, which likely exacerbates the underlying disease state. As such, sleep deterioration is a plausible contributor to protracted epileptogenesis. Outside of surgical intervention, available drugs for the treatment of epilepsy are generally limited to controlling seizures, and are not always effective, especially in temporal lobe epilepsy (TLE) (Téllez-Zenteno & Hernández-Ronquillo, 2012). While beneficial, symptom management falls short of treating the underlying pathologies of epilepsy. Conceptually, antiepileptogenic treatments are an attractive option for effective prevention, treatment, or perhaps even cure for epilepsy. Sleep could be one such avenue for effecting successful treatment of epilepsy that extends beyond symptom management.

In mouse models of acquired epilepsy, sleep architecture progressively deteriorates following initial SE (Matos et al., 2018). Typically, nocturnal mice consolidate their rest and active periods to times when the environment is light and dark, respectively. Lack of consolidated rest-active patterns in epilepsy models is indicative of a breakdown of circadian controllers. Circadian rhythms are centrally coordinated by the suprachiasmatic nucleus (SCN), which encodes external lighting cues from the retina and acts to synchronize circadian activity in the periphery (Moore, 2013). It is known that direct stimulation of SCN neurons during normally quiescent periods is adequate to phase shift

circadian behavioral activity (Jones et al., 2015). The SCN is connected to multiple effectors and relay nuclei to form an extended circadian neural network through which it receives information from many regions of the brain (Morin 2013). In epilepsy, the broadly synchronous activity of seizures may affect one or more regions feeding into this circadian network. In this case, seizures may be effectively integrating into SCN electrical activity, which would broadcast erroneous information and compromise circadian rhythms.

Additionally, hyperexcitability in the epileptic condition may be contributing to dysregulation network activity during slow-wave sleep. Slow-wave sleep is associated with alternating periods of high and low cortical activity that are termed Up- and Down-states, respectively (Nir et al., 2011). Up-state duration and frequency are directly impacted by neuronal excitability and network activity (Levenstein et al., 2019). In the hippocampus, cortical network state has been correlated with the development of sharp-wave ripples in the CA1, which is thought to be mediated by elevated dentate synchrony driven by cortical Up-states (Sullivan et al., 2011). If network and neurons become hyperexcitable, as they do in epilepsy, regulation of network activity would likely be impaired.

Hippocampus:

In temporal lobe epilepsy (TLE), the hippocampus a common site of seizure generation (focus) and route to secondary generalization (Téllez-Zenteno & Hernández-Ronquillo, 2012). Chronic hippocampal pathologies associated with TLE include interneuron and hilar neuron cell death and mossy fiber sprouting in the dentate gyrus (DG), which are often modeled with induced status epilepticus (SE) in rodents with

chemoconvulsants (Kanic acid or pilocarpine) or electric kindling (Reddy & Kuruba, 2013).

Under normal conditions, DG gates activity entering the hippocampus with strong local

inhibitory neurotransmission mediated by interneurons (Andersen, 1975; Ewell & Jones,

2010; Freundl & Buzsáki, 1996). Mossy fibers are the axons of DG excitatory granule

cells, which normally send unidirectional projections into the hilus and cornu amonis

(CA)3. These hippocampal pathologies mentioned act to impair DG-gating abilities by

reducing interneuron mediated inhibition, as well as forming recurrent excitatory mossy

fiber projections back into the DG (Franck et al., 1995; Krook-Magnuson et al., 2015).

These changes in local circuitry can lead to hyperexcitability of the DG and CA3, which

is likely a driver of epileptiform activity.

Inhibitory interneurons mediate synaptic and tonic inhibitory currents (Buzsáki,

1984). Synaptic inhibition mediates rapid, point-to-point inhibitory communication via the

release of gamma-aminobutyric acid (GABA) on a dense population of synaptic ionotropic

$GABA_A$ receptors ($GABA_A Rs$). GABA then rapidly diffuses out of the synapse into the

extracellular space (Glykys & Mody, 2007). Ambient extracellular GABA concentrations

are regulated by sequestration into surrounding astrocytes and GABAergic axon

terminals (Soudijn & van Wijngaarden, 2000). Ambient GABA also binds to $GABA_A$

receptors diffusely expressed in the neuronal membrane. Extrasynaptic $GABA_A Rs$ are

unique in their sensitivity to GABA, which allows relatively low GABA concentrations to

activate tonic inhibitory currents (Glykys et al., 2008). As discussed in Chapter 2, tonic

inhibition can be largely regulated by three elements: neurotransmitter release and

reuptake, and modulation of receptors (e.g., function, expression, localization). We have

demonstrated that transient sleep deprivation leads to reduced tonic inhibition in principal

neurons of both DG and CA1. This is likely a homeostatic response to prolonged potentiation of GABA neurotransmission from elevated neural activity associated with waking vigilance.

In the present study, we aim to determine if early epileptogenesis will lead to a similar down-regulation of tonic inhibition. Additionally, we aim to assess the dynamics of sleep response to acute sleep deprivation during early epileptogenesis.

Methods

Animals:

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin School of Medicine and Public Health (UWSMPH). Animals were housed in the vivarium at the University of Wisconsin. Male C57bl/6 mice 5-6 weeks of age (Envigo, Madison, WI) were used for chronic EEG and tonic inhibition experiments.

Epidural electrode implantation and electroencephalography recordings:

Electroencephalographic (EEG) electrode implantation was performed at P35-45 for all animals as previously described (Wallace et al., 2015). Briefly, mice were anesthetized with isoflurane (5% induction, 1-2% maintenance). After confirming the plane of anesthesia, the incision site was prepared by shearing and disinfecting the scalp with alternating betadine-70% ethanol swabs. After exposing the skull, 4 stainless steel screws were implanted (bregma +1.5 mm:lateral ±1 mm, bregma -3 mm:lateral -1 mm, and lamda -1 mm at midline). Stainless steel wire was attached to the left frontal, right parietal, and occipital screws for EEG acquisition, while the other two served as anchors for the head cap. Two stainless steel braided wires were placed in the nuchal muscles for electromyography (EMG) recording. After a 72-hour recovery, EEG/EMG was acquired in five sessions over 9 weeks, each lasting ~7 days (Figure 1A). EEG and EMG signals were digitized with an XLTek amplifier (XLTEK, USA) sampling at 512 Hz. All recordings were conducted in standard (12:12, light:dark) lighting. Access to food and water *ad libitium* was ensured throughout.

Kainate-induced status epilepticus:

Induction of status epilepticus (SE) was attained by repeated, low-dose administration of kainate receptor agonist, kainic acid (KA, Tocris cat no. 0222), as previously described. Briefly, a solution was made by dissolving KA in a saline vehicle with a working concentration of 1.25 mg/ml. Repeated low-dose treatment involved an initial dose of 10 mg/kg KA administered via intraperitoneal injection, followed by 5 mg/kg KA doses every 20 minutes until SE was observed. Criteria for SE was two Racine class 5 seizures occurring within a 20-minute window. Class 5 on the Racine scale for behavioral seizure classification is characterized by forelimb clonus, rearing, and loss of postural control (Racine 1972). Vehicle control mice were treated with volume-matched injections of saline vehicle.

Sleep scoring and NREM delta power analysis:

For sleep scoring, recordings were processed with bandpass (1-70 Hz for EEG and 3-500 Hz for EMG) and notch (60Hz) filters and imported into Sirenia Sleep Pro analysis software (Pinnacle Technology, Inc.). Vigilance states were manually scored in 4-second epochs. Waking states were identified by high EMG amplitude. Sleep states were identified as epochs with relatively quiescent EMG and further differentiated based on predominant EEG power; Non-rapid eye movement sleep (NREMs) consisted of large, slow EEG activity (1-4 Hz), while low amplitude, rhythmic theta activity (5-7 Hz) dominated rapid eye movement (REM) sleep. To calculate power spectral density, the bandpass filter was removed, and each 4-second segment of EEG that was scored as NREMs was

transformed with an FFT to determine its power spectral density. Power in the delta (1-4 Hz), theta (6-9 Hz), sigma (10-14), and gamma (25-100 Hz) bands was calculated. Delta power was normalized to the sum of each band calculated.

Acute sleep deprivation:

All animals were housed in individual EEG recording chambers. In each 7-day session, mice (saline n=3, KA n=7) underwent sleep deprivation after several days of baseline recording. Sleep deprivation began at the start of the light period and was achieved via gentle handling lasting 4 hours (4SD, Figure 1A). Briefly, animal behavioral and EEG activity was monitored for behaviors indicative of sleep onset (e.g. nesting, extended behavioral arrest, the onset of large-amplitude, slow waves in EEG). When these were observed, the animal was gently aroused or the recording cage was slightly shifted, thereby encouraging persistent waking vigilance. Due to the potential collateral effect of SD procedures impacting other animals being recorded, all animals were subjected to SD simultaneously.

Electrophysiology solutions:

Solutions were prepared including high-sucrose cutting solution (CS, containing in mM: 80 NaCl, 24 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 25 D-glucose, 0.5 $CaCl_2$, 4 $MgCl_2$, 75 sucrose, 1 Na-L-Ascorbate, and 3 NaPyruvate) and artificial cerebrospinal fluid (aCSF, containing in mM: 125 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 25 d-glucose, 2 $CaCl_2$, 1 $MgCl_2$ and adjusted to 325 ± 2 mOsmol with sucrose). High-chloride (KCl) intracellular solution was made in a batch to ensure constancy of the intracellular milieu across data

sets and contained in mM: 140 KCl, 10 EGTA, 10 HEPES, 2 Mg_2ATP, 0.3 NaGTP, and 20 phosphocreatine and was adjusted to pH 7.3 with 5 N KOH and 310 mOsmol. All solutions were aerated with a 95% O_2/5%CO_2 gas mixture.

Patch-Clamp tonic inhibition and mIPSC recordings:

Mice were anesthetized with isoflurane and perfused transcardially with ice-cold CS prior to decapitation and brain isolation. Horizontal hippocampal slices (400 μm) were prepared as described in (Bischofberger et al., 2006) in ice-cold CS with a vibratome (VT1000s Leica, Nußloch, Germany). Slices were then transferred to a holding chamber filled with a warm (37°C) 1:1 mixture of CS:aCSF for 1 hour, after which the temperature was allowed to equilibrate to room temperature (22°C).

After transfer to a submersion recording chamber, slices were equilibrated for 5 minutes. Cells were visualized with an upright microscope (Axioskop, Zeiss, Germany) equipped with infra-red differential interference contrast (IR-DIC) optics, CCD camera and controller (Hamamatsu, Japan). DGGCs were identified visually based on morphology and anatomical location. We acquired whole-cell patch recordings in the voltage-clamp configuration (-70 mV, room temp.) with a Multiclamp 700B amplifier (Molecular Devices, San Jose, CA) filtered with a 2 kHz low-pass, 4-pole Bessel filter, digitized at 20 kHz with a Digidata 1333 (Molecular Devices, San Jose, CA) and acquired with Axograph X on a Macintosh G4 computer. Borosilicate patch pipettes were filled with KCl intracellular solution and had a resistance of 2-5 MΩ when submerged in aCSF. GABAergic currents were isolated with a base solution containing 100 nM tetrodotoxin (TTX, Tocris, cat: 1069), 25 μM D-(-)-amino-5-phosphonovaleric acid (AP5, Tocris, cat: 0106), and 5 μM

6,7-dinitroquinoxaline-2,3-dione (DNQX, Tocris, cat: 2312). Cell stability was assessed with a 5-minute baseline voltage-clamp recording prior to the initiation of tonic current assays. Resting membrane voltage (V_m) and cellular parameters calculated from current responses to a -5 mV step command (i.e., membrane capacitance (C_m), series resistance (R_s), input resistance (R_{in})) were recorded before and after any drug application. Recordings were included in analyses if R_s was less than 10 MΩ and drifted less than 2 MΩs throughout the recording. To measure tonic inhibitory currents, 6-minute recordings were acquired, in which we perfused base solution for 1-2 minutes, followed by perfusion of the base solution with 100 μM Bicuculline Methiodide (BMI, Tocris, cat: 2503).

Analysis and Statistics:

Tonic currents were calculated from 60-second segments of steady-state voltage-clamp traces, converted to all-point histograms, to which a Gaussian function was fit to the outward half of the distribution, yielding a mean current and standard deviation. The current density was calculated by normalizing the difference between calculated mean steady-state holding currents from before and after bicuculline application to the membrane capacitance of the cell. Statistical differences between tonic currents of control and 4SD recordings were evaluated with unpaired, two-tailed t-tests.

Miniature IPSCs (mIPSCs) were detected using Axograph X with a variable amplitude template calculated by fitting a double exponential to a consensus IPSC waveform (rise: 0.48 ms, decay: 16 ms) and amplitude threshold 4 times the holding current standard deviation. Median values were determined for each cell before being combined into lists based on SD treatment. Unpaired, two-tailed t-tests were used to

compare median inter-event interval (IEI, latency previous mIPSC except for the first event, which is calculated as the latency from start) and amplitude values from each recording. Using the bootstrapping method, we created 1,000 probability distribution functions (PDFs) from lists drawn randomly and consisting of half the total number of detected mIPSCs for each metric and treatment group (e.g., amplitude*saline and IEI*KA, etc). From each set of bootstrap-generated PDFs, an estimated mean distribution was generated with 95% confidence intervals for each metric.

To evaluate longitudinal sleep dynamics and response to sleep deprivation, two-hour segments (ZT 4-6) were scored for vigilance states as described above and schematically represented in Figure 1A. Percent time in each vigilance state, as well as normalized EEG Delta (nDelta) power of the period following sleep deprivation, were compared to the same period the previous baseline day. For each metric collapsed across time, differences between groups at baseline and post-SD timepoints were assessed with an unpaired two-tailed t-test and between baseline and post-SD within treatment groups was tested with a paired two-tailed t-test. To compare the effect of treatment across time, each metric was analyzed with a two-way ANOVA with a Tukey-Kramer corrected post hoc multiple comparisons test. All statistical analyses were considered significant with p-values less than 0.05.
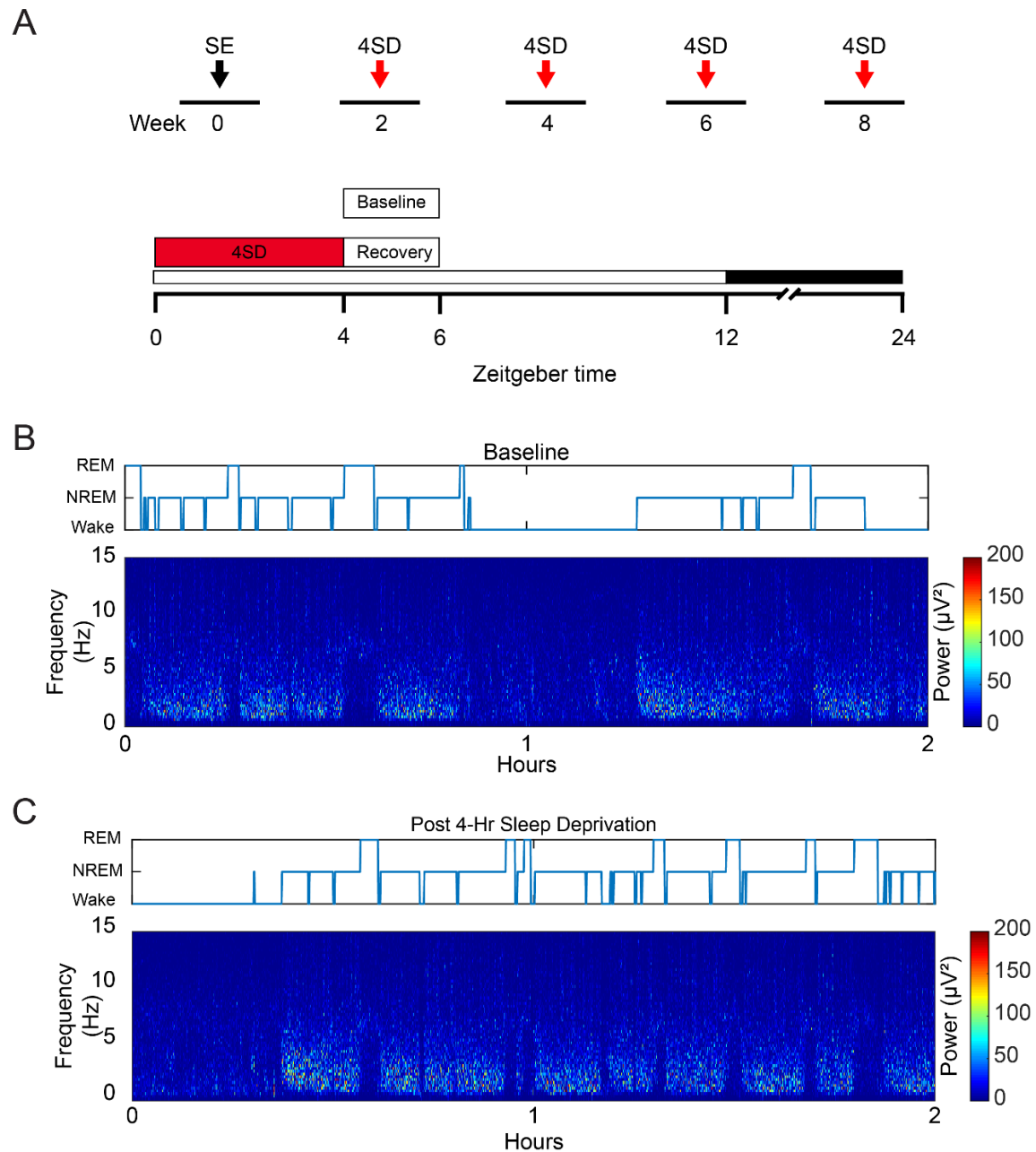
Figure 1: Experimental design of post-SE sleep deprivation and EEG analysis.

The effects of kainate-induced status epilepticus on sleep were assessed with chronic encephalography (EEG) recordings. Experimental design for recording periods and acute sleep deprivation (4SD) are shown in panel A. Shown above, EEG was recorded for several days before and following SE-induction during week 0, and 4SD on recording weeks 2, 4, 6, and 8. The bottom timeline indicates the timing of 4SD, as well as baseline and recovery sections used for the analysis of sleep and NREM Delta EEG. Representative graphs of scored vigilance states (i.e., hypnograms, top) and associated EEG power spectra across the 2-hour analysis window are shown for Baseline (B) and post-4SD recovery periods (C).

