ALTERED EXPRESSION OF BRAIN-DERIVED NEUROTROPHIC FACTOR IMPAIRS REM SLEEP ACTIVITY IN SPRAGUE-DAWLEY RATS: A SECONDARY ANALYSIS

A Thesis Presented for the
Master of Arts
Degree
The University of Tennessee, Knoxville

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May 2020

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To my very best good friend, Trey Copeland.

PREFACE

The purpose of this thesis is to conduct a secondary analysis of data that was originally published in the oxford journal of *Sleep* [1]. Some of the data collected for the original study was found to be in violation of research misconduct and IACUC compliance; therefore, a secondary analysis is needed. Data collected by procedures that were not approved by IACUC have not been used for this secondary analysis. The animals used for the secondary analysis along with the sleep-wake recording files for each animal have been included for the purpose of transparency (Tables A1-A2, filename BDNF_sleep_recording.zip Attachments).

ABSTRACT

Considering sleep is the most predominate state of a developing animal's life, the function of sleep must hold some significance. Learning and memory are functions of sleep that have been well established. Both non-rapid eye movement (nonREM) sleep and rapid eye movement (REM) sleep play distinct but complementary roles in the consolidation of memories. During memory consolidation, cellular modifications are regulated by brain-derived neurotrophic factor (BDNF). Animal studies have reported altered levels of BDNF expression along with impaired memory performance following REM sleep deprivation. Additionally, other studies have suggested intracortical injections of BDNF following total sleep deprivation enhances slow-wave activity (SWA) during nonREM sleep. These findings suggest a functional link between BDNF and sleep, but the precise nature of this role during normal sleep remains unclear. A secondary analysis of data collected from female and male knockdown *Bdnf* (+/-) Sprague-Dawley rats was used to investigate the role of BDNF expression in the regulation of sleep. Similar to the original findings, the secondary analysis revealed the knockdown *Bdnf* females and males exhibited significantly impaired REM sleep compared to the wild-type *Bdnf* (+/+) females and males; however, the secondary results were not as robust as the original findings suggested. Furthermore, in contrast to the original findings, the secondary analysis did not reveal any significant impact on the amount of time spent awake or in nonREM sleep. Given the knockdown animals were still able to achieve REM sleep, while time spent awake and in nonREM remained undisturbed suggest BDNF plays a causal role in the regulation of REM sleep.

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CHAPTER ONE: INTRODUCTION

The Sleeping Brain

Sleep is composed of two distinct stages: non-rapid eye movement (nonREM) and rapid eye movement (REM). The basic organization of normal sleep rhythmically oscillates between nonREM and REM with brief periods of arousal. During normal sleep, nonREM constitutes about 80 percent of total amount of time spent asleep with REM accounting for about 20 percent of remaining sleep. In humans, nonREM sleep cycles through four stages that increase in sleep depth. Animals such as rodents exhibit two stages of nonREM sleep that are physiologically similar to stage two, three and four nonREM sleep in humans.

A common instrument for measuring brain activity during sleep in humans and animals is the electroencephalogram (EEG). In humans, stage one nonREM sleep is marked by 8 to 13 Hz alpha waves in the cortex, indicating a transition from active wake into light sleep. Brain activity during stage two nonREM sleep shows low-voltage, mixed frequency oscillation patterns characterized by 8 to 14 Hz spindle oscillations in the cortical EEG. Stages three and four of nonREM sleep are collectively referred to as slow-wave activity (SWA) due to the spectral density in the frequency range 0.5 to 4 Hz. During this stage, the cortex and hippocampus exhibit synchronized EEG activity. Steriade and colleagues have shown in cats that this activity arises from widespread cortico-thalamic connections that coordinate bi-stable 0.25 to 1 Hz oscillations consisting of a brief depolarized up-state of sustained neuronal firing in the cortical network and a brief hyperpolarized down-state, characterized by neuronal silence [2].

During REM sleep, the cortical EEG resembles a highly desynchronized oscillation pattern similar to active wake. For this reason, REM sleep was also referred to as paradoxical sleep. In the hippocampus, sawtooth 4 to 10 Hz theta oscillations emerge. Theta oscillations arise from bidirectional interactions of cortical, hippocampal, and basal forebrain networks [3]. The medial septum is a well-established generator of theta oscillations. Medial septum nuclei are composed of GABAergic, glutamatergic, and cholinergic neurons that give rise to a local network that project to the hippocampus. Cholinergic projections from the medial septum exclusively terminate on the lacumosum-molecular (CA1) pyramidal neurons, whereas GABAergic and glutamatergic projections predominately terminate on hippocampal interneurons [4].

Sleep-Wake Regulation

The timing of this sleep-wake cycle is regulated by an interaction between circadian and homeostatic processes. The circadian process promotes states of active wakefulness by synchronizing environmental cues such as temperature and light exposure to daily biological rhythms. The suprachiasmatic nucleus (SCN) within the hypothalamus is one of the most well studied structures related to daylight's influence on the circadian process [5]. This is demonstrated best in nocturnal animals that typically display feeding and exploratory behavior during periods of darkness, whereas most of their time asleep takes place when exposed to light. SCN lesions in these animals not only disrupts the sleep-wake rhythm but also alters the distribution of sleep [6]. This is reflected in a decreased difference between the total sleep time

spent during their normal rest and active phase. Sleep deprivation has also shown to influence the SCN's circadian pacemaker function. In vivo studies that measured SCN activity following six-hours of total sleep deprivation in rodents reported a significant decrease in SCN activity compared to baseline suggesting the homeostatic response to sleep loss can function independent of the circadian process [7]. These findings also suggest that the amount of sleep is not determined by the circadian clock.

The homeostatic process describes the drive for sleep as a function of the preceding amount of time spent awake during a 24-hour cycle. During prolonged periods of wakefulness, the propensity for sleep increases resulting in a pressure to fall asleep. Slow-wave activity in the cortical EEG is a characteristic marker of sleep pressure that increases after prolonged periods of active wake [8-10]. This trend decreases as sleep is recovered with slow-wave activity returning to baseline levels. It remains to be fully understood how REM sleep contributes to sleep homeostasis; however, the amount of REM sleep lost during selective REM sleep deprivation is known to predict the subsequent REM sleep rebound during recovery sleep [11-13]. This suggests that in the absence of REM sleep, the pressure for REM sleep accumulates until homeostatically discharged to baseline levels.

Making Connections

Sleep is a reversible state of depressed consciousness that temporarily disengages the brain and body from the environment. The question for why we need sleep seems clear: without sleep mental and physical health declines, immune function and hormone synthesis are dysregulated, and synaptic plasticity for memory consolidation is impaired [14-18]. However, why does the brain need to temporarily disengage from the environment every day to ensure these processes function properly? The strength of synaptic connections depends on sleep. Synapses are the junctions between neurons that provide direct communication for information processing and storage within the brain.