Results

Sleep efficiency is not significantly altered by KA-induced status epilepticus:

While it is known that sleep is perturbed in epilepsy, it is largely unknown when those sleep disruptions begin in the process of epileptogenesis. In the present study, the response to 4SD was evaluated every 2 weeks for the first 2 months after KA-induced SE. We first compared sleep efficiency (i.e., the percentage of time spent awake, and in NREM and REM sleep) of baseline days between KA-treated mice and saline controls of all weeks combined and found that there was no significant effect of KA treatment in the amount time spent awake (Figure 2, Saline: 29.37±9.5%, KA: 29.23±14.9%, p>0.05), NREM (Figure 3, Saline: 61.96±7.9%, KA: 60.76±12.6%, p>0.05) or REM (Figure 4, Saline: 8.67±1.9%, KA: 10.0±3.3%, p>0.05). We also compared the percent time following sleep deprivation between KA-treated and control mice. Again, there was no significant effect of KA treatment in wake (Figure 2, Saline: 36.22±10.4%, KA: 31.13±13.4%, p>0.05), NREM (Figure 3, Saline: 55.17±9.5%, KA: 59.86±11.5%, p>0.05), or REM (Figure 4, Saline: 8.56±1.9%, KA: 9.0±3.1%, p>0.05). Within-group comparisons showed no significant differences from baseline to post-4SD (p>0.05). We then turned our focus to assessing the effects of SD on sleep efficiency within treatment groups as a function of time from seizure induction. Percentages of each vigilance state following 4SD were set as a ratio to that of the prior day. We did not observe any significant effect of treatment or time in SD-induced changes to post-4SD:baseline ratio of wake (Figure 2C, Time: $F_{(3,28)}=1.86$, p<0.05, Treatment $F_{(1,28)}=0.24$, p>0.05), NREM (Figure 3C, Time: $F_{(3,28)}=1.15$, p>0.05, Treatment $F_{(1,28)}=1.47$, p>0.05), or REM sleep (Figure 4C, Time: $F_{(3,28)}=0.06$, p>0.05, Treatment $F_{(1,28)}=0.96$, p>0.05). Multiple comparisons at each

time point revealed no significant differences (p>0.05). The results suggest that any changes to sleep efficiency that may occur chronically do not manifest within the first two months following KA-induced SE. This may be loose evidence that large disruptions in sleep architecture do not precede the establishment of chronic seizures in epilepsy.
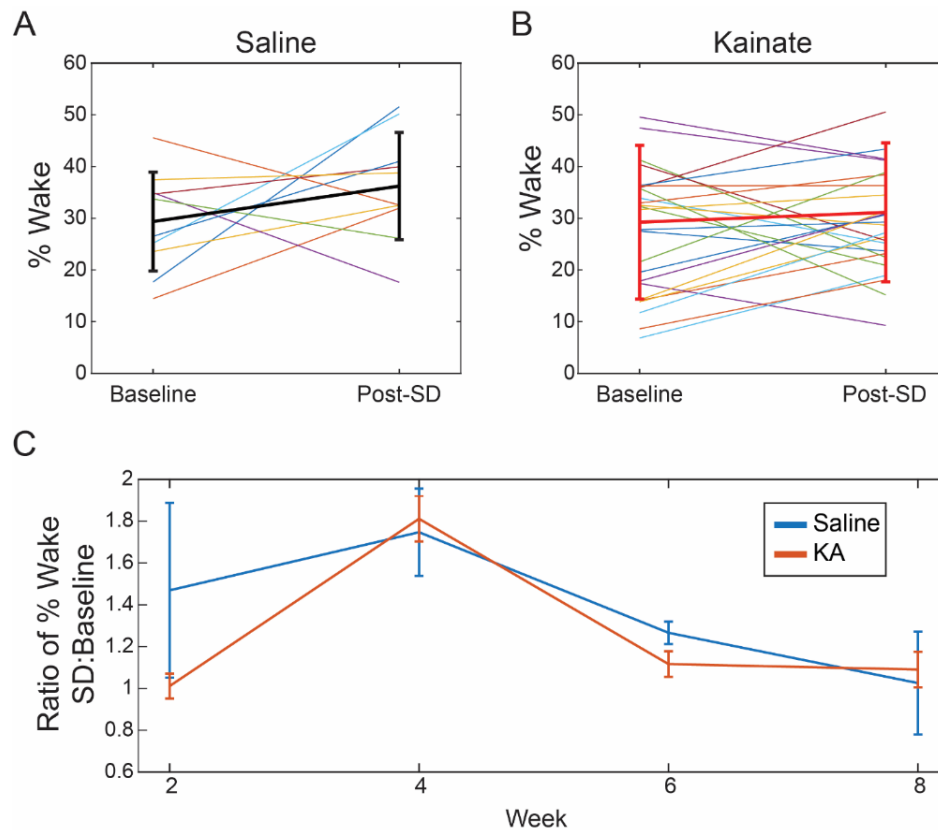

Figure 2: Post-SE analysis of 4SD-induced changes to wake.

Two-hour segments of EEG after 4SD and same period of the previous day were scored for vigilance states. Percentage of time awake of each week are pooled in for saline (A) and KA-treated (B) animals graphed individually with overlaid average ± standard deviation. Ratios of post-4SD:baseline percent time awake are graphed as average ratio ± standard deviation as a function of timepoint. Wake percentages between saline and KA-treated groups was assessed with unpaired, t-test at baseline (Saline: 29.37±9.5%, KA: 29.23±14.9%, t(34)=0.027, p>0.05) and recovery (Saline: 36.22±10.4%, KA: 31.13±13.4%, t(34)=1.08, p>0.05). Analysis of change in percent wakefulness from baseline to recovery periods within animals with a paired t-test did not reveal significant effects of 4SD for saline-treated (t(25)=1.31, p>0.05) and KA-treated (t(9)=0.85, p>0.05) mice. Two-way ANOVA analysis of recovery:baseline ratios of percent time awake did not significantly differ between treatment groups (F(1,28)=0.24, p>0.05) or across time (F(3,28)=1.86, p<0.05). Multiple comparisons showed that there were no significant differences at any time point (p>0.05).
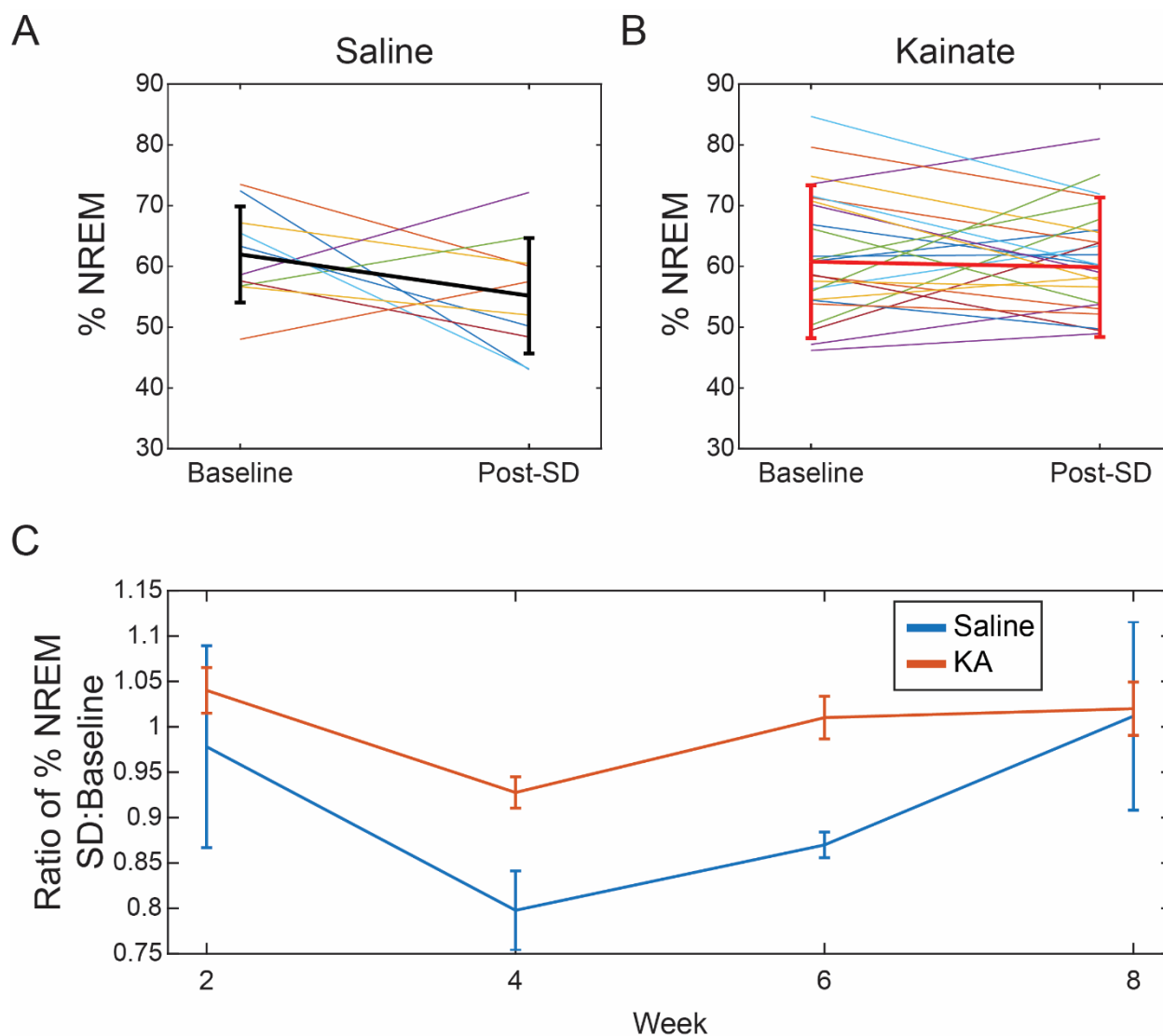
Figure 3: Post-SE analysis of 4SD-induced changes to NREM.

Two-hour segments of EEG after 4SD and same period of the previous day were scored for vigilance states. Percentage of time in NREM sleep of each week are pooled in for saline (A) and KA-treated (B) animals graphed individually with overlaid average ± standard deviation. Ratios of post-4SD:baseline percent time in NREM sleep are graphed as average ratio ± standard deviation as a function of timepoint. NREM percentages between saline and KA-treated groups was assessed with unpaired, t-test at baseline (Saline: 61.96±7.9%, KA: 60.76±12.6%, t(34)=0.28, p>0.05) and recovery (Saline: 55.17±9.5%, KA: 59.86±11.5%, t(34)=1.15, p>0.05). Analysis of change in percent NREM from baseline to recovery periods within animals with a paired t-test did not reveal significant effects of 4SD in saline-treated (t(9)=0.16, p>0.05) and KA-treated (t(25)=0.48, p>0.05) mice. Two-way ANOVA analysis of recovery:baseline ratios of percent time in NREM sleep did not significantly differ between treatment groups (F(1,28)=1.47, p>0.05) or across time (F(3,28)=1.15, p>0.05). Multiple comparisons showed that there were no significant differences at any time point (p>0.05).
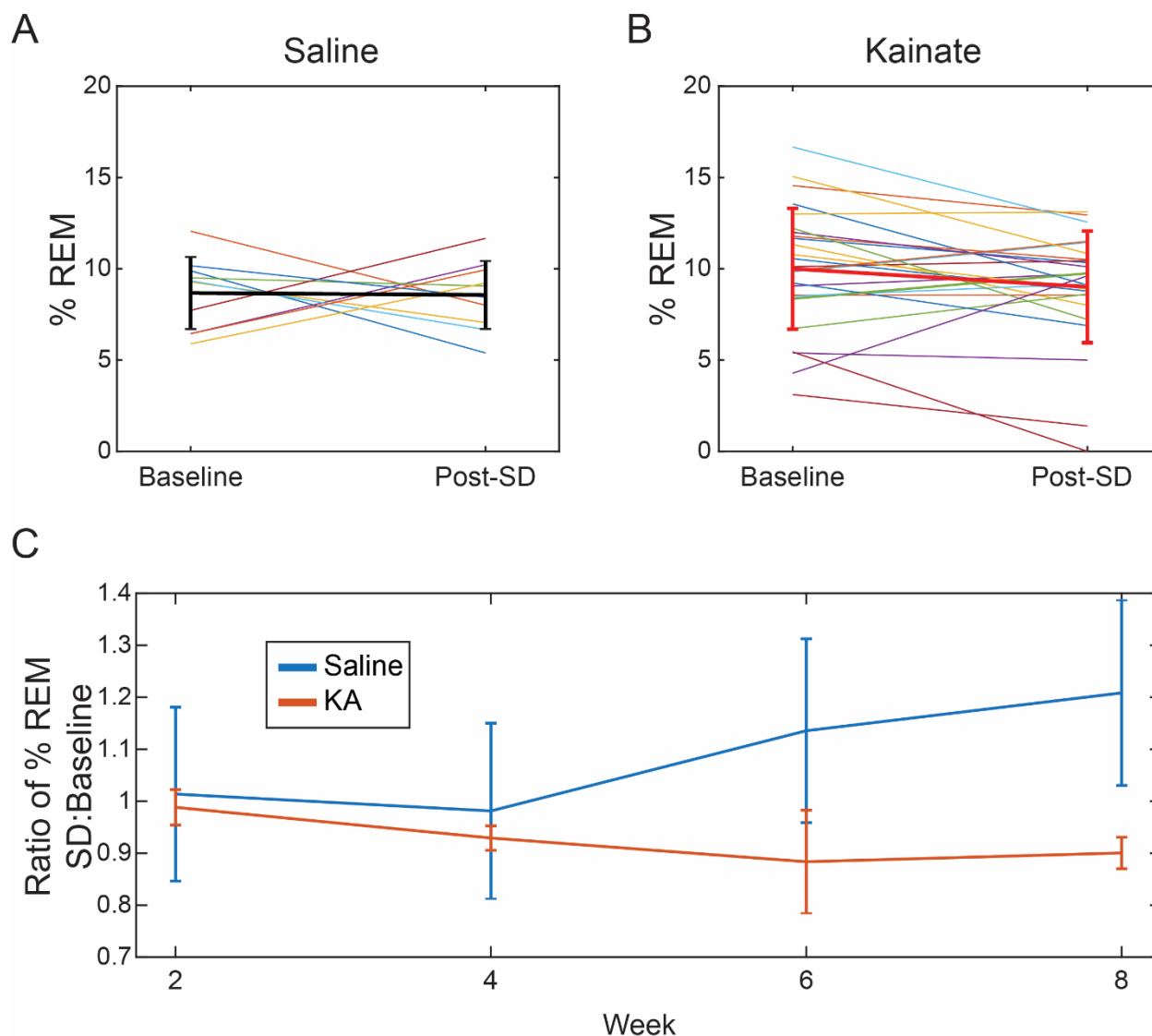
Figure 4: Post-SE analysis of 4SD-induced changes to REM.

Two-hour segments of EEG after 4SD and same period of the previous day were scored for vigilance states. Percentage of time in REM sleep of each week are pooled in for saline (A) and KA-treated (B) animals graphed individually with overlaid average ± standard deviation. Ratios of post-4SD:baseline percent time in REM sleep are graphed as average ratio ± standard deviation as a function of timepoint. REM percentages between saline and KA-treated groups was assessed with unpaired, t-test at baseline (Saline: 8.67±1.9%, KA: 10.0±3.3%, t(34)=0.25, p>0.05) and recovery (Saline: 8.56±1.9%, KA: 9.0±3.1%, t(34)=0.43, p>0.05). Analysis of change in percent REM from baseline to recovery periods within animals with a paired t-test did not reveal significant effects of 4SD for saline (t(9)=0.09, p>0.05) and KA-treated (t(25)=1.98, p>0.05) mice. Two-way ANOVA analysis of recovery:baseline ratios of percent time in REM sleep did not significantly differ between treatment groups (F(1,28)=0.96, p>0.05) or across time (F(3,28)=0.06, p>0.05). Multiple comparisons showed that there were no significant differences at any time point (p>0.05).

Dynamics of 4SD-induced potentiation of NREM delta power are altered by KA-induced SE:

Characteristics of vigilance states are an important piece to the puzzle of how epileptogenesis may affect sleep, however, another perspective is the potentiation of sleep intensity induced by prolonged periods of wakefulness. EEG delta power of NREM sleep has been correlated with the physiological response to wakefulness. Using the EEG designated as NREM sleep, we calculated normalized Delta (nDelta) power as described in the methods. Comparing mean NREM nDelta between KA-treated and saline controls, we saw comparable levels at baseline (Saline: 2.52±0.3 au, KA 2.43±0.7 au, $p > 0.05$) and after 4SD (Saline: 3.03±0.4 au, KA 2.64±0.8 au, $p > 0.05$). Within groups, we observed elevated NREM nDelta power in both saline-treated (Figure 5A, $p = 0.02$) and KA-treated (Figure 5B, $p = 0.04$) mice. We then assessed whether 4SD-induced potentiation of NREM nDelta power is dynamic over the 8 weeks of the experiment. We saw that NREM nDelta power potentiation was impaired in KA-treated animals as demonstrated by significant effects of time (Figure 5C, $F_{(3,28)} = 4.93$, $p < 0.05$) and treatment (Figure 5C, $F_{(1,28)} = 5.39$, $p < 0.05$), though multiple comparisons at each time point revealed no significant differences ($p > 0.05$). It is interesting to note that saline-treated animals had steadily increasing 4SD-induced potentiation of NREM nDelta power across time, while KA-treated animals exhibited much more variable responses over time.
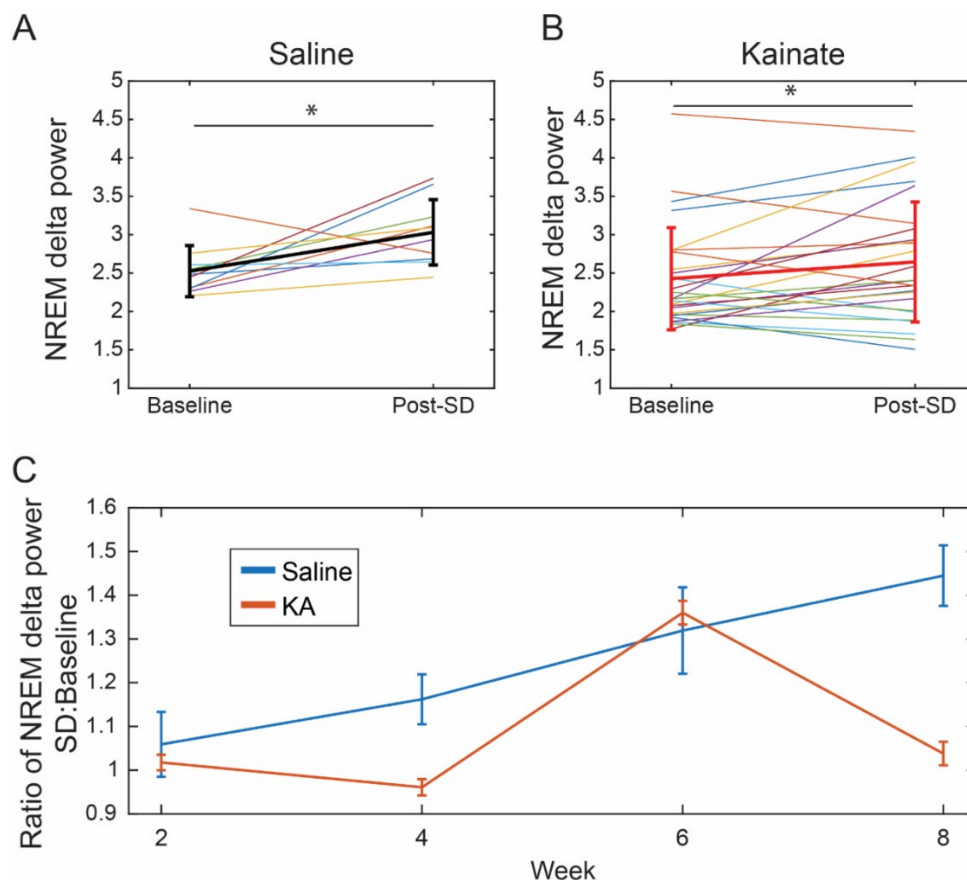
Figure 5: Post-SE analysis of 4SD-induced changes to NREM delta power.

Two-hour segments of EEG after 4SD and same period of the previous day were scored for vigilance states. EEG was parsed into 4-second segments and processed with a fast-Fourier transform to determine frequency spectral content. Segments correlating to NREM sleep were isolated and used to calculate normalized Delta (nDelta) power, by normalizing delta (1-4 Hz) power to summed power of delta, theta (5-9 Hz), sigma (10-15 Hz), and gamma (25-100 Hz) bands. NREM nDelta power of each week are pooled for saline (A) and KA-treated (B) animals graphed individually with overlaid average ± SD. Ratios of post-4SD:baseline NREM nDelta are graphed as average ratio ± standard deviation as a function of timepoint (panel C). NREM nDelta power between saline and KA-treated groups was assessed with unpaired, t-test at baseline (Saline: 2.52±0.3 au, KA 2.43±0.7 au, t(34)=0.44, p>0.05) and recovery (Saline: 3.03±0.4 au, KA 2.64±0.8 au, t(34)=1.47 p>0.05). We compared the change in NREM nDelta power from baseline to recovery periods within animals with a paired t-test and found significant potentiation of NREM nDelta power of 4SD for saline (t(9)=2.73, p<0.05) and KA-treated (t(25)=2.18, p<0.05) mice. Two-way ANOVA analysis of recovery:baseline ratios of NREM nDelta power demonstrated significant effects of treatment groups (F(1,28)=5.39, p<0.05) and across time (F(3,28)=4.93, p<0.05). Multiple comparisons showed that there were no significant differences at any time point (p>0.05).

Tonic inhibition is chronically reduced following KA-induced SE

Following the previous report that pilocarpine-induced SE impaired tonic inhibition in the DG, we investigated the state of GABAergic inhibition in the DG 8 weeks after the KA-induction of SE (Peng et al., 2004). We observed that tonic GABAergic current densities were significantly smaller in KA treated animals than saline controls (Figure 6B, Saline: 0.25±0.14 pA/pF, Post-KA: 0.13±0.11 pA/pF, p<0.05).
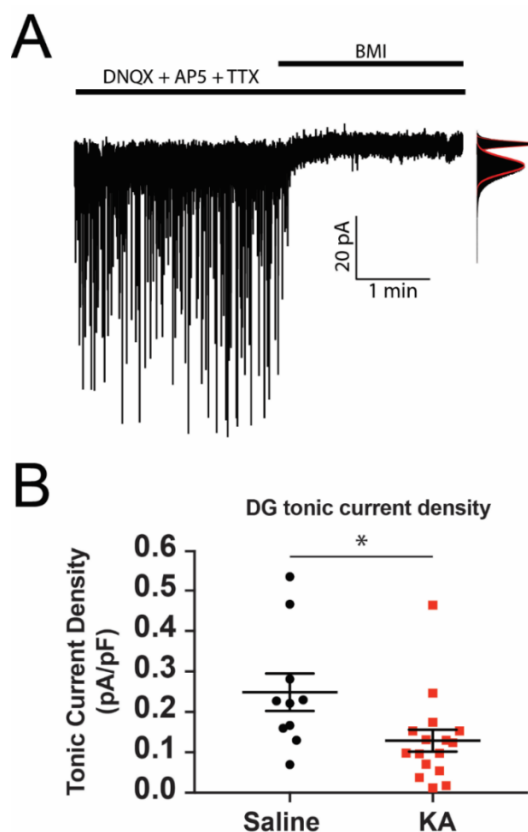

Figure 6: Analysis of SE-induced changes to tonic GABAergic currents in the dentate gyrus.

Tonic GABAergic currents were measured 8 weeks following saline or KA-induced SE.
A representative trace in panel A shows baseline recordings in the presence of DNQX, AP5, and TTX, followed by GABA$_A$R blockade with bicuculline methiodide (BMI). Steady-state currents were determined by fitting a Gaussian function to an all-points histogram generated with 60-second segments before and after BMI perfusion, shown to the right of the representative trace. The difference between mean currents was normalized to the membrane capacitance (C$_m$) to calculate the current density (pA/pF). Panel B summarizes mean current density ± SEM for recordings made in dentate gyrus granule cells of saline (black circles) and KA-treated (red squares) animals, demonstrating a significant reduction of tonic currents following KA-induced SE.

As mentioned, tonic inhibition is mediated by ambient GABA that is normally generated by synaptic release. We aimed to measure basal (non-spike driven) mIPSCs. From the detected mIPSCs (see methods above), we determined the median values of amplitude and IEI. We did not detect significant changes between the means of median amplitude (Figure 7A: Saline= 6.04±1.2 pA, KA= 5.63±1.2 pA, p>0.05) or IEI (Figure 7B: Saline=0.51±0.18 sec, KA=0.54±0.16 sec, p>0.05). Further bootstrap analysis of the underlying distribution of mIPSC amplitudes (Figure 7C) and IEIs (Figure 7D) revealed no substantial deviations of actual or approximated distributions, strengthening the results of the analysis of median values. Given the stability of mIPSC frequency and amplitude, the demonstrated reduction of tonic inhibition is difficult to explain in the current experimental schemata. Two other elements that are influential in regulating tonic inhibition (i.e., reuptake and receptor function) are the next likely candidates to mediate the loss of tonic GABAergic currents in KA-treated animals in the early stages of epileptogenesis.
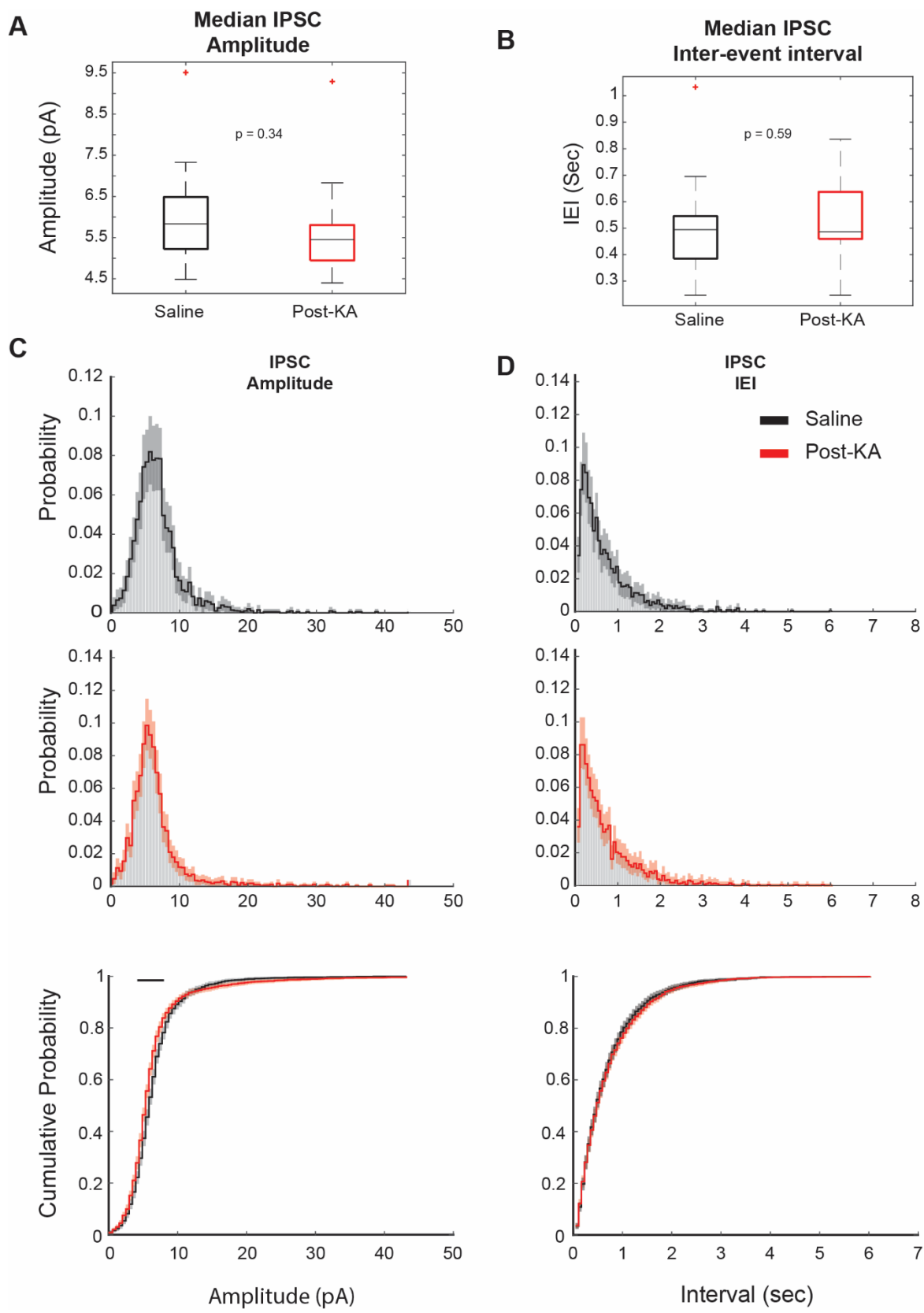
Figure 7: Analysis of SE-induced changes to synaptic GABAergic currents in the dentate gyrus.

Dentate gyrus granule cell miniature inhibitory postsynaptic currents (mIPSCs) were detected in voltage-clamp patch-clamp recordings in DG granule cells in the presence of AP5, DNQX, and TTX. Amplitude and inter-event interval were measured for each mIPSC detected from saline control (n= 2,019 mIPSCs, from 10 recordings) and KA-treated (n=2,814 mIPSCs, from 16 recordings). Median values of each metric and each recording were determined before pooling the data based on treatment type and plotted as box-and-whisker plots for amplitude (panel A) and IEI (panel B). We found no significant difference between control and KA-treated groups in mIPSC amplitude (panel A, Control=6.04±1.2 pA, n=10; Post-KA=5.63±1.2 pA, n=16, red, p>0.05) or median inter-mIPSC interval of DG granules cells (panel B, Control=0.51±0.027 sec, n=10; Post-KA=0.540.16 sec, n=16, red, p=0.06. Using combined lists, we assessed the overall distribution of amplitude and IEI of detected mIPSCs. We determined 95% confidence intervals using the bootstrap method with 1,000 iterations. We graphically represented these bootstrap analyses in probability distribution functions (PDFs) of amplitudes (panel C) and inter-event intervals (panel D). In PDFs, gray=original data, and overlaid lines are the mean bootstrap PDF with shaded 95CI range, (top = control, black, middle = 4SD, red) and compared the distributions of mIPSC metrics detected in 4SD to those acquired from saline treated controls by plotting mean ± 95CI in cumulative distribution functions (CDFs, bottom). Bins with non-overlapping 95CIs suggest statistical significance and are denoted as with bars above the CDF. Though several individual bins of the amplitude distribution showed statistical significance, we did not observe any substantial difference between distributions.

Discussion

In the present study, we characterized sleep during the early period of epileptogenesis, as well as evaluating GABAergic currents in the dentate gyrus of the hippocampus. We report that KA-induced SE does not significantly affect the proportion of wake, NREM, or REM at baseline, and neither do those proportions significantly change following 4-hour SD. Grouped analysis of NREM nDelta power demonstrates that sleep deprivation causes a significant potentiation of NREM nDelta power in both treatment groups. However, the pattern of 4SD-induced potentiation across early epileptogenesis was much more variable in animals that experienced KA-induced SE than saline-treated controls, which exhibited a consistent increase over the 2 months of the experiment.

The onset of sleep disturbances following induced SE may provide insight into the state of epileptogenesis and provide easily quantifiable biomarkers for disease progression. One study of sleep regulation after pilocarpine-induced SE (piloSE) has been conducted previously, assessing circadian rhythmicity of spontaneous locomotor activity (SLA) and transcription of core members of the circadian Clock. Matos et al. (2018) describe a progressive deterioration of SLA patterns and onset of seizures within two months of piloSE. Cosinor analysis showed that the general circadian pattern of SLA was not completely ablated in piloSE animals, though they exhibited increased overall SLA and increased variability in daily timing of peak SLA In the same study, they showed increased mean transcription of *Per1* at one month, and progressively decreasing *Bmal1* and *Clock* at one- and two-months after piloSE. *Per1* is a transcriptional repressor of the circadian Clock and is subject to regulation by electrical activity via CREB-mediate

transcription (Welsh et al., 2010). Elevated *Per1* transcription may be due to hyperactivity integrating into the extended circadian neural network and modulating the activity of the central circadian pacemaker in the suprachiasmatic nucleus (Morin, 2013), ultimately confusing appropriate SCN-mediated light entrainment and activity of local and peripheral physiology under circadian control.

NREM delta power is a measure of slow cortical synchrony and is associated with the intensity/depth of sleep. Cortical network activity during slow-wave sleep is characterized by Up- and Down-states, during which neuronal spiking frequency is elevated or low, respectively (Nir et al., 2011). Down-states become more frequent the deeper/more intense the NREM sleep is, which appears as an increase in delta power of cortical EEG signals (Levenstein et al., 2019). State frequency and duration are regulated by the intrinsic excitability of neurons as well as local and distant network activity. In the hippocampus, periods of hyper-synchronicity generate sharp wave-ripples, which are temporally correlated with the timing of cortical Up-states of slow-wave sleep (Levenstein et al., 2019). This points to the possibility that highly synchronous activity in the hippocampus and cortex can influence the occurrence of slow-waves or sharp wave-ripples, respectively. Indeed, stimulation of the DG has been shown to drive synchronous activity in the entorhinal cortex in urethane-anesthetized rodents (Bragin et al., 2012), but whether this occurs during spontaneous sleep has not been directly tested. Hippocampal pathologies of TLE driving hyperexcitability and deterioration of the DG's gating ability may drive an increase in sharp wave-ripple and cortical Up-state generation between seizures. This may underlie reports that focal epilepsy increases NREM delta power (Boly et al., 2017). In which case, our findings demonstrating variable, but *not* elevated, NREM

delta power suggest that major changes to sleep occur after the onset of spontaneous recurrent seizures. Circadian rhythms, however, may begin to deteriorate before seizure onset.

Hyperactivity after KA-induced SE may also be causative in the reduction of tonic inhibition that we showed in DG granule cells. GABA$_A$Rs are subject to activity-mediated downregulation of function and surface expression (Barnes, 2001). In the hippocampus, tonic currents are mediated by receptors containing either an α5 or δ subunit (Glykys et al., 2008). Indeed, it has been shown that surface expression of δ subunits was significantly decreased within 4 days of piloSE, which was sustained into the chronic seizure phase (Peng et al., 2004). In the KA-induced SE model, it has been shown that transcription of the δ subunit gene (*Gabrd*) is rapidly down-regulated, which would likely have a similar impact on surface expression (Khan et al., 2019). If the kainate model exhibits similar, long-term changes in δ subunit protein levels following transient repression of *Gabrd* expression, it would explain how tonic GABAergic currents are reduced while mIPSC amplitude and frequency remains unaffected in the 2 months after KA-induced SE.

Pathologies of chronic SE epilepsy models often include loss of inhibitory interneurons and hilar neurons. The latter is thought to be a trigger for the reorganization of mossy fibers, thinking that a loss of DG granule cell targets may lead to those axon fibers to attempt reacquisition of a synaptic connection (Dengler & Coulter, 2016). The physiological consequences of local rewiring of DG circuitry depend on which cell types are receiving recurrent projections. Both hyperexcitation and hyperinhibition are possible in post-SE models of TLE, with pro- and anti-seizure effects (Heng et al., 2013; Sloviter,

1992). The effects of mossy fiber sprouting on seizures have been directly tested, and blockade of mossy fiber reorganization was found to be insufficient in blocking seizures (Heng et al., 2013). This emphasizes the roles of intrinsic neuronal properties, as well as feedforward and feedback inhibitory neurotransmission that are altered early in epileptogenesis.

In summary, epileptogenesis is a spectrum of processes, incurring early and late effects on neuronal function and circuits in the hippocampus. We demonstrate that tonic GABAergic currents are significantly reduced following KA-induced status epilepticus. KA-treated mice also exhibit more variable 4SD-induced potentiation of NREM nDelta power during the first 2 months. Early changes in intrinsic excitability may be a mechanism of more chronic pathologies including selective neuron death and subsequent network reorganization often seen in TLE.

Limitations:

Our study of NREM nDelta power is limited to the assessment of how it responds to 4SD, without directly assessing dynamics across time and within NREM bouts. As such, we cannot make any conclusions about sleep homeostasis. Our understanding of sleep regulation during early epileptogenesis would benefit from an expanded investigation into the wake-sleep relationship in EEG NREM nDelta power. These analyses presented did not assess epileptiform activity (e.g., interictal spikes/high amplitude discharges), which limits our understanding of the effects that such activity has on sleep regulation. Future studies should include the detection of epileptiform activity and evaluation of associated effects on sleep (e.g., latency to sleep, NREM nDelta power and sleep efficiency).

Author Contributions

The author(s) declare their contributions as follows: Conceived and designed experiments: MJ, RM, EW. Performed experiments: EW. Analyzed Data: EW. Contributed materials/reagents/ analysis tools: RM, MJ. Wrote the paper: EW. All authors reviewed the manuscript.

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Chapter 4 – Altered Circadian Rhythms and Attenuated Oscillation of Clock Genes and Sirtuin1 in Epileptic Kcna1-null Mice

Abstract

Objectives:

Circadian rhythms are affected in many neurological disorders. While sleep disturbances are known in epilepsy, data on circadian rhythm disturbances in epilepsy are sparse. Here we examined light-entrained and circadian rest-activity and sleep-wake patterns in *Kcna1*-null mice which exhibit spontaneous recurrent seizures and is a model of sudden unexpected death in epilepsy (SUDEP). Furthermore, we sought to determine whether seizures or aberrant oscillation in levels of transcripts and proteins of core Clock members and a regulator, Sirtuin 1 (Sirt1), are associated with disrupted rhythms.

Methods:

We used passive infrared actigraphy to assess rest-activity patterns, electroencephalography for seizure and sleep analysis and Reverse Transcription PCR and Western blotting to evaluate transcription of Clock genes (*Bmal1*, *Clock*, *Per1/2*) and *Sirt1* in *Kcna1*-null and wild-type mice.

Results:

Epileptic *Kcna1*-null animals have disrupted light-entrained and free-running rest-activity patterns, tending to exhibit prolonged circadian periods. The electroencephalographic analysis confirmed disturbances in sleep architecture with more time spent awake and less in sleep. Though all epileptic mice manifested disrupted light-entrained and free-running rest-activity patterns, we found no correlation between actual seizure burden and degree of sleep disruption. However, we found attenuated oscillations of several Clock genes (i.e., *Clock, Bmal1, Per1, and Per2*) in the anterior hypothalamic region.

Interpretation:

Attenuated oscillation in the transcription of several core Clock genes correlates with, and may underlie, aberrant light-entrained and free-running rest-activity and sleep-wake patterns observed in *Kcna1*-null mice. This could contribute to late complications in epilepsy, such as SUDEP. Sirt1 may represent a useful therapeutic target for rescuing circadian Clock gene rhythmicity and sleep patterns in epilepsy.

Introduction

Epilepsy is a disorder that affects approximately 50 million people worldwide, with ~35% of patients remaining intractable, despite therapeutic advancements (Coan & Cendes, 2013; Laxer et al., 2014). Intractable epilepsy progressively alters brain function, developing complications that can include death (Coan & Cendes, 2013). Sudden unexpected death in epilepsy (SUDEP) is the leading cause of mortality related to epilepsy, the mechanisms of which are poorly understood (Devinsky et al., 2016).