Growth factors like brain-derived neurotrophic factors (BDNF), are proteins that play a key role in synaptic plasticity [19]. In vivo studies have demonstrated that BDNF mRNA expression in rodent hippocampus tissue increases after electrical stimulation [20,21]. Stimulating hippocampal neurons over a repeated period induces long-lasting increases in signal transmission between the two neurons. It is important to note, BDNF protein is first synthesized as a precursor, proBDNF form that is cleaved by proteases to form mature BDNF. A secreted mature BDNF has a high affinity for tropomyosin receptor kinase B (TrkB). Once activated, TrkB phosphorylation triggers an intracellular cascade of signals related to neuronal survival and synaptic plasticity [22-24].

Only a few studies have examined the effect sleep deprivation has on structural components associated with synaptic strength. Dendritic spines are a well-studied component of this system since they are thought to be important for synaptic regulation and cognition [25,26]. A study conducted with mice measured dendritic spine density in the hippocampus following five hours of sleep deprivation. The results showed a reduction in dendritic spine formation following sleep

deprivation; however, mice that were allowed recovery sleep were able to reverse dendritic spine loss [27].

Given the importance of both BDNF signaling and sleep in synaptic plasticity, it is interesting that BDNF expression is increased following sleep deprivation in rodents. A study examined BDNF expression in rats following selective REM sleep deprivation. The results immediately following the deprivation procedure showed an increased expression of BDNF compared to BDNF expression that was quantified several hours later suggesting BDNF plays a role in the homeostatic response of REM sleep [28]. Other studies have implicated BDNF in the regulation of nonREM sleep based on findings that showed intracortical administration of BDNF selectively increased slow-wave activity following total sleep deprivation in rats [29,30]. Together these findings suggest a functional link between BDNF and sleep; however, the precise role BDNF plays in the regulation of sleep is less clear.

Knockout studies using mice with both alleles removed from the *Bdnf* gene not only displayed impaired motor activity but also failed to survive beyond several weeks of birth [31,32]. For this reason, heterozygous *Bdnf* animals are used since they can maintain a normal lifespan and do not exhibit obvious neuronal modifications.

The original study hypothesized BDNF plays a role in sex-dependent sleep wake activity regulation. In line with the original findings we hypothesize that sleep will be impaired by altered BDNF expression; however, we predict that REM sleep will be selectively impaired compared to nonREM sleep. Sex differences are not taken into consideration due to a small sample size.

CHAPTER TWO: LITERATUR REVIEW

Early notions of sleep suggested the brain transitioned into an inactive state in response to decreased sensory input during wakefulness, however, advances in research have established that the activity of the brain during sleep suggest something different. The aim of this literature review is to provide a brief overview for how the fields' understanding of the phenomenon of sleep has evolved, taking into consideration the localization of sleep and wake promoting areas of the brain, and the neurotransmitters within these local areas that regulate sleep-wake states.

Standing on the Shoulders of Giants

One of the first studies to challenge the view of sleep as an inactive state of the brain was conducted in cats. Bremer showed that transection of the brainstem at the level of the medulla (*encephala insolé*) did not disrupt sleep-wake activity, whereas a transection made between the midbrain and pons (*cerveau insolé*) produced a chronic state of drowsiness [34]. These findings suggested that sensory input to the brain at the level of the spinal cord was not the switch that transitioned wakefulness to sleep. Rather, arousal was maintained by cortical and subcortical activity.

In subsequent experiments, electrical stimulation of neurons within the brainstem reticular formation of anesthetized cats evoked cortical EEG activity that resembled a state of arousal. However, when lesions were made to the same area, the low amplitude, high-frequency cortical EEG activity was replaced with slow-wave activity characteristic of sleep [35]. At the time, it was known that the brainstem reticular formation also received sensory input from the thalamus; therefore, it was thought that the observed effects produced by the lesion was due to the sensory pathway being disconnected from the brainstem. Taken together, their findings suggested sleep was not a passive process by the brain, but rather an active process that was generated by a network interaction between ascending neurons from the brainstem reticular formation and sensory pathways. Years later, work by Steriade and colleagues would confirm that gating of sensory input from the thalamus is a key component of sleep [36]. These findings developed the concept of the ascending reticular activating system (ARAS) as the control center for maintaining cortical activation.

Sleep was regarded as a unitary state until Aserinsky and Kleitman observed human infants exhibited slow eye movements at the beginning of sleep that became more rapid as sleep depth increased [37]. These rapid eye movements were also observed to coincided with reduced body movements. Shortly after their discovery, Jouvet used EEG to record brain activity of sleeping cats. He noted that cats would exhibit rapid eye movement (REM) with loss of muscle tone and wake-like activity reflected in the cortical EEG intermittently during sleep [38]. Collectively, their findings suggested sleep was composed of more than one stage. Rather, sleep was dissociated into nonREM and REM stages. What is more, the active cortical wake-like state observed by Jouvet further suggest the ARAS may also play a role in the activity of REM sleep.

The Neuroanatomy and Neurotransmitter Control of Sleep and Wakefulness

Wake-Generating Systems in the Brainstem

The ARAS is composed of a network of neurons that promote wakefulness via projections to forebrain targets. The cholinergic neurons of the pedunculopontine tegmentum (PPT) and laterodorsal tegmental nuclei (LDT) located in the upper pons of the brainstem project to the thalamus to facilitate transmission of sensory information to the cortex. Although cholinergic PPT and LTD neurons fire most rapidly during wakefulness, they are also active during REM sleep; therefore, these cell groups have been labeled as REM-OFF or REM-ON depending on their state [39,40]. Their distinct firing activity during periods of wakefulness have been linked to inputs from the hypothalamus. Immunohistological tracing studies have shown that neurons expressing the neuropeptides orexin within the lateral hypothalamus and histamine in the tuberomammillary nucleus (TMN) within the posterior hypothalamus (PH) differentially project to PPT/LTD neurons to promote wakefulness [41,42].

Orexin neurons have also been shown to receive inputs from noradrenergic neurons in the locus coeruleus (LC) as well as dopaminergic neurons in the ventral tegmental area (VTA) and serotonergic neurons in the raphe nucleus (RH) of the brainstem ARAS [43-45]. These monoaminergic neurons in the ARAS network innervate the lateral hypothalamus (LH) and posterior hypothalamus (PH) to promote wake by projecting to glutamatergic, GABAerigic and cholinergic inputs of the basal forebrain (BF) . The cholinergic neurons in the BF are well established contributors to this arousal network. In a study conducted in freely moving mice under optogenetic control and surface EEG recordings to measure brain activity, it was found that optogenetic activation of BF cholinergic neurons during EEG nonREM sleep induced cortical EEG wake activity [46].