We and others have shown that epileptic *Kcna1*-null mice, a model of SUDEP (Glasscock et al., 2010), have a circadian pattern of seizures, aberrant rest-activity rhythms, and progressively increasing seizure burden and rest deficit preceding death (K. A. Fenoglio-Simeone et al., 2009; Iyer et al., 2018; Wright et al., 2016). Such effects of seizures have not been as extensively defined in humans, despite the preponderance of sleep complaints among people with epilepsy (Baxter, 2005). Reciprocal interactions between seizures and sleep regulation may constitute a mechanism that drives the development of complications in epilepsy and possibly contribute to SUDEP. Understanding the mechanisms of disrupted circadian rhythms and sleep may serve to inform future therapies.

Circadian rhythms refer to any process with a cycle lasting about a day. Sleep is one of many physiological processes under circadian regulation. Circadian rhythms are regulated by a core circadian pacemaker in the suprachiasmatic nucleus (SCN). Activity in the SCN is strongly entrained by light via excitatory input from the retina but is also influenced by emotional and metabolic state (Mohawk & Takahashi, 2011; Yamaguchi et al., 2003). Central to SCN function is a transcription-translation autoregulatory feedback

loop of 'Clock' proteins, namely BMAL1, CLOCK, PER1-3, and CRY1/2. CLOCK:BMAL1 heterodimers bind to E-box enhancer regions in *Per* and *Cry* promoters, which drives transcription (Ko & Takahashi, 2006). Once translated, PER and CRY are subject to phosphorylation, allowing subsequent inhibition of the CLOCK:BMAL complex (Kondratov et al., 2003; Schibler & Sassone-Corsi, 2002). Many genes in the SCN show a circadian oscillation; such Clock-controlled genes (CCGs) affect many cellular functions including excitability via differential expression of several ion channels (Colwell, 2011; Kuhlman & McMahon, 2004). In the context of epilepsy, seizures may drive input to the SCN, potentially causing aberrant entrainment and neuronal hyperexcitability, ultimately contributing to disease state exacerbation.

Mechanistically, Clock-mediated transcriptional activation of CCGs via acetylation of histones and its binding partner BMAL1 enable access of transcriptional machinery and stability of the CLOCK:BMAL1 heterodimer at its promoter site (Etchegaray et al., 2003; Hirayama et al., 2007). BMAL1 acetylation can be counter-regulated by Sirt1, a nicotinamide (NAD+)-dependent deacetylase that is expressed in a circadian manner and whose absence is known to be associated with disrupted behavioral rhythms (Chang & Guarente, 2013). The acetylation state of BMAL1 has been shown to be important in CRY-mediated inhibition of the CLOCK:BMAL1 transcription factor and normal entrainment of circadian behavior (Xu et al., 2015). Several studies have shown that mutations or deletion in many of these core genes impact circadian rhythms, strengthening the notion that these genes are critically involved in circadian rhythm generation (Allada et al., 2001; Jones et al., 2013; Ko & Takahashi, 2006). In conditions where the amplitude of Clock gene oscillations are low or decreasing (as in aging),

circadian rhythms can be restored through positive modulation of SIRT1 (Chang & Guarente, 2013).

Here, we demonstrate that both light-entrained and free-running rest-activity and sleep patterns are altered in epileptic *Kcna1*-null mice. We found that seizure frequency did not correlate with the degree of sleep disruption, however, oscillations in transcript levels of several Clock genes were attenuated under light-entrained and constant dark (free-running) conditions. We hypothesize that perturbed regulation of Clock-controlled gene transcription may contribute to disrupted rhythms in *Kcna1*-null mice.

Methods

Animals:

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin School of Medicine and Public Health (UWSMPH). Animals were housed in the vivarium at the University of Wisconsin. Heterozygous mice (C3HeB.129S7-Kcna1[tm1Tem]/J, Jackson Labs) lacking one allele coding for the 1.1 isoform of voltage-gated potassium channels (Kv1.1) were used to produce mice of all genotypes. Homozygous knock-out mice (*Kcna1*-null animals) develop spontaneous seizures after the second postnatal week of life.

Lighting conditions:

We housed animals in either light-entrained (12hr-12hr, light-dark, [LD]) or constant-dark (free-running, DD) lighting conditions for experiments. Low-intensity red lighting was used for all necessary handling procedures and husbandry during lights-off.

Actigraphy:

We housed mice individually under either LD or DD in well ventilated, cylindrical Plexiglas cages, 6 inches in diameter and 10 inches in height. An infrared motion sensor suspended above the mice recorded gross locomotion. Individual movements were grouped in one-minute bins and plotted across time for analysis. As nocturnal animals, the greatest activity in LD-entrained mice is expected to occur during lights-off and decrease during lighted periods. When external light cues are removed, the organization

of locomotor activity reverts to a free-running pattern driven by internal pacemakers. Because the period of circadian rhythms in mice is shorter than the 24-hour entrained environment, the onset of active periods normally occurs earlier each subsequent day, appearing as a phase advance (Allada et al., 2001; Xu et al., 2015).

EEG electrode implantation, seizure recording, and sleep/wake cycle analysis:

Electroencephalographic (EEG) electrode implantation was performed at P34-36 for all animals as previously described (Wallace et al., 2015). Briefly, mice were anesthetized with isoflurane (5% induction, 1-2% maintenance). After confirming the plane of anesthesia, we sheared and disinfected the scalp. After making an incision to expose the skull, we implanted stainless steel screw electrodes: (bregma +1.5 mm and 1 mm right, bregma -3 mm and 1 mm left, and lamda -1 mm at midline). Two stainless steel braided wires were placed in the nuchal muscles for electromyography (EMG) recording. After a 72 hour recovery, we transferred mice into individual EEG acquisition chambers and allowed a >12 hour acclimation period. EEG and EMG signals were acquired continuously for two 5-day periods in light-entrained and constant darkness, with one day excluded from analysis while we transitioned animals between lighting conditions. Recordings were digitized with an XLTek amplifier (XLTEK, USA) sampling at 1024 Hz. We ensured ad libitium access to food and water.

Two independent reviewers identified seizures in the video-EEG records. For sleep staging, we processed recordings with bandpass (1-70 Hz for EEG and 3-500 Hz for

EMG) and notch (60Hz) filters and imported them into Sirenia Sleep Pro analysis software (Pinnacle Technology, Inc.). We manually scored vigilance states in 4-second epochs. We identified waking states as those epochs with high EMG amplitude, as shown in figure 1A. We scored epochs with relatively quiescent EMG as sleep and further differentiated them based on predominant EEG power; Non-rapid eye movement sleep (NREMs) consisted of large, slow EEG activity (1-4 Hz, Figure 1B), while low amplitude, rhythmic theta activity (5-7Hz, Figure 1C) dominated rapid eye movement (REM) sleep.
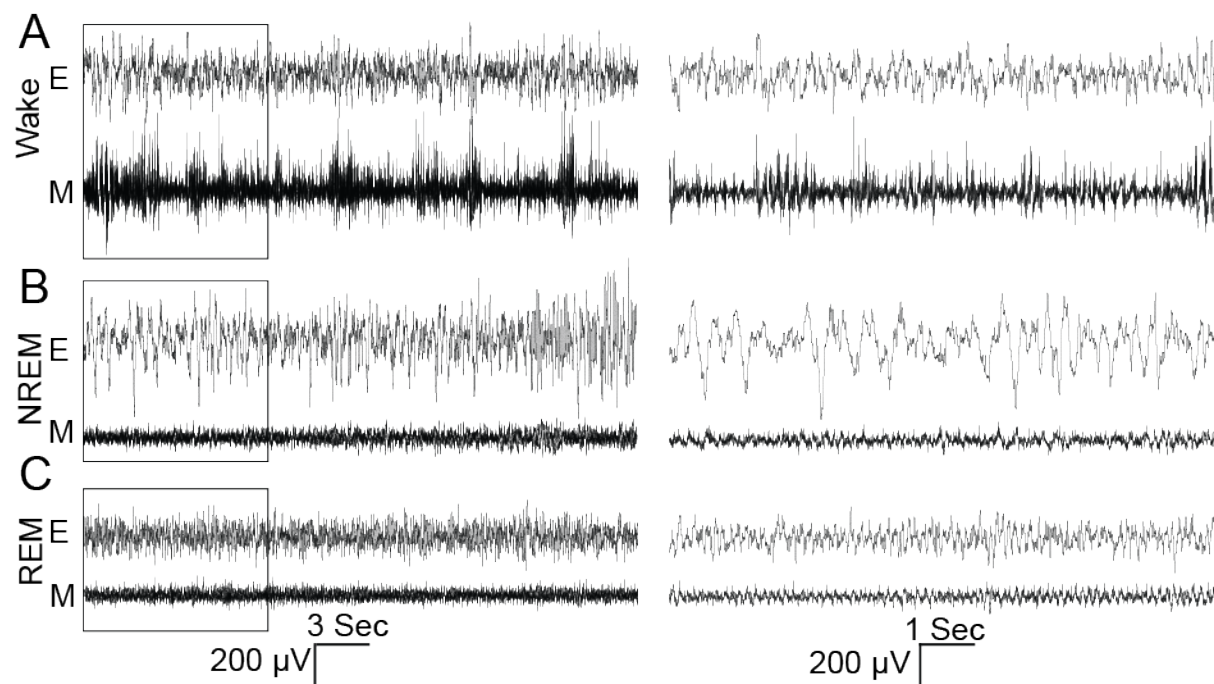

<u>Figure 1</u>: Criteria for EEG-based manual scoring of vigilance states.

Representative EEG and EMG traces of murine vigilance states are displayed in 30-second (left) and 10-second (right) windows. Four-second epochs which contained high EMG activity were scored as wake (A). Sleep was further divided into non-rapid eye movement (NREM) sleep where EEG was dominated by high-amplitude, delta activity (Figure 1B, 1-4 Hz), while epochs with low-amplitude, rhythmic theta (5-7 Hz) EEG activity were scored as rapid eye movement (REM) sleep (C).

RT-PCR and Western blotting:

We harvested tissue of WT and *Kcna1*-null mice (P30±1 or P45±3) at Zeitgeber times (ZT, hours from lights-on) 0, 6, 12, and 18 (n=4-6), using dim red light for any time-point when lights were off. Mice included in DD cohorts were held in constant darkness for 8 days prior to tissue procurement. The anterior hypothalamic region (~2 mm x 2 mm cube) immediately surrounding the SCN was rapidly isolated, bisected along the midline, and flash-frozen in liquid nitrogen. All samples were stored at -80ºC until processed simultaneously.

We isolated total mRNA using TRI Reagent (Sigma Aldrich) and standardized concentrations across samples. We synthesized cDNA from linearized total RNA with Superscript III First-Strand synthesis Supermix (Invitrogen) primed with random hexamers. We then conducted real-time PCR with SYBR green reagents and monitored amplification of target gene cDNA amplicons following a hot-start thermocycling protocol beginning with 2 minutes at 95°C, followed by 40 amplification cycles (15 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C, and 6-second imaging at 76°C). We confirmed primer specificity via melt-curve analysis (60-95°C: 0.5°C steps). Primer sequences are as follow: Bmal1-(fwd, 5'- CCCTAGGCCTTCATTGCACC-3'; rev, 5'-CATATTCTAACTGGTAGTCAGTGG-3'); Clock-(fwd, 5'-AAGATTCTGGGTCTGACAAT-3'; rev, 5'- TTGCAGCTTGAGACATCGCT-3'); mPer1-(fwd, 5'-GAAAGAAACCTCTGGCTGTTCCT-3'; rev, 5'-GCTGACGACGGATCTTTCTTG-3'); mPer2-(fwd, 5'-GCATATTCTAACTGGTAGTCAGTGG-3'; rev, 5'-

GTCTGAAGGCATCATCAGG-3'), Sirt1-(fwd, 5'-TCTGTCTCCTGTGGGATTCCT-3'; rev, 5'-GATGCTGTTCGAAAGGAA-3'), and Gapdh-(fwd, 5'-GACCTCAACTACATGGTCTACA-3'; rev, 5'-ACTCCACGACATACTCAGCAC-3'). We analyzed transcript levels with the $2^{\Delta CT}$ method, normalizing to the housekeeping gene Gapdh.

We isolated total protein using RIPA buffer containing phosphatase and protease inhibitors. Following isolation, we ran western blots, resolving protein samples in 4-15% gradient SDS-PAGE gels and transferring them to PVDF membranes. We blocked membranes with a 5% skim milk-TBST solution for 1 hour at room temperature. We then hybridized membrane-bound proteins overnight at 4°C with primary antibodies diluted in the above milk:TBST solution: αSIRT1 (1:1000 Millipore), αPER1 and αCLOCK (1:1000 and 1:100, respectively; Santa Cruz), αBMAL1 (1:1000, Abcam), αPER2 (1:1000, Millipore), and αβ-actin (1:10,000, MP Biomedical). We then incubated membranes with appropriate secondary antibodies conjugated with hydrogen peroxidase (Santa Cruz; 1:10,000) for one hour before chemiluminescent imaging (UVP ChemiDoc-it) with SuperSignal West Femto ECL reagents (Pierce Biochem). A common loading control was run in duplicate across all blots. We quantified target protein expression by normalizing signal intensity within samples relative to β-actin, then across blots relative to the median protein:β-actin ratio of the loading controls across all blots.

Data analysis:

We analyzed data using custom Matlab scripts, GraphPad Prism, and UVP VisionWorks LS imaging software. We calculated circadian period length using a χ^2 periodogram analysis built into the ActiView software (JD Instruments, Inc., Houston, TX). To test differences in period length and time spent in each vigilance state, we used multiple t-tests with a Holm-Sidak correction for multiple comparisons. We used the two-sample Kolmogorov-Smirnov (2K-S) test statistic to evaluate factorial differences in cumulative distribution functions (CDF) of bout lengths between epileptic and wild-type animals according to vigilance state (wake, NREM, and REM) and lighting condition. Expression of protein and mRNA levels were compared between Kcna1-null and WT mice with statistical significance of circadian oscillations and individual time-points determined via 2-way ANOVA with Sidak's correction for multiple comparisons. For all statistical analyses, we set the threshold of significance to p=0.05.

Results

Disrupted light-entrained and free-running rest-activity patterns:

In our wild-type controls held in LD, locomotor activity patterns are elevated during dark periods and decreased during the light periods, as had been previously reported (Oliverio & Malorni, 1979). When wild-type animals were placed in DD, the timing of locomotor activity reverted to the period of the internal circadian rhythm, which manifested as a daily advance of when active periods begin (figure 2A, left). In Kcna1-null mice, however, rest-activity patterns showed reduced consolidation of activity during dark periods when held in LD and consistent levels of activity in DD (figure 2A, right). Earlier studies established that circadian periods of rodents are slightly less than 24 hours (Benloucif & Dubocovich, 1996; Oliverio & Malorni, 1979). In our cohorts, using χ^2 periodograms, we found circadian period tended to be longer in epileptic mice, in both LD (KO: 29.38 ± 1.74, n=9, vs WT: 23.71 ± 0.13, n=6, p<0.05, Sidak t-test) and DD (KO: 27.26 ± 1.1627, n=9, vs WT: 23.68 ± 0.15, n=20, p<0.01, Sidak t-test) (figure 2C), demonstrating how light-entrainment and circadian control of behavior is significantly disrupted in *Kcna1*-null mice as compared wild-type littermates.
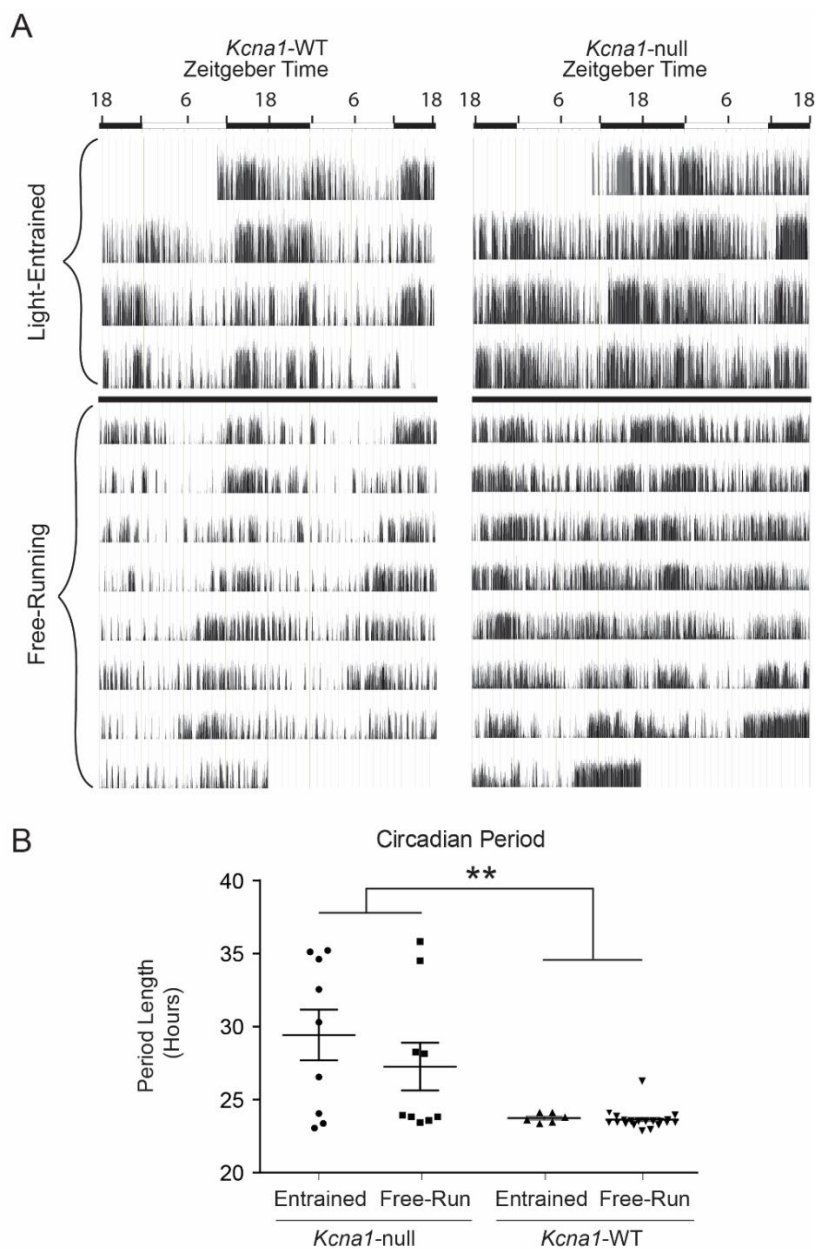
Figure 2: Analysis of circadian entrainment and period in light-entrained and free-running conditions.

Double-plot actigraphs (Panel A) show rest-activity patterns of wild-type (left) and *Kcna1*-null (right) mice held in light-entrained (12-12, light-dark, above) and free-running conditions (below). Wild-type mice entrained to light-entrained light conditions concentrate their locomotion to active periods during lights-off, the onset of which advances when they are transitioned to constant dark or free-running conditions (A, left). Locomotor activity of *Kcna1*-null mice was markedly elevated during the subjective day, indicating compromised entrainment to light cues and arrhythmic free-running behavior (A, right). Chi-squared periodograms of rest-activity patterns revealed that the mean circadian period in epileptic *Kcna1*-null animals was drastically increased over wild-type littermates in both light-entrained and free-running conditions (B, t-test, p<0.01).