Other optogenetic studies that selectively targeted GABAergic and glutamatergic BF nuclei also found that these cells promoted wakefulness; however, activation of paravalbumin-containing GABAergic cells pose a greater influence on wake compared to the other cells [47]. The purpose of the BF nuclei activating the cortex is to facilitate the processing of incoming sensory information during wakefulness.

The Hypothalamic Control of NonREM Sleep

Decades before the discovery of REM sleep, von Economo observed that patients who suffered from the virus *encephalitis lethargica* exhibited disturbances to their sleep-wake activity. He noted, upon post-mortem examination of their brains that patients who exhibited severe insomnia had lesions in the preoptic area (POA) of the anterior hypothalamus, whereas lesions in the posterior hypothalamus (PH) corresponded with patients who exhibited hypersomnolence [48]. These findings localized sleep and wake promoting areas to the hypothalamus even before the ARAS had been identified as the control of cortical activity and behavioral arousal. Surgical lesion experiments conducted in rodents would later verify von Economo's findings [49]. More recently, studies have identified sleep promoting neurons in the median preoptic area (MnPOA) and ventrolateral preoptic area (VLPO) in the anterior hypothalamus using c-Fos immunostaining techniques and electrophysiological methods to confirm c-Fos findings [50-53].

Saper and colleagues used retrograde and anterograde tracers to identify the pathway of the sleep promoting neurons in the VLPO in rodents. Their findings indicated the VLPO shares reciprocal connections to noradrenergic, histaminergic and serotonergic wake promoting neurons in the ARAS. Additionally, they found that the VLPO receives dense inputs from the MnPOA and lateral hypothalamus [54]. In previous studies, the inputs of the MnPOA and VLPO sleep promoting neurons were identified to be GABAergic and galaninergic; however, double-label immunohistochemistry of the retrograde projections to the ARAS showed galaninergic input from the VLPO [55,56]. In support of these findings, optogenetic activation of galaninergic VLPO neurons in rodents has been shown to promote sleep, suggesting the reciprocal interaction between wake and sleep promoting systems is under inhibitory control [57].

Saper and colleagues also showed in their anterograde tracing study that lateral hypothalamic neurons with orexin inputs project to the VLPO [54]. Previously, it has been mentioned that orexin neurons in the lateral hypothalamus are important for arousal stability; however, it had not yet been determined if activation of orexin neurons could induce arousal from a sleep state. In a study conducted in rodents, it was found that optogenetic activation of orexin neurons in the lateral hypothalamus increased the probability of transition to wakefulness from slow-wave sleep [58]. Based on these findings Saper later proposed orexin inputs from the lateral hypothalamus to both sleep and wake promoting systems acts like a switch to promote wake or sleep states [59].

REM Sleep Generator

The generation and maintenance of REM sleep is facilitated by a complex network interaction between neurotransmitters in the brainstem and the cortex. Jouvet was the first to localize REM sleep to the brainstem. He first showed in cats that REM sleep persisted following decortication, cerebellar abrasion, or rostral transection of the brainstem; however, when he transected the posterior boundary of the pons the cats did not exhibit signs of REM sleep. What is more, when he removed all of the structures rostral to the pons a state similar to REM sleep was still present suggesting the brainstem structures were necessary for REM sleep [60]. Jouvet was also the first to demonstrate in cats that cholinergic neurons play a role in generating REM sleep by systemically administering atropine. This resulted in the suppression of REM sleep, whereas administering an acetylcholinesterase inhibitor recovered the effects of the atropine [61]. In support of these findings, George and colleagues discovered that bilateral injections of a cholinergic agonist into the brainstem pontine reticular formation of cats produced atonia, desynchronized cortical EEG activity and theta hippocampus activity; suggesting a cholinergic mechanism in the brainstem reticular formation promotes REM sleep [62].

Selective optogenetic activation of cholinergic neurons in the PPT and LTD in rodents during nonREM sleep has been shown to increase the transition into REM sleep [63]. Recall that the cholinergic neurons in the PPT and LTD also play a role in promoting wakefulness through a REM-ON/REM-OFF switch. Several immunohistological studies that have found serotonergic and noradrenergic inputs from the LC and DRN project to the PPT and LTD [64-66]. Local activity from noradrenergic locus coeruleus (LC) neurons and serotonergic dorsal raphe (DRN) neurons exhibit a discharge pattern that is greatest during wake compared to during REM sleep. McCarley and colleagues suggested that this opposing discharge profile from cholinergic and monoaminergic brainstem neurons during wake and REM sleep produce the ON-OFF switch that

determines each state [67-69]. The decreased discharge activity of the monoaminergic during REM sleep is modulated by GABAergic input from forebrain and midbrain structures. Double-staining experiments combining glutamic acid decarboxylase (GAD) and cholera toxin-B (CTB) showed that GABAergic inputs to the LC and DRN diffusely project from the lateral hypothalamus and preoptic area as well as the ventrolateral periaqueductal gray (vlPAG) [70-73]. Recent consideration has been given to melanin-concentrating hormone (MCH) producing neurons in the lateral hypothalamus as contributing to the promotion of REM-ON neurons through their input onto REM-OFF neurons; however, the mechanism remains unclear [74-76].

CHAPTER THREE: MATERIALS AND METHODS

Animals and Housing

The secondary analysis was performed on data that are in accordance with the NIH Guidelines and the University of Tennessee Animal Care Committee (Protocol Number: 2311). The original experiment was conducted on male and female *Bdnf* wild-type (Charles River Laboratory, Wilmington, MA) and *Bdnf* heterozygous knockdown (Sage Labs, Boyertown, PA) Sprague Dawley rats, weighing 250-300 g. The study used a total of 27 animals for baseline sleep-wake recordings (WT-Females n =6, KD-Females n =8, WT-Males n =6, KD-Males n =7).

All animals were housed with a standard 12-hour light/12-hour dark cycle in the UT Medical Center Animal Facility with free access to food, water, and enrichment material. After animals were implanted with sleep recording electrodes, animals were individually housed to prevent cage mates from removing the implanted electrode. Until the recording sessions began, all animals were handled for at least 5 to 10 minutes each day by the experimenter to reduce protocol-related stress.

Surgical Procedure

Sleep Recording Electrode Implantation

As an additional measure to reduce stress, all surgical procedures started one week after arrival to animal facility. This allowed the animals time to acclimate to the animal facility. After this period, all animals were surgically implanted with chronic sleep-recording electrodes. Throughout the surgical procedure, Isoflurane (5% induction in 100% oxygen; 2%-3% maintenance in 100% oxygen) was used to anesthetize the animals. Both lidocaine and atropine were used for surgical and anesthetic support. These agents were not specifically cited in the approved protocol, but since they are considered part of normal support to improve the quality of animal care, and their use does not constitute protocol noncompliance; the agents were added by amendment to the approved protocol. Core body temperature was maintained during this time with a heating pad placed under the animal. Once under anesthesia, the animal's head was shaved and then positioned into a stereotaxic frame using blunted ear bars. A series of alternating povidone iodine and alcohol swabs were performed to clean the scalp before making a small midline incision. Once the facia was removed and the surface of the skull was exposed, 3% hydrogen peroxide followed by 70% ethyl alcohol were used to clean and dry the skull so the bregma suture could be more easily identified.

To record states of sleep and wakefulness, cortical EEG stainless steel electrodes were drilled bilaterally into the skull 3.0 mm anterior and 2.0 mm lateral to the bregma. An additional stainless-steel electrode was drilled into the skull 3.0 mm posterior and 3.0 mm lateral to the bregma to act as a reference electrode. To record EMG activity, a pair of teflon-coated stainless-steel wire electrodes were bilaterally implanted into the neck muscles. Lastly, to record hippocampal EEG, a hole was drilled into the skull 3.8 mm posterior and 2.5 mm lateral to the bregma. Once the dura was visible, it was punctured to allow a Formvar-coated stainless-steel

bipolar electrode to be sterotaxically implanted at a depth of 4.0 mm into the brain. All electrodes were crimped to mini-connector pins and pushed into a plastic electrode connector, which was secured to the skull with dental acrylic. Once the dental acrylic was completely bonded, antibiotic ointment (Triple antibiotic: Combination of bacitracin, neomycin, and polymyxin) was applied to the perimeter of this newly-formed head cap. Additionally, normal saline (5 cc, s.c) was administered to prevent dehydration.

Post-surgical Recovery

At the end of the surgical procedure, animals were returned to their home cage and observed until fully recovered from anesthesia. All animals were given buprenorphine (0.05 mg/kg, I.M) to manage post-operative pain. Once fully recovered, animals were transferred to the housing facility and signs for post-surgical complications were monitored daily.

Sleep Recording Procedure

Habituation

All animals were habituated to the sleep- recording system following post-surgical recovery. During habituation, each animal was placed into a recording chamber with free access to food, water, and enrichment for 6 hours (9:00 a.m to 3:00 p.m). A customized, multichannel preamplifier with a flexible cable was mated to the head cap mounted on the animal's head. The other end of this flexible cable was connected to a commutator mounted above the cage. The commutator allows the animal unrestricted movement around the chamber.

Recording Settings

The preamplifier mated to the head cap performed 100X amplification on the recorded signals. The cortical and hippocampal EEG signals were sampled at 1000 Hz and band-passed filtered between 0.5 Hz and 100 Hz. The EMG signal was sampled at 2000 Hz and band-passed filtered between 10 Hz and 200 Hz. A data-conditioning and acquisition system performed secondary amplification and digitization before sending signals to a computer located outside of the sleep recording-room. This allowed the experimenter to monitor the animals without disturbing them.

Sleep Recording

Each animal was connected to the sleep-recording chamber as previously described and allowed undisturbed sleep for 6 hours (9:00 a.m to 3:00 p.m) while being recorded. After recordings were finished, animals were returned to their home cage and transferred to the housing facility.

Data Analysis

Sleep-Wake Scoring Criteria

All collected sleep recording data was scored for three sleep-wake states: Wakefulness, NonREM sleep, and REM sleep (Figure 3.1). To establish EEG correlates related to each state, wakefulness was determined when the cortical EEG exhibited low amplitude (40-60 μV) and

high frequency (35-50 Hz) oscillations. During this state, the EMG exhibits bursts of activity indicating movement or exploration by the animal while the hippocampal EEG exhibits mixed oscillations that tended to be higher in frequency. NonREM sleep was established when the cortical EEG activity began to exhibit high amplitude (200 – 400 μV) and low frequency (0.1-10 Hz) oscillations. EMG tone is diminished, and the hippocampal EEG begins to exhibit sharp waves and associated ripples; a trait characteristic of delta slow-wave activity (SWA). During REM sleep, the cortical EEG exhibited low amplitude (50-80 μV) and high-frequency (20-40 Hz) oscillations. Due to atonia during this state, the EMG exhibited very low levels of activity. The hippocampal EEG during REM sleep exhibits distinct sinusoidal theta (4-8 Hz) oscillations. This qualitative feature provides an obvious distinction between wakefulness and REM sleep despite the cortical EEG exhibiting fast, de-synchronized oscillations during both states.

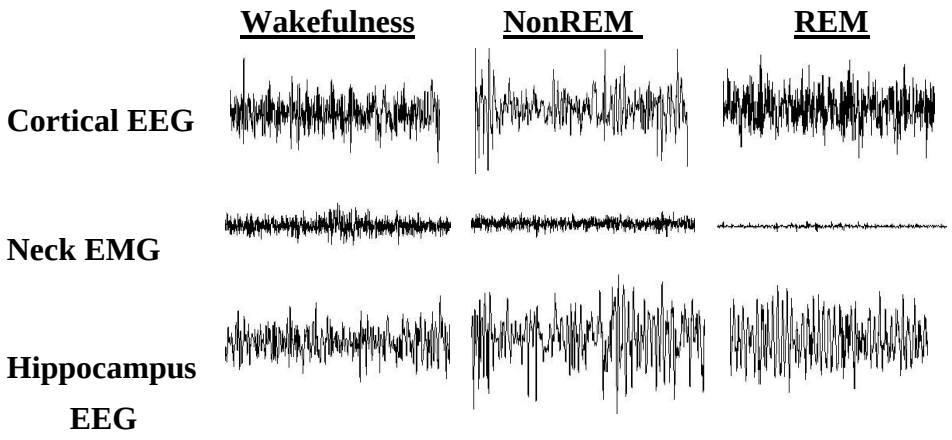


Figure 3.1. Representative Sleep-Wake EEG Oscillation Patterns

Recordings of brain oscillations for each sleep-wake state occurring over approximately 6 seconds. Note the difference in brain wave amplitude and frequency across states.

Statistical Analyses

To assess the effect of altered BDNF expression on baseline sleep-wake states, the total percentage of time spent in wakefulness, NonREM sleep, and REM sleep were analyzed using two-way (time x strain) ANOVA with repeated measures. Additionally, we conducted univariate (strain) ANOVA to analyze the total number of episodes for each state, and their average duration as well as REM sleep latency.

Pairwise, comparisons were used to determine significant interactions for all analysis. Mauchly's Test of Sphericity was used to test for homogeneity of variance in all analyses of the repeated measures. When sphericity was violated, the greenhouse-Geisser correction was applied.

CHAPTER FOUR: RESULTS

Sleep-Wake Architecture is Impaired in Knockdown Animals

The aim of this secondary analysis was to examine the functional link between BDNF expression and sleep-wake activity using data collected from heterozygous *Bdnf* and wild-type rats. All rats were allowed six hours of undisturbed sleep while sleep-wake activity was recorded. The presentation of results illustrates both time and strain differences and are also collapsed across time to emphasize any differences between strain.