Altered vigilance patterns and sleep architecture in Kcna1-null mice:

Because actigraphy only provides an indirect measure of sleep patterns, we then employed video-EEG based analysis of sleep in *Kcna1*-null mice and wild-type controls. Consistent with our observation of aberrant actigraphy, we found that *Kcna1*-null mice spent significantly more time awake than wild-type littermates in both LD and DD (figure 3A, WT: 53.24 ± 0.73%, n=28 days, 5 mice, vs KO: 66.53 ± 1.54%, n=50 days, 7 mice, p<0.0001, Sidak t-test; figure 3B, WT: 50.47 ± 0.97%, n=29 days, 6 mice vs KO: 70.0±1.258%, n=35 days, 7 mice, p<0.0001, Sidak t-test). As shown in the daily values of a representative animal (figure 3C and 3D), the increase in time spent awake is consistent across days of recording. This imbalance of sleep is mainly due to decreased time in non-rapid eye movement sleep (NREM), which is decreased in *Kcna1*-null animals, again independent of lighting condition (figure 3A, LD: WT:40.57±0.69% vs KO:29.68±1.51%, p<0.0001, Sidak; DD: figure 3B, WT:43.64±0.92% vs KO:26.84±1.18%, p<0.0001, Sidak). Time spent in REM was also significantly decreased in epileptic animals in both LD (figure 3A,WT:6.18±0.26% vs KO:3.794±0.1983%, p<0.0001, Sidak) and DD (figure 3B,WT:5.89±0.27% vs KO:3.153±0.1496%, p<0.05, Sidak) conditions. When we evaluated sleep architecture by compiling bout lengths according to genotype, vigilance state, and lighting condition, we found that all categories except light-entrained REM had significantly altered distributions. It is important to note that maximal bout lengths (indicated by triangles above the CDF plots in figure 3E-J) are comparable within condition, suggesting that differences are chiefly in bouts with less than maximal durations. As seen in figure 3E and F, wake bout lengths of mutant animals in both conditions are right-shifted, indicating a greater likelihood of longer bouts

(p<0.0001, 2K-S test). Distributions of NREM bout lengths for LD and DD are shown in figure 3G and H, respectively. While the distribution of NREM bout lengths of *Kcna1*-null mice shifted to the left of WT controls in DD (p<0.0001, 2K-S test), they were right-shifted for LD conditions, indicating generally longer bout lengths (p<0.0001, 2K-S test) despite reduced overall time in NREM. However, considering the total percentages (figure 3A), NREM is likely occurring in fewer, albeit longer, bouts in *Kcna1*-null mice under LD. Finally, REM bout distribution was not altered in *Kcna1*-null mice compared to WT littermates in LD (figure 3I, p=0.91, 2K-S test), but was affected in LD (figure 3J, p<0.0001, 2K-S test).
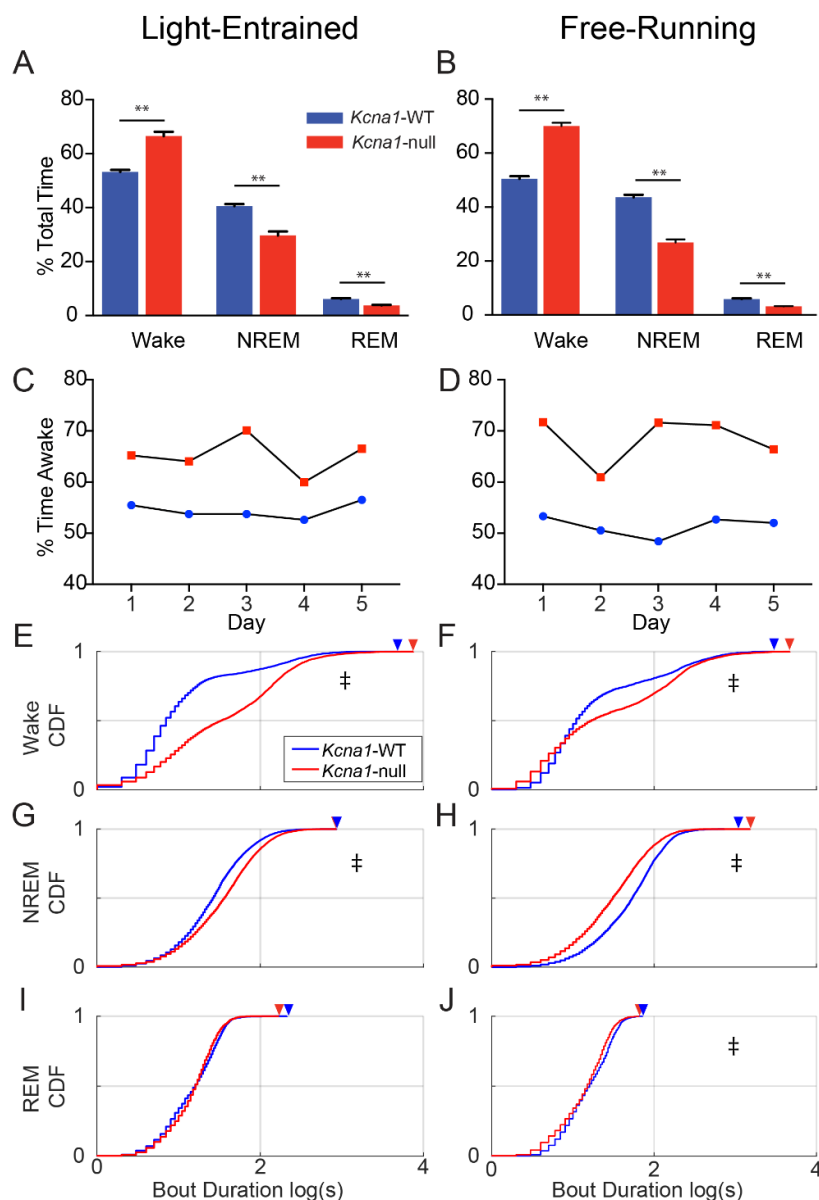
Figure 3: Analysis of sleep efficiency and architecture.

EEG-based sleep scoring shows perturbed sleep architecture in epileptic *Kcna1*-null mice (red fill), that spend more time awake and less time in either NREM or REM than wild-type (blue fill) in both light-entrained (A) and free-running (B) conditions (p<0.01). Daily time awake for representative animals over 5 days of light-entrained (C) and free-running (D) conditions show consistently increased time spent awake in epileptic animals (red) over age-matched wild-type (blue). Bout length distributions were also altered in epileptic mice (red lines) when compared to controls (blue lines). Two-sample Kolmogorov-Smirnov analysis of cumulative distribution function (CDF) of bout lengths in *Kcna1*-null mice are altered for Wake (E) and NREM (G) in light-entrained conditions and wake (F), NREM (H), and REM (J) in free-running conditions (‡ = p<<0.0001). Triangles above CDF plots indicate maximum bout lengths of *Kcna1*-null (red) or wild-type (blue).

Correlation between seizure frequency and sleep-wake patterns:

We have previously reported that *Kcna1*-null mice can have up to 78 seizures over a period of 10 days (Wright et al., 2016). Given the aberrant circadian rest-activity patterns and sleep architecture, we sought to determine the extent to which seizure frequency affects sleep architecture by first correlating daily seizure counts with several metrics of sleep, including mean length and number of bouts in each vigilance state. As illustrated in figure 4, we discovered that the number of daily wake bouts in epileptic mice increased slightly with the number of seizures detected in the same period, although the correlation was not statistically significant (R^2=0.045, p=0.14). Similarly, we found no relationship between seizures and bout counts of other vigilance states (data not shown). While we consistently observed extended, post-ictal periods of wakefulness, this analysis suggests that seizure frequency alone is insufficient in explaining the severely disrupted locomotor and vigilance patterns of our *Kcna1*-null animals.
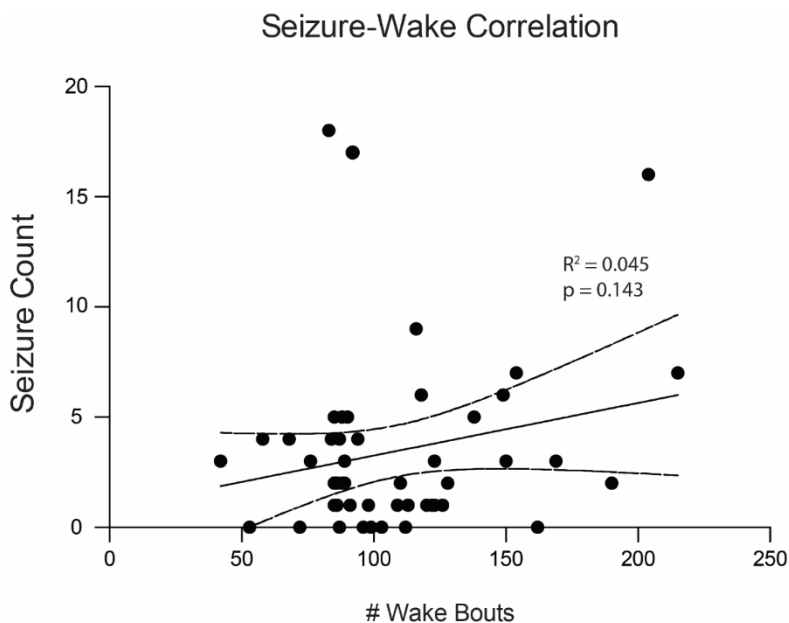
Figure 4: Analysis of seizure burden and sleep fragmentation.

Linear regression analysis of the number of wake bouts versus seizure within 24-hour periods did not reveal a significant correlation (R= 0.045, p=0.14), overlaid are the best fit line with 95%-confidence intervals for the correlation. Additional analysis of other sleep metrics showed no significant effect of seizure count on mean bout length or bout count for Wake, NREM nor REM.

Aberrant oscillation in core Clock members:

We then investigated other mechanisms of sleep regulation and asked whether the expression of Clock genes is altered. To this end, we investigated mRNA levels of *Clock*, *Bmal1*, *Per1*, and *Per2* in *Kcna1*-null and wild-type mice (n=3-4 /time point/ genotype/lighting condition). In LD, we found that all genes except *Bmal1* had significant oscillations indicated by a main effect of time (figure 5C: *Clock* $F_{(3,17)}=4.863$, $p<0.05$, figure 5E: *Per1* $F_{(3,16)}=66.28$, $p<0.0001$, figure 5G: *Per2* $(F_{(3, 17)} = 9.592$, $p<0.001$, 2-way ANOVA). Additionally, we found a main effect of genotype on the LD expression of *Clock* (figure 5C: $F_{(1,17)}=24.25$, $p=0.0001$, 2-way ANOVA), *Per1* (figure 5E: $F_{(1,16)}=20.90$, $p<0.001$, 2-way ANOVA), and *Per2* (figure 5G: $F_{(1,17)}=7.957$, $p<0.05$, 2-way ANOVA), where transcription was diminished in *Kcna1*-null mice when compared to wild-type controls. Assessment of individual time-points revealed a decrease in levels of *Clock* at ZT 0 and 6 (figure 5C: $p<0.001$, Sidak's multiple comparisons) and *Per1* at ZT 6 (figure 5E: $p<0.001$, Sidak's multiple comparisons) in mutants compared to wild-type mice housed in LD. Interestingly, these time-points often corresponded to peak daily expression, emphasizing the diminished drive of transcription in epileptic mice. In DD, we found that oscillations in transcription persisted in *Per1* (figure 5F: $F_{(3,21)}=35.42$, $p<0.0001$, 2-way ANOVA) and *Per2* (figure 5H: $F_{(3,21)}=9.014$, $p<0.001$, 2-way ANOVA). We also found that only *Per1* (figure 5F: $F_{(1,21)}=5.159$, $p<0.05$, 2-way ANOVA) showed a main effect of genotype, where again *Kcna1*-null animals showed reduced transcription when compared to controls.
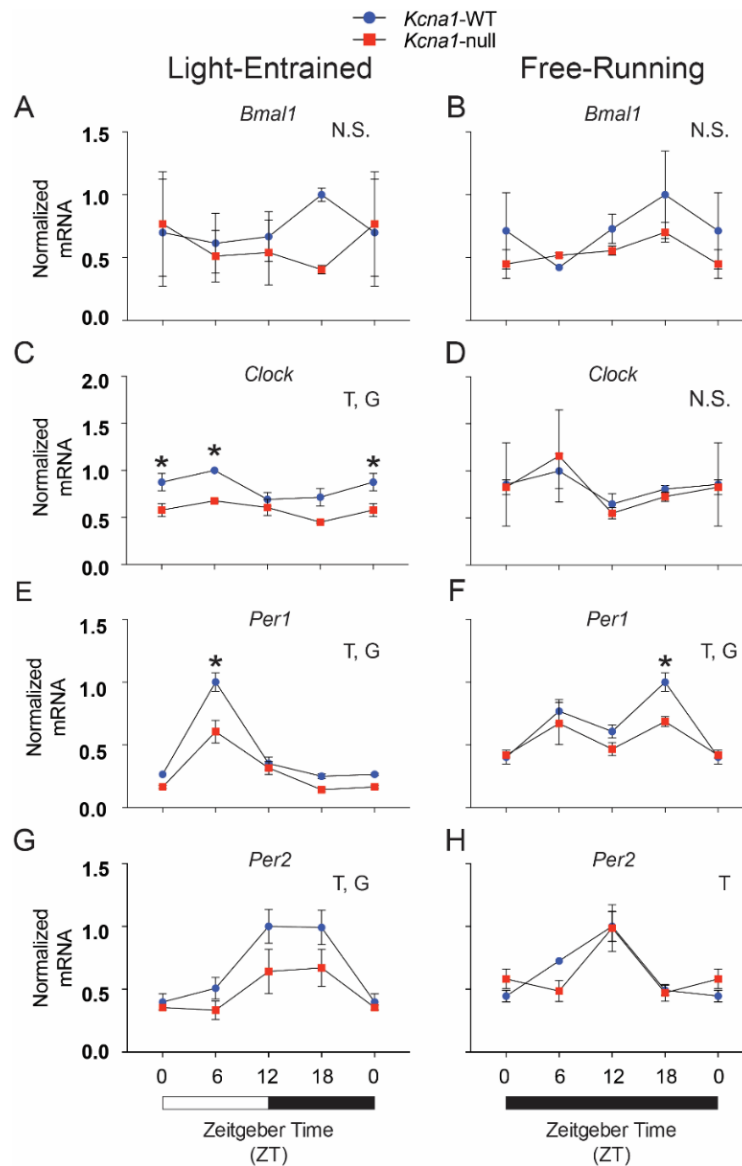
<u>Figure 5</u>: Analysis of circadian Clock mRNA expression patterns.

Transcript levels of four clock genes (*Bmal1*, *Clock*, *Per1,* and *Per2*) relative to *Gapdh* were measured in the SCN of epileptic *Kcna1*-null (red squares) and wild-type (blue circles) mice at four time-points (ZT 0, 6, 12 and 18) in light-entrained (A, C, E, G) and free-running conditions (B, D, F, H). Graphs included repeated values of ZT 0 at the right of each figure. Two-way ANOVA with Sidak-corrected multiple comparisons showed a main effect of time on the expression of *Clock*, *Per1,* and *Per2* (C, E, and G) in light-entrained and *Per1* and *Per2* (F and H) in free-running condition (annotated with a 'T' above). Of these, we saw a main effect of genotype of *Clock*, *Per1,* and *Per2* (C, E, and G) under light-entrained and *Per1* (F) under free-running conditions, indicating a reduced overall expression of mRNA (indicated above with a 'G'). Point-wise comparisons showed several time-points that were particularly diminished as indicated with an asterisk on the graph above (p<0.01, Sidak). Lighting conditions are denoted by bars at the bottom (filled=lights-off, open = lights-on). N.S. = not significant.

Given the changes in transcriptional profiles of several Clock genes in our *Kcna1*-null model, we then examined whether associated protein levels were disrupted as well. We found significant oscillations over time in PER2 in both LD (figure 6E: $F_{(3,40)}=3.79$; $p<0.05$, 2-way ANOVA) and DD (figure 6H: $F_{(3,32)}=3.856$; $p<0.05$, 2-way ANOVA). We found a main effect of genotype only in PER2 expression of mice held in DD (figure 6H: $F_{(1, 32)}=4.761$, $p<0.05$, 2-way ANOVA). Other Clock proteins tests (i.e., BMAL1, CLOCK, and PER1) did not exhibit significant oscillations across time or significant effect of genotype ($p>0.05$, 2-way ANOVA) Representative western blots are shown in figure 6I.
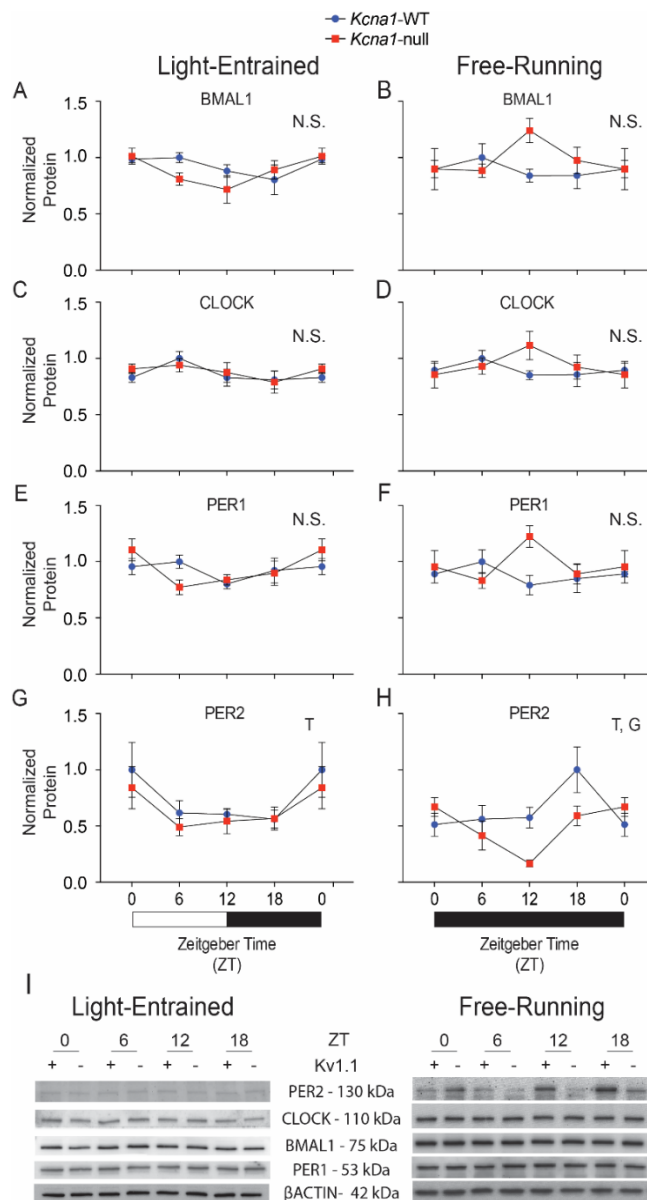
Figure 6: Analysis of circadian Clock protein expression patterns.

Protein expression patterns of four Clock proteins (BMAL1, CLOCK, PER1, and PER2) measured in the SCN of *Kcna1*-null (red squares) and wild-type (blue circles) at four time-points (ZT 0, 6, 12 and 18) in light-entrained (A, C, E, G) and free-running (B, D, F, H) conditions are shown above. Panel I shows representative western blots for each Clock gene protein and β-actin, zeitgeber time (ZT) and genotype (wild-type with a '+' vs *Kcna1*-null with a '-') are labeled above each lane. Two-way ANOVA with Sidak-corrected multiple comparisons showed a main effect of time in PER1 and PER2 in light-entrained conditions and PER2 in free-running conditions, annotated with a 'T' above. Of these, we saw a main effect of genotype in PER2 (H) in free-running conditions, indicated with a 'G'. Lighting conditions are denoted by bars at the bottom (filled=lights-off, open = lights-on). N.S. = not significant. Representative western blots for each core Clock protein and the internal control (β-actin) are shown in panel I.