Time Spent in REM Sleep

Two-way ANOVA with repeated measures revealed a significant main effect of strain in the amount of time spent in REM between the knockdown and wild-type animals (Females: $F_{(1,14)}$= 31.712, $p < 0.0001$; Males: $F_{(1,13)}$= 13.277, $P = 0.004$) , but no effect of time (Females: $F_{(5,60)}$ = 1.418, $p = 0.231$; Males: $F_{(5,55)}$= 1.118, $P = 0.362$) and no interaction between time and strain (Females: $F_{(5,60)} = 2.310$, $p = 0.055$; Males: $F_{(5,55)} = 0.692$, $p = 0.631$) (Figure 4.1.A,B)

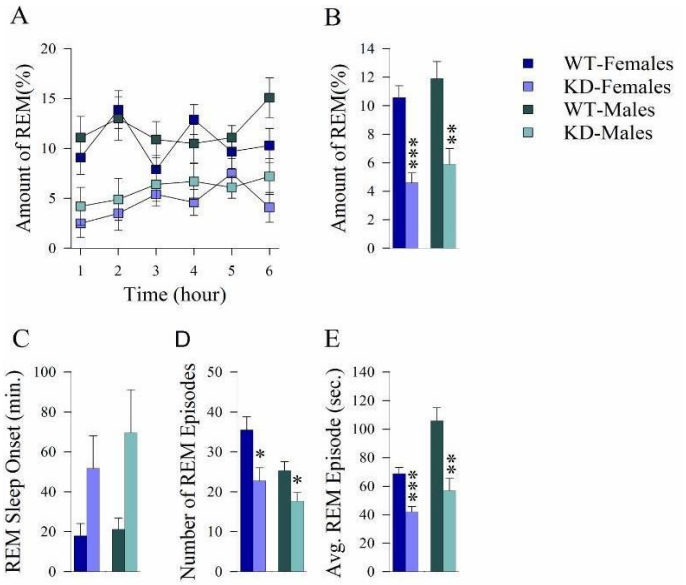


Figure 4.1. Differences in Amount of Time spent in REM Sleep

(A) Amount of time spent in REM sleep during consecutive one-hour intervals of the six-hour sleep recording (9:00 am to 3:00 pm) for all groups of animals (WT-Females (n = 6) vs KD-Females (n = 8); WT-Males (n = 6) vs KD-Males (n = 7)). (B) Total percentage of time spent in REM Sleep collapsed across time to emphasize the significance between strain. (C) REM Sleep latency. Note that compared to the wild-type female and male strain, the knock-down females and males rats took longer to reach the first REM sleep episode. (D) The total number of REM episodes. (E) Average duration of a REM sleep episode. All values are represented as mean ± SEM. Asterisk indicates level of significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

Although univariate analyses of the amount of time required to transition into the first REM episode did not reveal a significant difference between the knockdown and wild-type animals (Females: $F_{(1,14)} = 2.936$, p =0.112;Males: $F_{(1,13)} = 4.058$, p = 0.069), there was a trend for knockdown animals to require more time to achieve the first REM episode compared to the wild-type animals (Figure 4.1C. Additionally, the knockdown animals exhibited significantly fewer REM episodes (Females: $F_{(1,14)}= 7.123$, p = 0.02; Males: $F_{(1,13)}= 6.275$, p = 0.029) that were shorter in duration (Females: $F_{(1,14)}= 21.452$, p = 0.001; Males: $F_{(1,13)}= 15.044$, p = 0.003) compared to the wild-type animals (Figure 4.2D,E). Taken together, these findings indicate the knockdown animals had difficulty initiating and maintaining REM sleep.

Time Spent Awake

Two-way ANOVA with repeated measures revealed a significant main effect of time ($F_{(3.018, 36.213)} = 4.034$, p = 0.14) , but no effect of strain ($F_{(1,14)} = 1.160$, p = 0.303), and no interaction between strain and time ($F_{(3.018, 36.213)} = 0.906$, p = 0.448) in the amount of time spent awake between the knockdown and wild-type females . Pairwise comparisons did not reveal a significant difference between a specific time point between the knockdown and wild-type females' amount of time spent awake (1-hour: t = -1.563, p = 0.179; 2-hour: t = -0.950, p = 0.386; 3-hour: t = - 0.394, p = 0.710; 4-hour: t = -0.993, p = 0.366; 5-hour: t = 1.546, p = 0.183; 6-hour: t = -0.257, p = 0.807). The two-way ANOVA with repeated measures for males did not reveal a significant main effect of time ($F_{(2.799,30.791)} = 1.721$, P = 0.186), strain ($F_{(1,11)} = 3.800$, p = 0.077), or time by strain interaction ($F_{(2.799,30.791)} = 0.563$, p = 0.632) in the amount of time spent awake. Additionally, the number of wake episodes between the knockdown and wild-type females and males did not significantly differ (Females: $F_{(1,14)} = 0.034$, p = 0.857; Males: $F_{(1,13)} = 1.373$, p = 0.266) nor did average duration of a wake episode (Females: $F_{(1,14)} = 1.603$, p = 0.230; Males: $F_{(1,13)} = 1.633$, p = 0.228) (Figure 4.2A-D).

Time Spent in NonREM

Similarly, a two-way ANOVA with repeated measures for the amount of time spent in nonREM revealed a significant main effect of time between the knockdown and wild-type females ($F_{(3.355, 40.256)} = 4.187$, p = 0.009) but no effect of strain (Females: $F_{(1,14)} = 0.032$, P = 0.861), and no interaction between strain and time ($F_{(3.355,40.256)} = 1.118$, p = 0.356). Pairwise comparisons did not reveal a significant difference between a specific time point between the knockdown and wild-type animals in the amount of time spent in nonREM (1-hour: t = 0.941, p = 0.390; 2-hour: t = -1.442, p = 0.209; 3-hour: t = 0.137, p = 0.896; 4-hour: t = -0.363, p = 0.731; 5-hour: t = -1.701, p = 0.150; 6-hour: t = - 0.529, p = 0.619). The two-way ANOVA with repeated measures for males did not a reveal a significant main effect of time ($F_{(5,55)} = 1.912$, P = 0.107), strain ($F_{(1,11)} = 0.002$, P = 0.968), or time by strain interaction ($F_{(5,55)} = 0.561$, P = 0.729) in the amount of time spent in nonREM (Figure 4.3E,F). Additionally, the number of nonREM episodes (Females: $F_{(1,14)} = 0.008$, p = 0.928; Males: $F_{(1,13)} = 0.944$, p = 0.352) and average duration of a nonREM episode (Females: $F_{(1,14)} = 0.178$, p = 0.680; Males: $F_{(1,13)} = 0.008$, p = 0.929) did not significantly differ between the knockdown and wild-type females and males. Collectively, these findings indicate the quantity and quality of time spent awake and, in NonREM sleep, were not affected by altered expression of BDNF (Figure 4.2C,D).

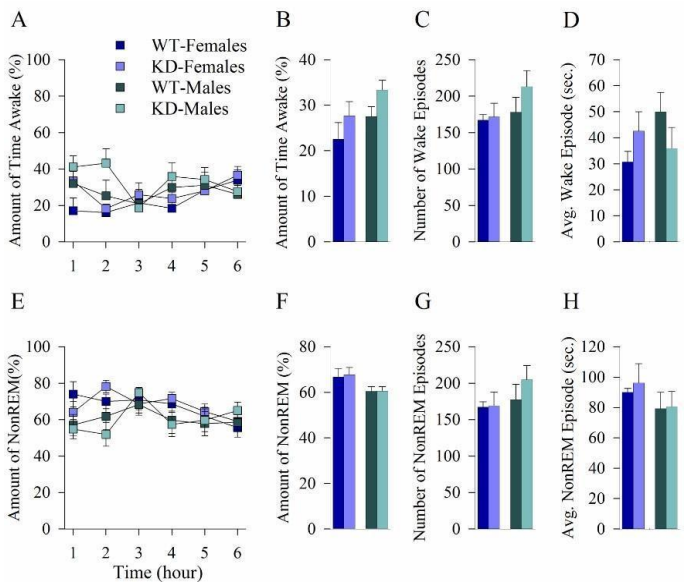


Figure 4.2. Differences in the Amount of Time Spent Awake and in NonREM

(A) Amount of time awake during consecutive one-hour intervals of the six-hour sleep recording (9:00 am to 3:00 pm) for all groups of animals (WT-Females (n = 6) vs KD-Females (n = 8); WT-Males (n = 6) vs KD-Males (n = 7)). (B) Total percentage of time spent in wakefulness collapsed across time to emphasize the lack of significance between strain. (C) The total number of wake episodes. (D) Average duration of a wake episode. (E) Amount of time in NonREM during consecutive one-hour intervals of the six-hour sleep recording for all groups of animals. Like wakefulness, no significant differences were found between all groups. (F) Total percentage of time spent in NonREM sleep collapsed across time to emphasize the lack of significance difference between strain. (G) Total number of NonREM episodes. (H) Average duration of a NonREM episode. All values are represented as mean ± SEM.

CHAPTER FIVE: DISCUSSION

The aim of the original study was to investigate the functional link between BDNF expression and sleep-wake activity using *Bdnf* knockdown and wild-type *Bdnf* rats. Secondary analysis of the original data found that compared to the wild-type rats, altered expression of BDNF in the knockdown rats had a profound effect on REM sleep expression during the rest phase of the light cycle. The knockdown rats were still able to achieve REM sleep but at a significantly reduced total amount compared to the wild-type rats. Compared to the original findings, the results of the secondary analysis did not find a significantly reduced amount of time spent in REM sleep across each consecutive hour of the sleep-wake recording [1]. Interestingly, the knockdown rats did not require a significantly longer amount of time to achieve the first REM episode as the original data suggested; however, once REM sleep was initiated, there were significantly fewer episodes that were shorter in duration, suggesting altered expression of BDNF significantly decreased the quality of REM sleep. Although the variability within the knockdown rats' REM sleep onset is high, it is important to note that the sample size for the original study was underpowered. Therefore, it is reasonable to suggest that increasing the sample size would not only reduce the variability but also verify if the trend we are observing is significant. Furthermore, given decreased REM sleep quality is associated with psychiatric disorders such as depression these findings may provide insight into the mechanism underlying the disorder.

Similar to the original findings, the secondary analysis did not reveal any significant differences in the total amount of time spent in nonREM sleep between the knockdown and wild-type rats. Considering both the knockdown and wild-type rats were able to achieve about 60 percent of nonREM during the light period suggest circadian processes regulating nonREM sleep were intact. In contrast to the original findings, the number of nonREM episodes and the average duration of a nonREM episode did not significantly differ between the knockdown and wild-type rats. This suggest the quality of nonREM sleep was not affected by altered expression of BDNF. Given REM sleep onset could be affected by altered BDNF expression despite nonREM sleep being unaffected suggest the timing of these two distinct stages may be independent of one another.

Similarly, the secondary analysis of the amount of time spent awake showed no significant difference between the knockdown and wild-type rats. This is reflected in the similar number of wake episodes and average episode duration when awake; however, the original findings suggested the knockdown animals exhibited a significantly higher number of wake episodes compared to wild-type rats despite having a similar duration. In both analyses, altered BDNF did not impact the normal microarousals that occur during transitions between sleep cycles.

There were several shortcomings in the original study that deserve consideration. The original study analyzed changes in EEG slow-wave activity during nonREM sleep but did not analyze theta power during REM sleep. Inspection of the recording signals will reveal that multiple animals had damaged EEG cortical or EEG hippocampal signals that likely resulted from damage caused by surgical implantation of electrodes (Figure A1,A2). In these cases, the signals were good enough to score each sleep-wake state, yet unreliable for generating power. Considering hippocampal theta activity is an EEG hallmark trait of REM sleep, this would have

been a useful measure to demonstrate how theta activity would change in response to changes in REM sleep. Logically, it would follow that the amount of hippocampal theta activity in the knockdown animals would also be significantly reduced to reflect the reduction of REM sleep. Additionally, the original study conducted a three-hour selective REM sleep deprivation procedure to investigate the role BDNF plays in the homeostatic regulation of REM sleep. Due to concerns for the method used to conduct the deprivation procedure the data has been left out of the current analysis; however, future investigation into the homeostatic process of sleep could provide insight into the role BDNF plays in regulating REM sleep homeostasis.

Female and male rats were used to conduct the original study; however, the secondary analysis did not examine any potential sleep related sex differences. Alternatively, these results should serve as the basis for preliminary findings to motivate future investigation. Considering most of sleep research has been conducted in males until recently, sex differences in sleep have not been fully explored. There is evidence to suggest BDNF interacts with sex steroid signaling [77,78]. Gonadectomized male pups at postnatal day 0 (P0) showed significantly reduced BDNF mRNA levels a week later. Estrogen treatment in the gonadectomized male pubs at postnatal 0 (P0) resulted in BDNF mRNA levels similar to intact animals suggesting estrogen regulates the production of BDNF in the rat hippocampus [79]. Female reproductive hormones are known to alter sleep architecture, and therefore a female *Bdnf* knockdown model may provide insight into how developmental changes impact sleep.