In summary, we found that the majority of genotype effects were at the transcript level, PER2 being the only tested protein altered in epileptic animals. Overall, epileptic *Kcna1*-null mice showed dampened amplitude of oscillation and decreased average expression for *Clock*, *Per1*, and *Per2* in LD and *Per1* and PER2 in DD, when compared with age-matched wild-type littermates.

Sirt1 expression patterns in Kcna1-null mice:

Considering the dampened transcription of several Clock genes in *Kcna1*-null mice, and that similar decreases have previously been correlated with reduced Sirt1 in aging, we sought to determine if Sirt1 expression is altered in epileptic *Kcna1*-null mice (Chang & Guarente, 2013; Orozco-Solis & Sassone-Corsi, 2014). In light-entrained animals, we found a main effect of genotype and time in transcript levels (figure 6A, $F(1,16)=21.19$ and $F(3,16)=14.19$, $p<0.001$, respectively, 2-way ANOVA). Again, the expression level of *Sirt1* mRNA in *Kcna1*-null animals was lower than wild-type, especially at ZT 6 ($p<0.05$, Sidak) in LD. However, we did not find any significant differences in SIRT1 protein expression levels under LD or DD (Fig 7 C and D).
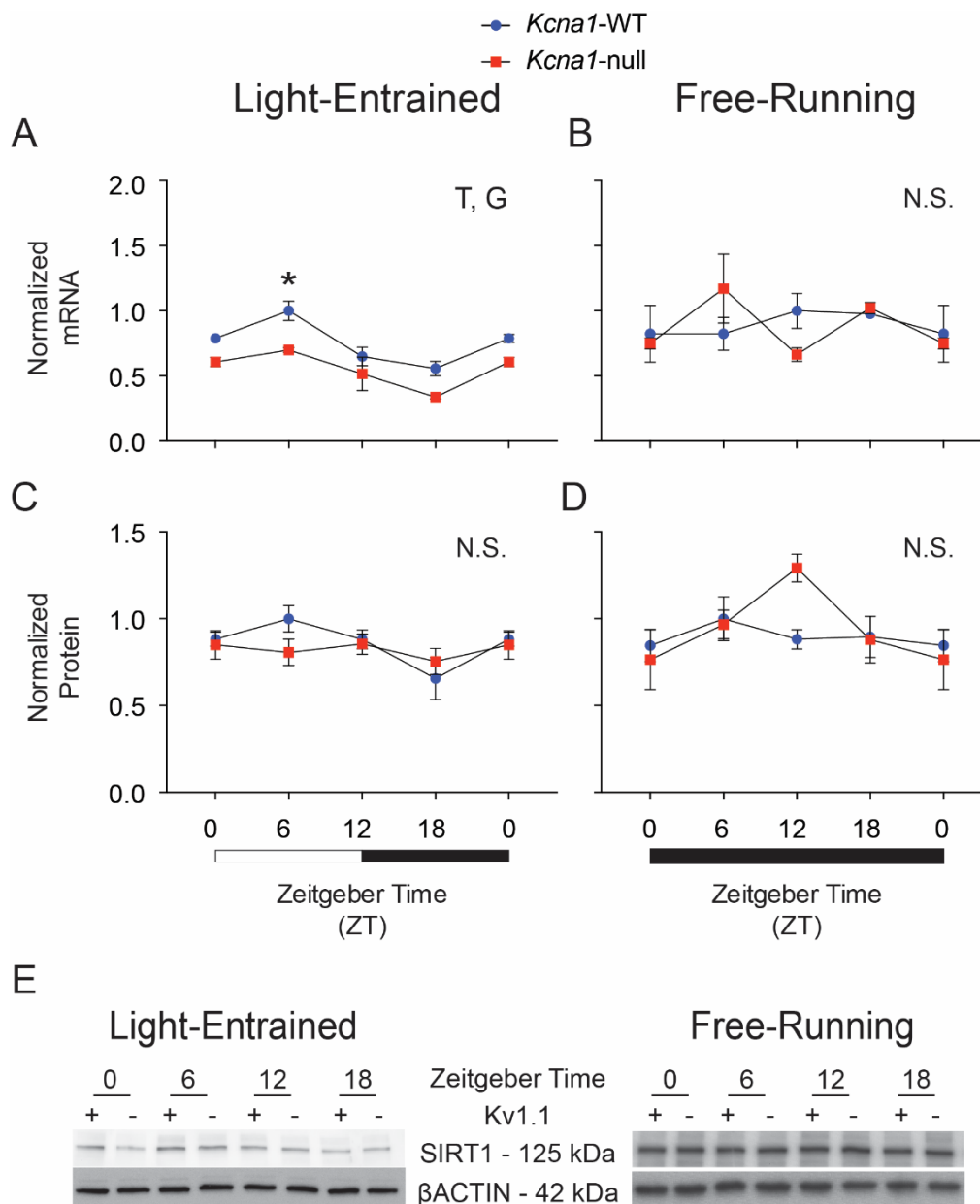
Figure 7: Analysis of circadian regulator Sirtuin 1 expression patterns.

Sirtuin 1 expression patterns of *Kcna1*-null (red squares) and wild-type (blue circles) are shown above for mRNA (A: light-entrained and B: free-running) and protein (C: light-entrained and D: free-running). Two-way ANOVA with Sidak-corrected multiple comparisons showed main effects of time and genotype in the expression of mRNA in light-entrained conditions (A), indicating that overall reduced expression in epileptic mice. Point-wise comparisons showed ZT 6 was particularly diminished, which is indicated with asterisks on the graphs above ($p<0.01$). Representative western blots for SIRT1 and β-actin are shown in E. Lighting conditions are denoted by bars at the bottom (filled=lights-off, open = lights-on). N.S. = not significant.

Clock gene and Sirt1 expression are not affected in young Kcna1-null mice:

Voltage-gated potassium channels are integral in shaping the excitability and firing dynamics of neurons, constituents of the circadian pacemaker within the SCN being no exception. As an important control to ensure that the absence of Kv1.1 did not play a significant role in Clock gene expression alterations, we assessed gene transcription patterns at post-natal day 30, an age at which seizures are uncommon. Indeed, we found no significant main effects of genotype in the mRNA expression of any Clock genes or *Sirt1* at this younger age (Figure 8 A and B, 2-way ANOVA, $p > 0.05$), although single-timepoint analysis did indicate an increase in *Per2* levels in epileptic animals over wild-type littermates ($p < 0.05$, Sidak). Similar Clock gene and Sirt1 expression at P30 suggests that genotype alone does not affect circadian rhythm maintenance, which is consistent with previous findings that rest-activity patterns in *Kcna1*-null mice did not differ from wild-type controls at this age (Iyer et al., 2018).
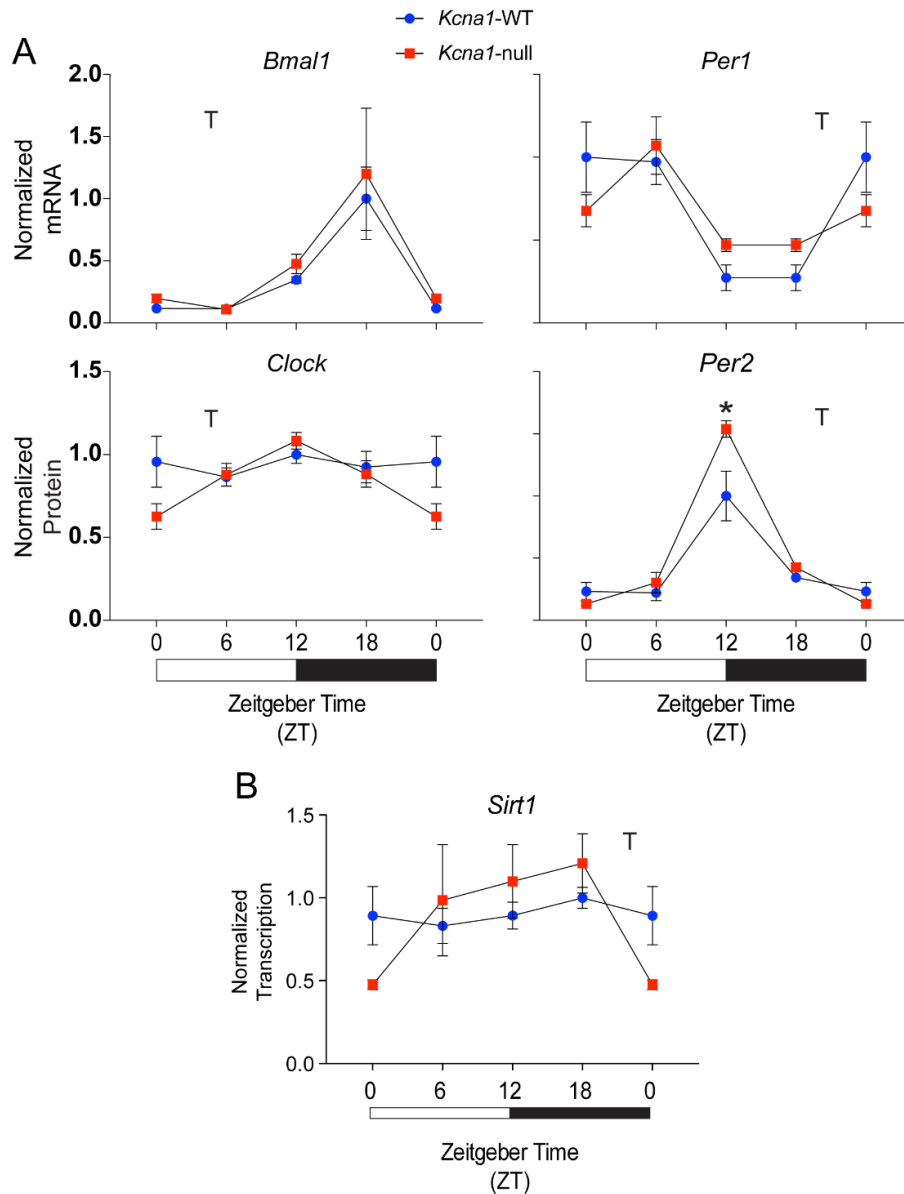
Figure 8: Analysis of pre-epileptic circadian Clock mRNA expression.

The effect of genotype on transcript levels of four Clock genes (*Bmal1*, *Clock*, *Per1,* and *Per2*) and *Sirt1* relative to *Gapdh* measured in the SCN of young (P30) *Kcna1*-null (red squares) and wild-type (blue circles) mice at four time-points (ZT 0, 6, 12 and 18) in light-entrained conditions. Graphs included repeated values of ZT 0 at the right of each figure. Two-way ANOVA with Sidak-corrected repeated comparisons showed a main effect of time on the expression of *Clock*, *Bmal1*, *Per1*, *Per2* (A)*,* and *Sirt1* (B), annotated with a 'T'. Of these, we saw no main effect of genotype in mRNA levels of any gene tested, though multiple comparisons revealed *Per2* expression is elevated in *Kcna1*-null mice at ZT12 (Sidak $p < 0.05$). Lighting conditions are denoted by bars at the bottom (filled=lights-off, open = lights-on).

Discussion

There are several key observations in the present study. First, we found that epileptic *Kcna1*-null mice have altered locomotor rest-activity patterns in both LD and DD as measured by actigraphy. The mean circadian period was prolonged in *Kcna1*-null mice compared to wild-type controls, regardless of the lighting condition. Electroencephalographic sleep-wake analysis also showed that epileptic mice spent significantly more time awake and less time in NREM and REM sleep, with altered sleep architecture as shown by differences in bout length distributions. We found that oscillations in transcript expression of several Clock genes (*Clock*, *Per1*, and *Per2*) as well as a regulator, *Sirt1*, were attenuated in epileptic mice, especially under light-entrained conditions. Lack of any direct correlation between daily seizure burden and sleep parameters suggests that other mechanisms are responsible for observed behavioral rhythm and sleep disturbances. The dampened oscillation of Clock controlled genes may be such a mechanism in this model.

Sleep patterns and circadian rhythms are known to be disturbed in many neurological conditions such as Parkinson's and Alzheimer's diseases (Wulff et al., 2010). Among patients with epilepsy, several reports have shown an association between circadian rhythms and seizures. For example, frontal lobe seizures tend to occur more out of sleep and studies of local hippocampal activity in temporal lobe epilepsy showed that spike probability and synchrony are highest during slow-wave sleep (Herman et al., 2001; Staba et al., 2002). In animal models, studies have shown that susceptibility to kindled seizures peaks late in the day, with post-kindling seizures occurring more at

subjective night and post-kainate seizures during inactive periods (Hellier & Dudek, 1999; Weiss et al., 1993). We previously showed that seizures in *Kcna1*-null have a periodicity peaking at around ZT7, a pattern that exists in both light-entrained and free-running conditions 6. Clinically, it has been reported that seizures commonly have patterns of occurrence related to circadian rhythms, which vary depending on the type of seizure and epilepsy 8. Also, the circadian rhythm of sleep is disrupted among patients with epilepsy, especially in those whose seizures are not well controlled (Unterberger et al., 2015). In an actigraphy-based study, children with epilepsy were found to have reduced sleep duration compared to age-matched controls (Holley et al., 2014). In animal models of epilepsy, several biological processes with circadian rhythmicity have been shown to be disrupted (K. A. Fenoglio-Simeone et al., 2009; Quigg et al., 2000; Stewart et al., 2001). We show that the circadian rest-activity rhythms are disrupted in epileptic mice independent of the lighting condition. In addition, the mean circadian period of epileptic mice is prolonged in both LD and DD, a finding consistent with previous studies (K. Fenoglio-Simeone et al., 2009; K. A. Fenoglio-Simeone et al., 2009). In people with epilepsy, the potency of disrupted sleep in triggering seizures coupled with evidence that seizures disrupt circadian sleep patterns supports the idea of a vicious cycle in which reciprocal interactions between seizures, aberrant circadian rhythms, and sleep loss contribute to disease progression.

In our genetic model of epilepsy, we found that *Kcna1*-null mice have severely affected circadian locomotor patterns, varying from altered entrainment and circadian periodicity to near-total arrhythmia in behavior. We further confirmed this finding with EEG

assessments of sleep architecture, showing reduced time asleep and altered distributions of bout lengths in epileptic mice. One factor potentially responsible for the observed disruption in circadian rest-activity patterns and sleep were the seizures themselves. However, we found no significant correlation between seizure burden and degree of sleep fragmentation. Another potential mechanism is the altered circadian rhythmicity of Clock gene expression.

Under normal circumstances, *Bmal1*, *Per1/2* transcript levels oscillate in the SCN, whereas *Clock* typically has constitutive expression across light/dark cycles (Duong et al., 2011; C. Liu et al., 2007) Abnormalities in circadian rest-activity rhythms and attenuation in the oscillation of Clock genes in SCN including *Clock, Per1* and *Per2* had been reported in aging and neurogenerative disease literature (Bonaconsa et al., 2013; Duncan et al., 2012; Wang et al., 2016). Clock gene regulation also changes in response to acute sleep deprivation as evidenced by altered CLOCK:BMAL1 E-box occupancy, and changes in relative mRNA abundance of several Clock genes, though the consequences of sustained sleep perturbations (such as occur in epilepsy) on Clock gene expression remains unclear (Mongrain et al., 2011; Wisor et al., 2008). In *Kcna1*-null animals, we found a dampened amplitude of transcription of multiple Clock genes, especially under LD. As the amplitude of core Clock gene expression positively correlates with the strength of circadian rhythms, we propose that perturbed transcriptional regulation may result in the altered circadian behaviors observed in our model. Interestingly, the effects of epilepsy on Clock genes were primarily limited to mRNA. Although protein levels seemed to be largely unaffected in this epilepsy model, it is possible that patterns of post-

translational modifications affecting DNA binding, protein stability, and nuclear localization account for the changes in transcription levels (Y. Liu et al., 2016). As is the case with *Sirt1*, the effects of transcription we see in the core Clock genes may extend to transcription of numerous other 'Clock-controlled genes' which would have ramifications on cellular processes. Stabilization of circadian CLOCK:BMAL1-mediated transcription may help to normalize sleep patterns and defend against lasting effects of seizure activity.

Modulators of the circadian Clock vary from light exposure to dietary habits and cellular metabolism. While the latter affects Clock gene regulation through multiple pathways (i.e. ROR/REVERB, AMPK, and mTORC1), SIRT1 has been shown to dramatically affect circadian Clock gene machinery through interactions with the CLOCK:BMAL1 transcription factor and PER2. Mitigating metabolic stress with diet-based therapies has existed for centuries (Wheless, 2008). Both the ketogenic diet, (KD) a therapy for pharmaceutically intractable epilepsy, and caloric restriction have been shown to up-regulate Sirt1 expression in the brain (Maalouf et al., 2009). Enhancing SIRT1 function has further demonstrated its utility in restoring circadian rhythms in conditions where SIRT1 expression is diminished, such as aging (Chang & Guarente, 2013). Indeed, a prior study showed that KD can rescue entrainment of light-entrained rest-activity patterns in *Kcna1*-null mice, though the role of SIRT1 has not been directly investigated (K. A. Fenoglio-Simeone et al., 2009). Enhancement of SIRT1 activity may constitute part of the therapeutic effectiveness of KD, complementing its anti-seizure effects by assisting the recovery of normal circadian rhythms. Additionally, because *Kcna1*-null mice exhibit a progressive disruption in rest-activity patterns preceding death,

it is possible the disruptions in sleep and circadian rhythms we observe may constitute an early phenotype of compromised sleep that may worsen concomitantly with seizure burden, potentially contributing to disease progression and mortality (Iyer et al., 2018). Although the significant changes we observed in light-entrained *Sirt1* mRNA did not extend to protein levels, the changes we see in mRNA levels may be a broader result of a reduction in transcriptional activation by the circadian Clock, as seen in aging (Chang & Guarente, 2013). Considering the therapeutic efficacy of leveraging SIRT1 activity to restore circadian rhythms in conditions where Clock gene expression and circadian rhythms are weak (e.g., aging), SIRT1 modulation may be an effective tool to mitigate progressive sleep perturbations in our *Kcna1*-null mice. Future studies will be designed to address these questions directly.

There are several limitations to our study. First is the global deletion of *Kcna1*; its influence on mechanisms outside of electrophysiological function remain unclear and, as yet, untested. This limitation does not negate the utility of this model, especially considering such syndromes where genetic mutation or autoimmunity ablate the expression of such channels, often leading to convulsions (Irani et al., 2010). Further studies in models not confounded by genetic mutations are needed to clarify this limitation, though we did not find that mRNA expression patterns of *Clock*, *Bmal1*, *Per1/2*, and *Sirt1* were affected young (P30) *Kcna1*-null mice. The observation that transcription patterns of core Clock genes are unaffected by genotype at an age where seizures rarely occur supports the conclusion that the alterations that we see in Clock gene expression in older animals are likely due to spontaneous epileptiform activity. Technical

complications excluded simultaneous actigraphy-EEG data acquisition, prohibiting us from correlating activity counts with EEG-based seizure frequency or sleep metrics. We had multiple individuals scoring sleep stages, which added variability in bout analyses. However, we believe the impact of multiple scorers to be minimal, considering great effort was made to standardize the implementation of our scoring criteria. Finally, it is not clear how the aberrant oscillation of Clock genes is disrupting behavioral patterns. It may be possible that targeting sleep (with sleep aids for example) may restore Clock gene oscillations which we have not investigated here. Future experiments will aim to evaluate further the reciprocal nature of the relationship between seizures and SCN functions, as well as determining the therapeutic effects of enhancing SIRT1 activity.

Conclusion:

In conclusion, light-entrained and circadian rest-activity patterns and sleep-wake cycles are aberrant in epileptic *Kcna1*-null mice, and these abnormalities persist under both light-entrained or free-running conditions. Seizure frequency in animals had no clear impact on the degree of sleep disruption. Patterns of transcription of several Clock genes and *Sirt1* are attenuated in epileptic mice. *Sirt1* may represent a useful therapeutic target for rescuing circadian Clock gene rhythmicity and sleep patterns in epilepsy.

<u>Author Contributions</u>

The author(s) declare their contributions as follows: Conceived and designed experiments: RM, JR, EW. Performed experiments: EW, SW. Analyzed Data: EW. Contributed materials/reagents/ analysis tools: RM, AR, BS. Wrote the paper: RM, EW. All authors reviewed the manuscript.

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Chapter 5 – Discussion and Future Directions

<u>Summary of findings</u>

The work described in this dissertation was intended to disentangle the detrimental interaction of sleep loss and seizures in temporal lobe epilepsy (TLE). Central questions that this dissertation attempted to address were 1) How does sleep deprivation impact hippocampal physiology to affect excitability? 2) What occurs first? Are sleep problems a component of latent epileptogenesis or do they come into play after the onset of spontaneous recurrent seizures? and 3) How are sleep and regulators of sleep impacted in individuals with epilepsy?

In the first study (Chapter 2), we investigated the effects of 4-hour sleep deprivation (4SD) on GABAergic inhibition in the hippocampus. We reported that sleep deprivation caused a significant reduction of tonic GABAergic currents in principal excitatory cells within the dentate gyrus (DG) and cornu amonis (CA)1 (Chapter 2, Figure1. We also found that the frequency and amplitude of miniature inhibitory postsynaptic currents (mIPSCs) were not substantially altered by 4SD (Chapter 2, Figure 4). We then evaluated populations of ionotropic $GABA_A$ receptors that are associated with tonic currents in the hippocampus, which usually contain either an α5 or δ subunit (Glykys et al., 2008). The first was evaluated by measuring the currents sensitive to L-655,708, a negative allosteric modulator with high specificity for α5-containing $GABA_A$ receptors, relative to total tonic current magnitude. In the DG, we found that currents blocked by L-655,708 did not change following 4SD treatment (Chapter 2, Figure 3B), whereas 4SD caused a

significant decrease in L-655,708 sensitive currents in CA1 pyramidal neurons (Chapter 2, Figure 3C). We then measured currents activated by THIP, an agonist of GABA$_A$ receptors with high selectivity for those containing a δ subunit. In DG, we found that THIP activated currents were not affected by 4SD, whereas they were increased in CA1 (Chapter 2, Figure 2).

In the second study (Chapter 3), we began to evaluate the timeline of sleep disruptions following kainate-induced status epilepticus (SE) by measuring vigilance distribution and 4SD-induced potentiation of NREM EEG power, a correlate of sleep pressure. We observed no significant differences of proportions of time spent awake and in NREM and REM sleep (Chapter 3, Figures 2-4), however we reported that the dynamics of post-4SD:baseline ratio of NREM EEG delta power differed over the first 2 months with variable ratios in KA-treated animals as compared to a consistent increase in NREM delta power ratio in saline-treated controls (Chapter 3, Figure 5C). We also showed that tonic GABAergic currents are smaller in KA-treated animals 2 months after SE-induction (Chapter 3, Figure 6B).

In the third study (Chapter 4), we assessed sleep architecture and circadian rhythms of locomotor activity, as well as patterns of core Clock genes in the central circadian pacemaker, the suprachiasmatic nucleus (SCN) in *Kcna1*-null mice, a genetic model of temporal lobe epilepsy (TLE). With continuous passive motion detection, we show that epileptic mice have significantly altered circadian rhythms and impaired entrainment of circadian rhythms (Chapter 4, Figure 2). Epileptic *Kcna1*-null mice spent significantly more time awake and less time in NREM and REM sleep, with a significantly different distribution of bout durations in both light-entrained and free-running conditions

(Chapter 4, Figure 3). Circadian rhythms are mediated by the activity of SCN, which coordinates Clock-gene expression throughout the body. We found that transcription patterns of several core Clock genes were affected in light-entrained (Chapter 4, Figure5: *Clock*, *Per1*, and *Per2*) and free-running conditions (*Per1*). Core Clock protein levels were less affected in epileptic animals, with PER2 altered in free-running conditions (Chapter 4, Figure 6). We also evaluated expression patterns of a transcriptional corepressor, Sirtuin 1, and found that its transcription was affected by genotype in light-entrained animals (Chapter 4, Figure 7.

Considering a direct role of neural hyperactivity in epilepsy and sleep deprivation

In our experiments measuring the effects of 4SD on tonic inhibition, we reported a reduction of tonic inhibitory currents and went on to investigate the mechanism(s) causing the reduced currents. Conceptually, regulation of GABAergic neurotransmission is mediated by three elements: release, reuptake, and reception (i.e., receptor function, expression, and localization). Our experimental design allowed us to assess the frequency and amplitude of miniature IPSCs, and the function of GABA$_A$ receptors responsible for tonic currents. While changes to α5 and δ subunit-containing GABA$_A$ receptors in CA1 could potentially explain the finding of diminished overall tonic inhibition, the underlying effects of 4SD in DGGCs remain unknown.

Miniature IPSCs could speak to the number of synapses and basal synaptic release, but a limitation to our experiments is the inability to measure spontaneous activity in the slice or occurring in the time leading up to slice preparation. The process of preparing slices for patch-clamp recordings cuts dendrites and axons, especially long-range excitatory

projections (Hahn et al., 2012). Extensive pruning of connections in slice preparations likely contributes to the drastic reduction of spontaneous activity within the CA1 as compared to *in vivo* recordings (Okamoto et al., 2014). An important aspect to keep in mind when conducting and interpreting *in vitro* slice work is how the properties of the isolated cell may have been influenced in the uninterrupted system. We know that the activity of the entorhinal cortex and hippocampus are significantly elevated during waking (Staba et al., 2002) and that excitatory projections from the cortex preferentially activate the dense population of inhibitory interneurons of the dentate gyrus (Ewell & Jones, 2010). So, it is possible that elevated spontaneous activity in the cortex during wakefulness would drive elevated feed-forward inhibition in the DG and initiate the activity-mediated downregulation of $GABA_A$ receptor function, surface occupancy, or expression (Eugene M Barnes, 2001). This hypothesis could be tested with *in vivo* depth electrode placement in the hippocampus and cortex to monitor network activity while sleep deprivation is occurring.

A shortcoming of this study was that we were unable to determine the mechanism underlying the 4SD induced reduction of tonic inhibitory currents in DGGCs. Elements of tonic inhibition regulation that were not assessed in this study include the activity of membrane GABA transporters, the contribution of spontaneously gated $GABA_A$ receptors to tonic inhibition, and extracellular concentrations of GABA. GABA transporters (GAT1 and -3), are responsible for clearing GABA from the extracellular space, which has a significant impact on tonic inhibitory currents (Jin et al., 2011; Moldavan et al., 2017). Spontaneous gating of $GABA_A$ receptors has also been shown to contribute to tonic inhibitory currents (O'Neill & Sylantyev, 2018; Wlodarczyk et al., 2013), which we were

unable to measure because of our use of the GABA$_A$ receptor inverse agonist, bicuculline. Future directions for this project include experiments to assess reuptake and spontaneously gated currents in epileptic circuits and following sleep deprivation.

Following the logic of increased spontaneous neuronal activity in the cortex and hippocampus, neuronal hyperactivity and epileptiform activity could be playing a role in sleep dynamics and tonic inhibition following kainate-induced status epilepticus. In this study, we first demonstrated that NREM EEG delta power was altered in kainate-treated animals. Perhaps the most striking finding is the variability of NREM EEG delta power potentiation across time (Chapter 3, Figure 5C), which may suggest that SE is affecting the coordination of cortical patterns of activity during SWS. Cortical activity during slow-wave sleep is differentiated into so-called 'Up-' and 'Down-' states, during which the frequency of network activity is high and low, respectively. This consolidation of electrical activity creates electrical fields that can be detected by electrodes positioned at the surface of the cortex, such as were implanted for post-SE EEG acquisition. Conceptually, the greater the population of active neurons and temporal consolidation of cortical activity, the greater the amplitude of slow EEG waves (Nir et al., 2011). The temporal precision of cortical 'Up-Down' states depends on tightly regulated local inhibition and excitation (Niethard et al., 2018). GABA$_A$ receptor subunit expression (i.e., δ and β2/3) is known to be downregulated in models of epilepsy and hyperactivity (Goodkin et al., 2007; Peng et al., 2004). In patients with epilepsy, activity (i.e., mean firing rates and bursts per minute) of cortical and hippocampal neurons is elevated above what is recorded in non-epileptic cortices in all vigilance states (Staba et al., 2002). This highlights a potential caveat in our assessment of NREM EEG delta power and the conclusions that we draw about how

sleep characteristics respond to sleep restriction in individuals undergoing epileptogenesis. It is reasonable to associate dynamics of NREM delta power sleep homeostatic responses to sleep deprivation (Nelson et al., 2013), but a limitation of EEG is that we can only passively observe underlying cortical activity that generates the field potentials that are recorded. Staba et al. (2002) reported mean cortical activity levels, which provides no information of any temporal patterns of single-unit activity. If cortical hyperactivity is consistent through Down-states, one effect would be a diminished amplitude of cortical EEG. Research correlating cortical network activity and field potential slow waves in induced-SE models of TLE is lacking. One study by (Bragin et al., 2012) that may provide some insight evaluated Up-Down states and pathologic high amplitude discharges (i.e., interictal spikes) in epileptic and non-epileptic mice under anesthesia. They determined that the durations of urethane-induced Up- and Down-states were prolonged in epileptic mice, changing the average period of a slow wave from ~1.3 seconds in normal animals to ~3 seconds in epileptic animals. The study also reported that the normally high neuronal activity of Up-states was rapidly suppressed by the coincidence of high amplitude discharges detected in the dentate gyrus or cortex, regardless of whether the discharge was synchronized between regions. These data suggest that local and extended epileptic activity (i.e., seizures or interictal spikes), can disrupt local network Up-Down states and affect slow waves. It remains to be determined if these Up-down state pathologies exist in spontaneous NREM sleep in unanesthetized epileptic mice. If so, it may explain the variability of 4SD potentiation of NREM EEG activity in the 1-4 Hz range we detected because 1) high-amplitude discharges often manifest prior to the onset of electrographic seizures in the kainate model and vary in

frequency across time (Pfammatter et al., 2018), and 2) predominant frequency of NREM sleep might have moved below the band included for NREM delta power analyses. Direct evaluation and correlation of epileptiform activity, neuronal activity, and slow-wave EEG activity is a future direction of study in the sleep and epileptogenesis project.

In contrast to our findings during early epileptogenesis, a study of high-density EEG in patients with focal epilepsies demonstrated a global increase in the amplitude of NREM slow-wave sleep (Boly et al., 2017). This is consistent with the idea that elevated excitability in epileptic brains could contribute to increased synchrony or neuronal recruitment during Up states, enhancing NREM sleep. The discrepancy may be because the timeframe of our study was early in post-kainate epileptogenesis and that increases in NREM delta power are a feature of later disease progression. Another possibility is that limiting our analysis to 2-hour segments prohibited us from detecting differences across the entire light-dark cycle.

There are several levels of future study that would benefit our understanding of sleep dynamics during the latent and protracted periods of epileptogenesis. Firstly, conducting a more comprehensive assessment of NREM EEG would help us determine if differences exist at other time points in the day. Secondly, extending EEG acquisition into the chronic phase of epileptogenesis would allow us to determine if elevated NREM delta power is a feature of chronic kainate-induced epilepsy. Thirdly, evaluating NREM delta power as a function of time and characteristics of preceding wake activity (e.g., duration, locomotor activity), would bring insights into sleep homeostasis and how it may be affected at by induced SE. Lastly, direct evaluation and correlation of epileptiform activity, neuronal activity, and slow-wave EEG activity would provide insights into sleep

dysregulation in epilepsy and characterize the effects of epileptiform activity on cortical activity underlying spontaneous slow-wave sleep.

The second finding of the early epileptogenesis project is a reduction of tonic inhibitory currents in DGGCs in animals that have undergone kainate-induced SE compared to saline-treated controls. This loss of tonic inhibition could be driven, or perhaps sustained, by increased neural activity. It has been shown that acute effects of status epilepticus include the rapid downregulation of $GABA_A$ receptor δ subunits (Peng et al., 2004). Hyperexcitability has also been shown to trigger the internalization of β2/3 subunits in an in vitro model of seizure activity (Goodkin et al., 2007). As with the study of 4SD and tonic inhibition, elevated rates of spontaneous cortical and hippocampal activity associated with the epileptic condition may be driving the downregulation of $GABA_A$ receptor function and surface expression (E M Barnes, 1996; Goodkin et al., 2007; Peng et al., 2004; Staba et al., 2002). This would go toward explaining why tonic inhibition is impaired in animals undergoing epileptogenesis, though additional experiments are necessary to assess $GABA_A$ receptor function and subunit expression in greater detail.

Continuing the theme of direct effects of elevated firing rates in epilepsy, it is plausible that impaired circadian rhythms in *Kcna1*-null mice (genetic model of TLE) may be due to aberrant excitability in the extended circadian networks in the brain. Our study is the second to evaluate light-entrained behavior and Clock gene expression in a mouse model of temporal lobe epilepsy. Similar to our findings, (Matos et al., 2018) show that

pilocarpine-induced status epilepticus causes progressively deteriorating patterns of spontaneous locomotor activity, as well as differential transcription patterns of genes encoding several members of the core Clock. Our study confirms aberrant locomotor activity patterns and altered Clock gene transcription in our genetic model of TLE. Where it differs is how the genes were differentially expressed. Matos et al. (2018) showed a transient increase in *Per1* and a decrease in *Bmal1* and *Clock* expression, while differences observed in our genetic model of epilepsy were all decreased relative to non-epileptic controls. These differences may be due to disparate severity between the pilocarpine and genetic models of epilepsy affecting different levels of input, mimicking RHT-input projected into the circadian pacemaker (SCN) and extended circadian network. The idea would be that the effect of seizures on Clock gene expression depends on when (relative to lighting conditions) and how frequently the seizures occur; frequent seizures during periods of darkness could promote a 'day-like' profile of the circadian transcriptional control. If seizures are pervasive enough, this could drive expression patterns from circadian to constitutive, and if seizures are mimicking RHT activity and favoring the circadian transcriptional repression, average expression of Clock-controlled genes would eventually be reduced.

Light entrained circadian rhythms require temporally isolated input into the SCN, which is normally provided by activation of intrinsically photosensitive retinal ganglion cells (ipRGCs) and their projections into the brain along the retinohypothalamic tract (RHT). The SCN also receives input from several other regions that form the extended circadian network (Morin, 2013). The current hypothesis is that epileptiform activity affects activity within this extended network, which relays the erroneous entrainment cues into

SCN. Depending on the timing and the nature of the projection into the SCN (excitatory or inhibitory), these pathologic inputs would act as false entrainment cues. The basic mechanisms of SCN entrainment converge on in influx of calcium into the cell which upregulates CREB phosphorylation and subsequent promotion of the Periodicity 1 gene transcription. This electrical activity-dependent signaling is the mechanism through which masking (i.e., light-exposure during normally dark periods) or direct optogenetic stimulation of SCN neurons work to modify the timing of behavioral and physiological processes that are under circadian control (Jones et al., 2015; Nakamura et al., 2008). If our hypothesis is correct, seizures modify the electrical activity in the SCN which translates to aberrant transcriptional repression of circadian controlled processes. As discussed above, chronic effects could include an ablation of rhythmic expression of Clock-controlled genes and deteriorating entrainment of behavioral circadian patterns.

While informative, assertions made about circadian rhythms using light-entrained animals are limited because SCN activity is heavily influenced by light-reception. To the best of my knowledge, this study is the first to assess Clock genes and circadian rhythms of epileptic animals in free-running (i.e., constant dark) conditions. While light-entrained studies of circadian systems can provide insights into an individual's ability to respond to external timing cues, experiments in free-running animals allow us to isolate the effects of seizures on circadian rhythms. In mice, the period of the circadian oscillations is slightly shorter than 24 hours, which can be appreciated by a gradual advance of the onset of locomotor activity periods when mice are held in constant darkness (Partch et al., 2014). In epileptic *Kcna1*-null mice, patterns of free-running locomotor activity are largely arrhythmic (Chapter 4, Figure 2), which demonstrates the impact that random and

erroneous entrainment cues imparted by seizures or other epileptiform activity have on the regulation of circadian rhythms (Fenoglio-Simeone et al., 2009). While our analyses of activity and Clock gene expression can determine gross perturbations of the circadian system output (i.e., behavior), they lack the sensitivity to thoroughly test the hypothesis that epileptiform activity is integrating into the firing rates of the SCN. Future experiments for this project should include experiments designed to simultaneously record cortical EEG and local field potentials in the SCN and key members of the extended circadian network (e.g., intergeniculate leaflet and paraventricular nucleus). Such experiments would allow us to detect seizures and determine if seizures are affecting the activity of SCN or connected relay nuclei during and after the seizure.

Sleep disturbances are not unique to epilepsy. Many neurological disorders are associated with comorbid sleep problems including Alzheimer's disease, Parkinson's disease, autism spectrum disorder, multiple-sclerosis (Ju et al 2017, Mazzone 2018). The coincidence of sleep problems and multiple neuropathological states suggest that disturbances in sleep patterns could driven by general neuronal hyperexcitability. As such, research focusing on models of these diseases, as well as kainate-treated animals that do not develop spontaneous recurrent seizures but do exhibit circuit damage and hyperexcitability, could begin to elucidate the frank effects of hyperexcitability on sleep dynamics.

Strange bedfellows- intermediaries of seizure activity and sleep regulation

Up this point, this discussion has focused on the direct implications of prolonged neuronal hyperactivity in our investigations of GABAergic neurotransmission and sleep

regulation. Indirect effects of seizures on the circadian rhythms are also possible because there is an extensive overlap between the mechanisms that underlie circadian Clock synchronization and signaling systems that are both sensitive to epileptiform activity and are capable of modifying seizure susceptibility.