In summary, the results of this secondary analysis demonstrate BDNF plays a causal role in the expression of normal REM sleep. It is interesting that the knockdown animals were still able to achieve REM sleep although it was altered. This suggest that there is more to the mechanisms regulating REM sleep than simply BDNF signaling. There is a wealth of insight to be gained from using heterozygous *Bdnf* rats as a model for investigating the role BDNF plays in regulating REM sleep. Future studies could locally administer BDNF into sleep promoting areas within the brain to see if observed effects could be rescued in the knockdown rats. Additionally, neuronal tissue quantification of BDNF in knockdown and wild-type rats would provide a beneficial measure for determining the levels of BDNF present in generating the observed effects. Furthermore, the original study recorded across a 6-hour period during the light cycle, which limits the interpretation of results. Future investigations should consider recording over a 24-hour period to account for differences in behavior during the dark-cycle when rats are active.

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APPENDIX

Table A-1: Animals used for Data Analysis

STRAIN	SEX	ANIMAL ID	DATE REC'D	VENDOR
Knockdown	Male	BDNF M1 KD	2/24/2016	Sage
Knockdown	Male	BDNF M3 KD	2/24/2016	Sage
Knockdown	Male	BDNF M5 KD	2/24/2016	Sage
Knockdown	Male	BDNF M6 KD	2/24/2016	Sage
Knockdown	Male	BDNF M7 KD	2/24/2016	Sage
Knockdown	Male	BDNF M23 KD	9/27/2016	Sage
Knockdown	Male	BDNF M24 KD	9/27/2016	Sage
Knockdown	Female	BDNF F1 KD	2/24/2016	Sage
Knockdown	Female	BDNF F2 KD	2/24/2016	Sage
Knockdown	Female	BDNF F3 KD	2/24/2016	Sage
Knockdown	Female	BDNF F4 KD	2/24/2016	Sage
Knockdown	Female	BDNF F5 KD	2/24/2016	Sage
Knockdown	Female	BDNF F7 KD	2/24/2016	Sage
Knockdown	Female	BDNF F8 KD	2/24/2016	Sage
Knockdown	Female	BDNF F21 KD	Unknown	Sage
Wild-type	Male	BDNF M9 WT	5/26/2016	Charles River
Wild-type	Male	BDNF M12 WT	5/26/2016	Charles River
Wild-type	Male	BDNF M13 WT	5/26/2016	Charles River
Wild-type	Male	BDNF M14 WT	5/26/2016	Charles River
Wild-type	Male	BDNF M15 WT	5/26/2016	Charles River
Wild-type	Male	BDNF M16 WT	5/26/2016	Charles River
Wild-type	Female	BDNF F9 WT	5/26/2016	Charles River
Wild-type	Female	BDNF F10 WT	5/26/2016	Charles River
Wild-type	Female	BDNF F12 WT	5/26/2016	Charles River
Wild-type	Female	BDNF F13 WT	5/26/2016	Charles River
Wild-type	Female	BDNF F14 WT	5/26/2016	Charles River
Wild-type	Female	BDNF F15 WT	5/26/2016	Charles River

Table A-2: Animal Procedure Dates and Sleep Recording Dates

ANIMAL ID	SURGERY DATE	RECORDING DATE
BDNF M1 KD	4/7/2106	4/19/2016
BDNF M3 KD	4/8/2016	4/19/2016
BDNF M5 KD	5/10/2016	5/19/2016
BDNF M6 KD	5/10/2016	5/19/2016
BDNF M7 KD	5/11/2016	5/19/2016
BDNF M23 KD	10/5/2015	10/26/2016
BDNF M24 KD	10/6/2016	10/26/2016
BDNF F1 KD	4/11/2016	5/6/2016
BDNF F2 KD	4/12/2106	4/23/2016
BDNF F3 KD	4/18/2016	5/10/2016
BDNF F4 KD	4/19/2016	5/10/2016
BDNF F5 KD	5/17/2016	5/30/2016
BDNF F7 KD	5/16/2016	5/30/2016
BDNF F8 KD	5/16/2016	5/30/2016
BDNF F21 KD	10/7/2016	11/10/2016
BDNF M9 WT	6/23/2016	7/5/2016
BDNF M12 WT	6/20/2016	7/5/2016
BDNF M13 WT	6/28/2016	7/12/2016
BDNF M14 WT	6/29/2016	7/9/2016
BDNF M15 WT	6/30/2016	7/19/2016
BDNF M16 WT	7/1/2016	7/19/2016
BDNF F9 WT	7/12/2016	8/1/2016
BDNF F10 WT	7/12/2016	8/1/2016
BDNF F12 WT	7/13/2016	8/1/2016
BDNF F13 WT	7/14/2016	8/16/2016
BDNF F14 WT	7/14/2016	8/22/2016
BDNF F15 WT	7/15/2016	8/18/2016

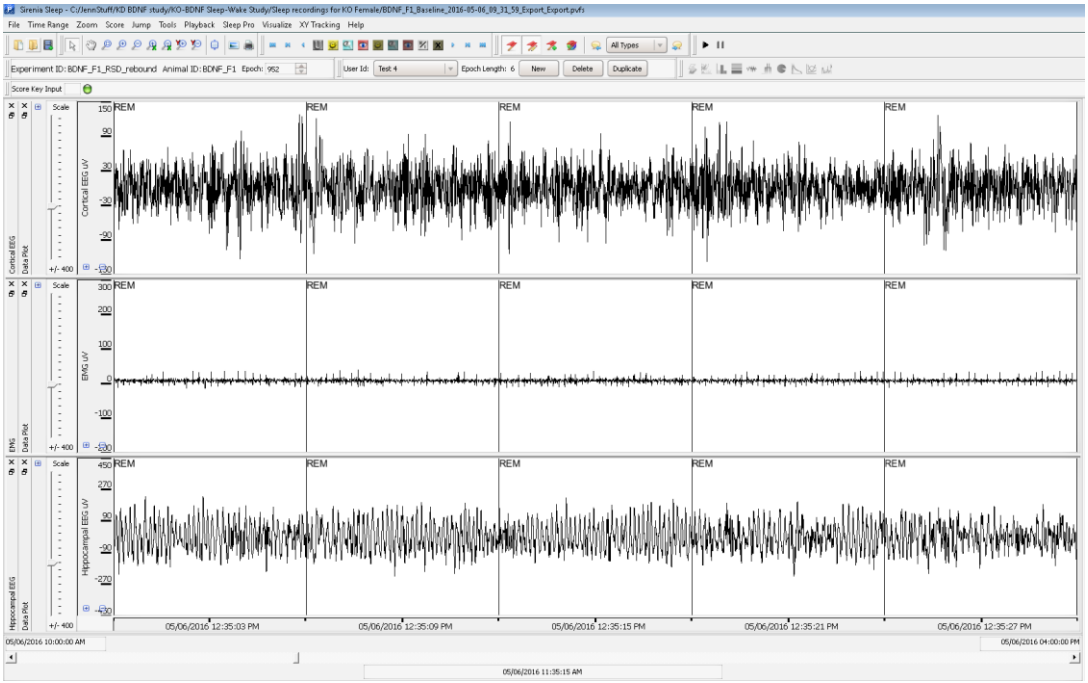


Figure A1. Representative image of normal EEG hippocampal theta activity

Recordings of EEG activity from surgical electrodes implanted into the cortex and hippocampus. Note the hippocampal activity during REM sleep produces district sawtooth waves with theta (4 to 10Hz) frequency.