Hypothalamus-Pituitary-Adrenal axis activity in sleep, circadian rhythms, and epilepsy:

Perhaps the most important route of SCN control over peripheral physiology is the modulation of the hypothalamus-pituitary-adrenal (HPA) axis. The activity of SCN neurons influences the release of corticotropin-releasing hormone (CRH) from neurons in the paraventricular nucleus in the hypothalamus (Balsalobre et al., 2000). The pituitary gland releases adrenocorticotropic hormone (ACTH) in response to CRH, which in turn stimulates the production of corticosterone by the adrenal glands. Corticosterone is a glucocorticoid, so named for its effects on glucose homeostasis and neuronal function (Coutinho & Chapman, 2011; Garabedian et al., 2017). In epilepsy, the HPA axis is known to be hyperactive, which in turn drives up the susceptibility for subsequent seizures (O'Toole et al., 2014). Considering that circadian Clock and metabolism are both affected by glucocorticoid signaling (Dickmeis, 2009), it is worth a deeper look into how glucocorticoid signaling may affect both circadian rhythms and epilepsy. Transcriptional regulation of Period genes is the mechanism employed in the central circadian pacemaker in the SCN, in peripheral tissues, and in cell culture experiments to synchronize circadian rhythms. It seems that one point of convergence in *Per1* transcriptional control is the regulation of cAMP-response element-binding protein (CREB). In the SCN, CREB is activated by calcium influx promoted by axons of the

retinohypothalamic tract releasing glutamate and pituitary adenylate cyclase-activating peptide (PACAP). Glutamate receptor activity leads to the influx of calcium through NMDA receptors, which leads to phosphorylation of CREB via calcium/calmodulin-dependent protein kinase (CaMK) (O'Neill et al., 2008). PACAP receptor signaling upregulates protein kinase A (PKA) activity which mediates CREB activity directly and indirectly, by upregulating the function of calcium-permeable channels and CaMK signaling. *In vitro* cell culture studies of circadian rhythms commonly use treatments that modify adenylate cyclase activity (e.g., forskolin) or approaches that affect cell metabolism like serum shock to synchronize circadian Clocks (Balsalobre et al., 1998; Rawashdeh et al., 2016; Yagita & Okamura, 2000). Alternatively, glucocorticoid receptor (GR) signaling has been used to synchronize circadian rhythms in vitro with the same effect on Period1 transcription (Balsalobre et al., 2000). GRs are nuclear transcription factors that are thought to directly affect the transcriptional promotion of period 1, but may also have effects on circadian rhythms similar to serum shock by repressing transcription of REVERBα (Dickmeis, 2009; Scheschowitsch et al., 2017). These experimental methods demonstrate a link between circadian Clock function and cellular metabolism. Seizures are metabolically demanding events and are known to increase levels of cyclic nucleotides, including cAMP, which subsequently increases seizure susceptibility (Boulton et al., 1993; Wasterlain & Csiszar, 1980). Seizure-induced increases of cAMP would also impact the patterned oscillations of core circadian Clock genes by the CREB-mediated control of period 1 expression discussed previously. In the context of epilepsy, metabolic stress and the resulting dysregulation of the circadian Clock might be an integral aspect of the vicious cycle between sleep and seizures.

Taking a step back, HPA hyperactivity could be reinforcing this cellular phenomenon through pathological glucocorticoid signaling. This idea would explain why some forms of epilepsy exhibit circadian patterns of epileptiform activity because circadian rhythms of HPA activity could be driving the variable seizure susceptibility across the circadian period (Foldvary-Schaefer & Grigg-Damberger, 2009; Gowers, 1902; Pitsch et al., 2017).

Neuroimmune regulation, sleep, and seizures:

Another intersection between sleep, the HPA axis, and seizures is the immune system. Immune response has been shown to fluctuate based on sleep state and time of day (Comas et al., 2017). Seizures have been shown to contribute to activation of the immune system through increased expression of cytokines and their receptors in neurons and activated microglia, and subsequent deterioration of the blood-brain barrier and immune cell migration to seizure foci (Aronica & Crino, 2011). One signaling molecule, tumor necrosis factor (TNF)α, stands out because of its dual effects on sleep and proliferation of the inflammatory response. In one study, TNFα produced during acute systemic inflammation was shown to downregulate the expression of Orexin, which is a wake-promoting signaling molecule (Kapás et al., 2008). In addition to promoting sleep, inflammation has been shown to increase seizure susceptibility in individuals with epilepsy and conceivably plays a role in continuing processes of epileptogenesis (Nasiri et al., 2017; Rana & Musto, 2018). Inflammation can be regulated by several systems, including the central inflammation reflex and HPA activity. The inflammatory reflex is a compensatory response to systemic immune activation mediated by cholinergic neurons

that surveil systemic inflammation (Kin, 2006; Kobrzycka et al., 2019). The activity of the Vagus nerve has been shown to mediate the inflammatory reflex by releasing acetylcholine and stimulating the production of glucocorticoids in the adrenal glands, both of which fight inflammation by suppressing the expression of proinflammatory cytokines in macrophages and microglia (Bonaz et al., 2017; Borovikova et al., 2000; Carrillo-De Sauvage et al., 2013). The activity of the vagus nerve is associated with the parasympathetic nervous system, which is the predominant arm of the autonomic nervous system active during NREM sleep (Zoccoli & Amici, 2020). So, reciprocal interactions between inflammation and sleep are well-positioned to mediate the effects between seizure activity and sleep deficits.

In addition to the interactions between inflammation and sleep, seizures and neuroinflammation also directly impact each other. Elevated levels of proinflammatory molecules have been reported in patients and animal models of epilepsy following seizures (Ravizza et al., 2011). In epilepsy, sustained neuroinflammation and deterioration of the blood-brain barrier are associated with neurotoxicity, hyperexcitability, and elevated seizure susceptibility and severity (Dey et al., 2016). Feedback between neuroinflammation's effect on neuronal excitability and the proinflammatory effects of seizures have highlighted anti-inflammatory therapies as a target to combat the development and potentially reverse pathologies already present. One class of proinflammatory molecules that has received particular interest are prostaglandins (PGs). Crucial to PG production and signaling are the enzymes cyclooxygenase (COX) 1 and 2 (Dey et al., 2016). Unlike the constitutively active COX-1, COX-2 is induced during injury (e.g., TBI, seizures) and infection and proliferates inflammatory response (Ravizza et al.,

2011). Administration of COX-2 inhibitors in the hours and days following induced SE has been shown to improve outcome in animal models of acquired epilepsy by reducing neuronal cell death and the frequency of spontaneous recurrent seizures (Dey et al., 2016; Ravizza et al., 2011). Interestingly, an opposing effect was seen when COX-2 inhibitors were administered prior to the induction of SE, which suggests opposing effects of COX-1/2 products. The effects of the prostaglandin PGE_2 have been shown to exacerbate pentylenetetrazol-induced seizures, even in the presence of COX-2 inhibitors, whereas the prostaglandins PGD_1 and $PGF_{2\alpha}$ have been shown to be seizure-suppressive (Chung et al., 2013; Kaushik et al., 2014; Oliveira et al., 2008). PGE_2 signaling has also been shown to promote wakefulness and to increase after total sleep deprivation (Haack et al., 2009). Conversely, PGD_2 has been shown to exert sleep-promoting effects, as inhibition of PGD_2 production or reception has been shown to decrease NREM and REM sleep during the normal rest period of mice (Hayaishi, 2011). The differences in the effects that PGE_2 and PGD_2 have on inflammation and sleep may explain the unique responses of pre- or post-SE treatment with COX-2 selective inhibitors, where pre-administration might be disrupting sleep and affecting neural excitability and post-administration would be opposing the seizure-induced immune response. These preclinical studies demonstrate the potential that anti-inflammatory therapies (e.g., COX and cytokine inhibition) have when applied during or after SE induction to reduce seizure susceptibility and to inhibit disease progression (Ravizza et al., 2011). Clinical trials of the COX-2 selective inhibitor celecoxib and non-selective COX inhibitors non-steroidal anti-inflammatory drugs (NSAIDs) such as asprin and ibuprofen have been conducted and yielded mixed results (Rawat et al., 2019). Considering the anti-seizure and pro-sleep

effects of PGD$_2$, research into the effectiveness of sleep promotion through non-immunomodulatory mechanisms as a therapy for chronic, seizure-induced inflammation and epilepsy is another area of interest for future study.

<u>My two cents on sleep and epilepsy</u>

My thesis work aimed to characterize the negative interactions between sleep loss and epileptiform activity, to disentangle the vicious cycle of sleep deprivation and seizures and to assess individual components. I provide evidence that acute (4-hour) sleep deprivation directly impacts GABAergic neurotransmission by reducing tonic inhibitory currents in the dentate gyrus and CA1 of seizure naive mice. Interestingly, we see a similar reduction of tonic inhibition in the dentate gyrus of animals that experience kainate-induce status epilepticus but were not subjected to sleep deprivation. I hypothesize that these two results are mediated by a common cause: prolonged elevation of network activity causing activity-dependent downregulation of GABA$_A$ receptors. It is conceivable that depriving individuals with TLE of sleep would compound this effect, driving tonic inhibition in the hippocampus down even further and transiently exacerbating seizure susceptibility. The impact of seizures on sleep regulation, especially circadian rhythms, involves much more than acting as an erroneous cue for SCN entrainment. Indeed, even if disrupted firing patterns of SCN neurons do prolong the effects of epileptiform activity in circadian rhythms, it is clear the interactions are much more complex and extend beyond synaptic connectivity to include endocrine elements in the periphery. Our experiments were focused on electrophysiological and behavioral sequelae of sleep deprivation and epilepsy. However, consideration of the vastly complex network of

systems affected by, and affecting epileptiform activity, sleep, and circadian rhythms should be included in the assessment of processes driving epileptogenesis and in the development of therapies directed at stopping, or even reversing epilepsy. Targeting sleep may have the net effect of normalizing multiple factors that contribute to a pro-epileptic environment, including inflammation and activity of the HPA axis; a therapeutic angle that could extend beyond epilepsy and benefit many other diseases of hyperexcitability.

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Appendix

Mouse EEG Headcap Fabrication

Materials:

        Dremel with a thin cutting wheel
        2x50 header strip – Digikey (ED1316-100-ND)
        Stainless steel wire (insulated solid, AM Systems Inc., Cat: 791400)
        Stainless steel wire (insulated braided)
        Smooth-jawed hemostat
        Tabletop vice
        Fine forceps
        Soldering iron
        Lead-free solder

1. Fabrication protocol:

2. Pre-heat soldering iron

3. Cut 3 lengths of solid wire for each head cap.

4. With fine forceps, strip both ends, leaving ~4 mm section of insulation.

5. Cut 2 lengths of braided wire for each head cap.

6. Strip ~2mm of insulation of one end.

7. Remove every fourth row of pins from the connector strip and trim the cuts sides so that they are flat

8. Cut the strip into 2x3 segments.

9. Cut the pins down to ~3-4 mm.

10. Set the 2x3 header strip in a tabletop vice with the pins up.

11. Solder wires into hollow pins in the order shown in Figure 1.

12. Note: After the solder cools, test the connection by firmly pulling on the wire.

13. After all wires are soldered into position, move the section of insulation on the solid wire down to the joint.
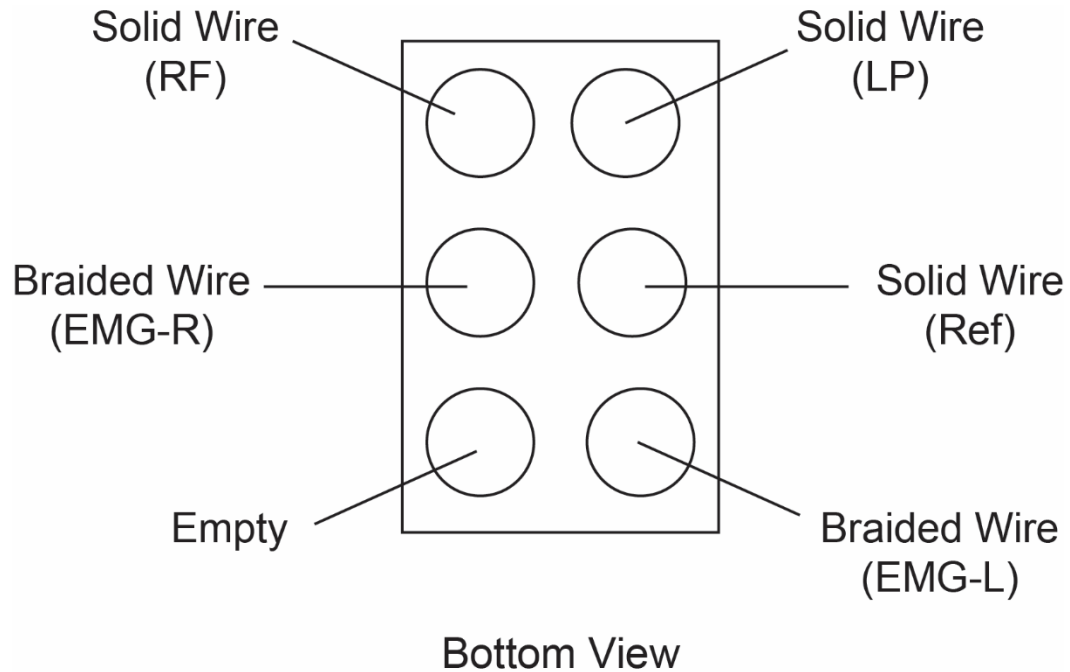

Figure 1: Headcap wiring schematic

Diagram showing the bottom aspect of a mouse EEG head cap. Annotations show wire types (solid or braided) to be soldered into position, as well as connection assignments during head cap implantation.

Mouse EEG surgery

Materials:

     Stainless steel screws (Antrin Miniature Specialties Inc., cat: NAS721CE90-100)
     Coarse forceps (WPI Straight, German, Cat: 15914-G)
     Fine forceps (WPI Economy tweezers #2, Cat: 501975)
     Fine angled forceps (WPI Angled, Fine, Cat: 504482)
     Fine iris scissors (WPI 10.0 cm, SuperCut, Straight, Cat: 14395)
     Scalpel handles and #10 blades
     Fine slotted screwdriver
     Metal spatula (small)
     18Ga needles – BD (MDS supply)
     Silver epoxy or paint: optional
     Dental acrylic: (e.g, AM Systems Inc. 525000)
     Hydrogen peroxide (H_2O_2) 3%
     Betadine/povidone-iodide solution
     70% Ethanol (EtOH)
     Sterile gauze
     Sterile cotton-tipped swabs
     1 mL syringes with needles suitable for subcutaneous injection
     Bupivacaine
     Meloxicam
     Sterile saline
     Nitrile gloves
     Hot bead sterilizer
     Isoflurane vaporizer and manifold
     Stereotactic frame
     Far-infrared warming pad (intra-procedural) and battery
     Warming pad (recovery)
     Water (gelatin)

Prior to first surgery:

1. Autoclave all surgical utensils and head caps

2. Charge batteries for warming pad

Surgical protocol:

1. Begin the flow of 100% oxygen at 0.5 L/min to the isoflurane vaporizer.

2. Set isoflurane mixture to 5% and direct vaporizer outflow to the induction chamber and allow ~5 minutes to charge the chamber

3. Record the weight of the mouse

4. Transfer to the induction chamber until loss of righting reflex.

5. Remove from the induction chamber and clip hair from the surgical area (from neck to eyes, between ears).

    Note: This may take several rounds in the induction chamber to complete.

6. Begin isoflurane flow to the nose cone and reduce to 2%.

7. Transfer mouse to stereotactic frame, supporting the body with a warming pad covered with a soft paper towel

8. Ensure stability of the mouse head in the frame: adjust ear bars if necessary

9. Clear hair clippings from head and body with a cotton swab dipped in 70% EtOH

10. Apply Betadine to the surgical area with a cotton swab and let sit for ~10 seconds, then clear with EtOH. (repeat 3x)

11. Check plane of anesthesia with bilateral toe pinch (if the withdrawing reflex is present, wait for 1-2 minutes and retest, if reflex persists, increase isoflurane content)

    Note: If at any point the animal's breathing slows drastically, reduce the concentration of isoflurane administered.

12. Using Iris scissors, cut a ~1 in incision along the midline from the base of the skull (just caudal to the line of the ears) extending forward to reveal the skull ~0.5 cm anterior to bregma

13. Reflect skin to the sides, exposing the skull.

14. Clear the skull of connective tissue:

15. With a dry, sterile cotton swab, work any connective tissue from the skull

16. With sterile cotton swab dipped in hydrogen peroxide (H_2O_2), clear any residual connective tissue.

17. Clear H_2O_2 with a dry sterile cotton swab.

18. Wash the skull with a sterile cotton swab dipped in EtOH.

19. Dry with a sterile cotton swab:

    NOTE: Be sure to make a mental note of major blood vessels under the skull prior to completely drying the skull, either mark them with a Sharpie or remember approximate sites for screws that will avoid these as much as possible.

20. Score the exposed skull with either scalpel or 18 ga needle, scratching a fine diamond pattern into the bone. This will aid in the adhesion of the dental acrylic to the skull and improving the longevity of the EEG head cap.

21. Drill 3 holes for SS screws with an 18 ga needle, avoiding skull sutures.

   a. Right frontal: Bregma +1-2mm, lateral 1-2mm

   b. Left Parietal: Bregma -1-3 mm, lateral 1-2 mm

   c. Occipital reference: Lambda -2 mm

   Note: Additional screws can be placed for increased stability of the head cap. It is

   suggested for chronic (>2 weeks) recordings.

22. Using the angled forceps to hold screws, place SS screws into the pilot holes.

   Note: For this and steps 26-29, take care to offset force applied to the implants.

   (e.g., Use the forceps to stabilize and offset the downward force applied to the

   screwdriver).

23. Place EEG head cap in holder and tilt ~45 degrees towards you

   Pro-tip: Tuck EMG wires into the space between the head cap and holder to get

   them out of the way.

24. Starting with the RF screw, wrap the right front wire around the screw 3-4 times,

   and trim excess wire

25. Repeat step 25 with LP (left front wire)

26. Rotate the head cap holder to an upright position, adjusting the stereotactic arm

   to keep a constant distance between the head cap and skull and minimize tension

   on the screws.

27. Repeat step 25 with the occipital reference (Left middle wire)

28. Move the head cap-holder to an upright position and lower as far as you can

   without allowing contact between head cap solder points and screws

29. Free EMG wires from the previously stowed position

30. Shape them to follow midline then bend 90 degrees lateral at the insertion point of nuchal muscles

31. Cut to length (should be able to reach the nuchal muscle with a little extra)

32. Lightly cut with a scalpel and remove the insulation from the last 2-4 mm of braided wire, bend exposed ends back over the wire

33. Separate the muscle fibers in the lateral nuchal muscle and insert wire such that the exposed ends are secured into muscle tissue

34. Optional: Apply conductive silver colloidal paint or acrylic, bridging wire and screw.

35. Position edges of the scalp so they cover most of the EMG placement areas and do not contact the EEG screws or wires

36. Secure the wires and head cap with a dental acrylic mixture that has a viscosity thin enough to flow off the spatula readily, cover the skull and wires.
    Note: I recommend making several new mixtures, getting a new one when the dental acrylic starts getting stringy

37. Slowly build up the dental acrylic, taking care not to overflow.
    Note: If acrylic runs away from the head cap, wait a couple of minutes for the acrylic to harden slightly. The higher cohesion will allow you to pick off the wayward acrylic with fine forceps.

38. Build up the acrylic on the back and front of the head cap

39. Reduce Isoflurane to 0% and continue O_2 flow to the animal while finishing the head cap cementing, note time you stopped to record in the surgical log.

40. Make a new batch of dental acrylic for finishing. Place a bead around the base to overlap with the skin around the incision, and on any uneven surfaces. The finished head cap should be smooth, as a grooming mouse may catch the surface of an uneven head cap and may shorten the longevity of the head cap.

41. Allow several minutes for the final hardening of acrylic cement.

42. Inject a dose of Meloxicam (5 mg/kg) and Bupivacaine (5 mg/kg) subcutaneously just caudal to the neck.

43. Remove the animal from stereotaxic frame and return to a recovery cage (cage should have either cob bedding or a soft paper towel and be positioned half on a heating pad set to medium heat), placing the mouse on its side, with its head away from cob bedding.

44. Note the time when sternal recumbency is restored (mouse turns itself upright)