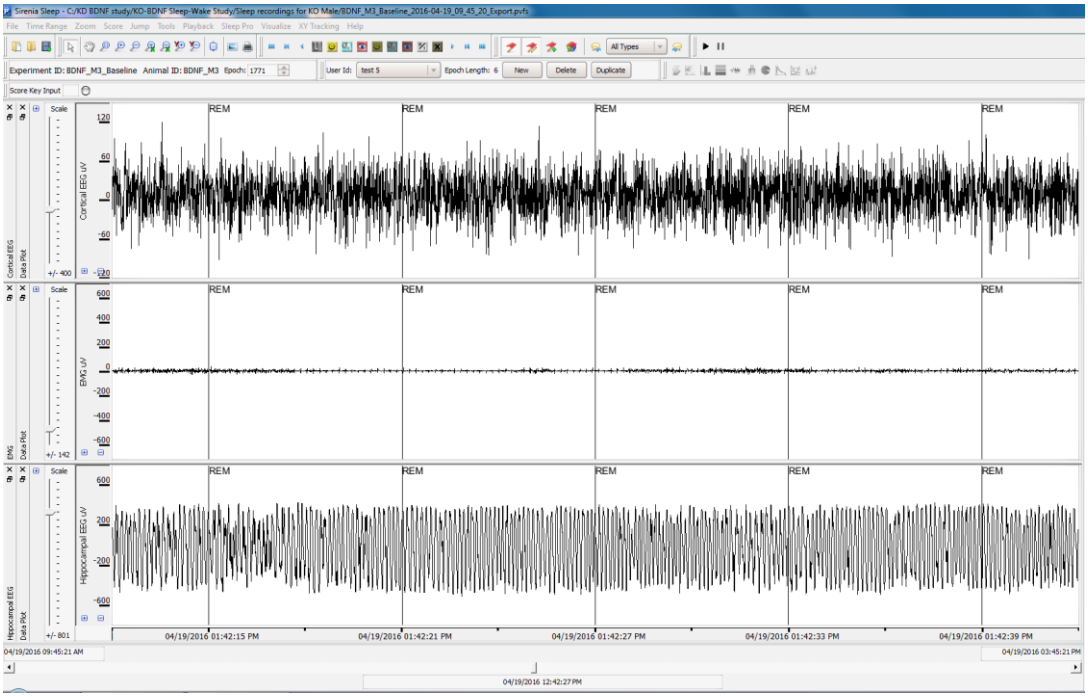


Figure A2. Representative image of damaged EEG hippocampal electrode signal

Recordings of EEG activity from surgical electrodes implanted into the cortex and hippocampus. Note the hippocampal activity during REM sleep produces uniform waveforms. This indicates a bad reading from the electrode suggesting an issue with the quality of the electrode.

LIST OF ATTACHMENTS

Description:

The accompanying folders contain the files of the sleep recordings scored for the secondary analysis. Each file has been compressed from its original size. The files can be viewed by downloading a free version of Sirenia Sleep software. The latest version of Sirenia can be found at: https://www.pinnaclet.com/sirenia-download.html. The scores for the secondary analysis are under the username "test" followed by a number. Each file represents a 6-hour sleep recording for the animal ID.

Filenames:

BNDF_M1_Baseline_2016-04-19_09_45_21_Export.pvfs
BNDF_M3_Baseline_2016-04-19_09_45_20_Export.pvfs
BNDF_M5_Baseline_2016-05-19_09_29_54_Export_Export.pvfs
BNDF_M6_Baseline_2016-05-19_09_29_52_Export_Export.pvfs
BNDF_M7_Baseline_2016-05-19_09_29_53_Export_Export.pvfs
BNDFKO_M23_Baseline_2016-10-26_09_10_48_Export.pvfs
BNDFKO_M24_Baseline_2016-10-26_09_10_50_Export.pvfs
BDNF_F1_Baseline_2016-05-06_09_31_59_Export_Export.pvfs
BDNF_F2_baseline_2016-04-23_09_58_40_Export.pvfs
BDNF_F3_baseline_6hr_2016-05-10_09_19_59_Export.pvfs
BDNF_F4_Recording_2016-05-10_09_20_00_Export.pvfs
BDNF_F5_Baseline_6hr_2016-05-30_09_19_35_Export.pvfs
BDNF_F7_Baseline_6hr_2016-05-30_09_19_34_Export.pvfs
BDNF_F8_Baseline_6hr_2016-05-30_09_19_32_Export.pvfs
BDNFKO_F21_Baseline_2016-11-10_10_10_46_Export.pvfs
BDNF_M9_baseline_2016-07-05_09_33_43_Exports.pvfs
BDNF_M12_baseline_2016-07-05_09_33_41_Exports.pvfs
BDNF_M13_baseline_2016-07-12_09_59_38_Exports.pvfs
BLS_BDNF_WT_M14_2016-07-09_09_51_42_Exports.pvfs
BDNFWTM15_BLS_2016-07-19_09_09_49_Export_Export.pvfs
BDNFWTM16_BLS_2016-07-19_09_09_51_Export_Export.pvfs
BDNF_WT_F9_baseline_2016-08-01_09_44_37_Export.pvfs
BDNF_WT_F10_baseline_2016-08-01_09_44_34_Export.pvfs
BDNF_WT_F12_baseline_2016-08-01_09_44_38_Export.pvfs
BDNF_WT_F13_baseline_2016-08-16_09_27_04_Export.pvfs
BDNF_WT_F14_baseline_2016-08-25_09_51_10_Export.pvfs
BDNF_WT_F15_baseline_2016-08-18_09_40_20_Export.pvfs

VITA

Jennifer Garner holds two undergraduate degrees in the arts and the sciences. In 2005, she graduated from San Francisco State University with a Bachelor of Arts degree in Drawing and Painting, then later pursued a degree in Biochemistry and Molecular and Cellular Biology from the University of Tennessee in 2016. Following graduation, Jennifer spent the summer working as a research associate in the Sleep and Cognitive Neuroscience Laboratory in the Anesthesiology Department at University of Tennessee Medical Center until joining the Experimental Psychology graduate program at University of Tennessee in the Fall of 2016. She continued to purse sleep-related research in the Sleep and Cognitive Neuroscience Laboratory and graduated with a Master of Art degree and a minor in Neuroscience. She aims to apply her knowledge of neuroscience to the field of medicine.