THE ROLE OF HYPOCRETIN IN POST-TRAUMATIC BRAIN INJURY SLEEP-WAKE DISTURBANCE

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Abstract

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Traumatic brain injury (TBI) is a major cause of disability, affecting millions of individuals in the United States alone. Disorders of sleep and wakefulness occur in over half of people who sustain a TBI. Sleep-wake disturbances predict poorer cognitive, social, and functional recovery from TBI and negatively impact quality of life. Currently, there is a dearth of highly effective therapies, largely due to a fundamental lack of understanding of the causes of increased sleep need and excessive daytime sleepiness after TBI. In the present set of experiments, I used a controlled cortical impact (CCI) model of TBI to investigate the role of hypocretin in post-TBI sleep-wake disturbance. In the first set of experiments (Chapter 2), I determined the effects of mild and moderate TBI on sleep-wake behavior using electroencephalographic (EEG) recordings and on neuronal populations important for regulating sleep and wake behavior using immunohistochemistry (IHC). I found that moderate TBI resulted in chronic decreases in wakefulness and increases in non-rapid eye movement (NREM) sleep during the dark period. Moderate TBI also chronically decreased numbers of hypocretinergic neurons in the hypothalamus and cholinergic neurons in the basal forebrain without affecting numbers of melanin-concentrating hormone neurons in the hypothalamus or histaminergic neurons in the tuberomammillary nucleus. In the second set of experiments (Chapter 3), I analyzed the effects of TBI on sleep-wake behavior over a 30-day period in intact and hypocretin knockout (KO) mice. Hypocretin KO mice did not display significant changes in sleep-wake behavior, either in terms of percent time spent in different sleep-wake states or in length of wake bouts. In intact animals, post-

TBI sleep-wake disturbance and reductions in hypocretin neuron numbers persist out to 30 days post-surgery. I conclude that in this injury model, TBI produces chronic deficits in wakefulness and in numbers of hypocretin neurons and that a change in the hypocretinergic system is necessary for post-TBI changes in sleep wake behavior. Thus, this project has important implications for the use of hypocretin-based therapeutics in chronic TBI.

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Chapter 1: Introduction

Traumatic brain injury

Traumatic brain injury (TBI) is a major public health problem. In the United States alone, it is estimated that as many as 5.3 million individuals are living with long-term disability as a result of experiencing a TBI and total medical costs from TBI may be as high as $76 billion per year. [1,2]

TBI is an incredibly complex disease state, on a cellular and behavioral level. Behavioral symptoms of TBI include deficits in attention and memory, neuromotor disorders, sleep disturbances, depression, psychosis, and personality changes.[3–7] On a cellular level, widespread neuronal loss occurs, even in brain areas remote from the injury site.[8–11] Excitotoxic, ischemic, and inflammatory processes can greatly increase the extent of neural injury.[12–14] Microglia and astrocytes become activated and have various effects on functional and cellular outcomes, some neuroprotective and others detrimental.[15–20] Many pro- and anti-inflammatory cytokines are upregulated.[21–23] White matter is lost and neural connectivity is impaired.[24] Glymphatic function is also impaired, resulting in reduced clearance of interstitial solutes from the brain.[25] The integrity of the blood-brain barrier may be compromised, allowing peripheral proteins and immune cells to enter the brain parenchyma.[26,27] The events that trigger various post-TBI detrimental or neuroprotective processes are not well understood, nor are ways that these processes interact.

Despite the vast amount of spending on care for TBI patients, currently available treatments for TBI have limited efficacy. Clinical trials of pharmacotherapies such as progesterone, citicoline, and modafinil, as well as various cholinesterase inhibitors and dopaminergic agents have produced mixed or negative results.[8,28–32] Lack of efficacy is likely due, at least in part, to a lack of understanding of the disease processes underlying post-TBI deficits.

Traumatic brain injury and sleep: human studies

One of the most common and debilitating symptoms of TBI is sleep-wake disturbance.[4,33–38] This high rate of sleep-wake disturbance (SWD) may lead to markedly reduced quality of life: post-TBI SWD predicted poor social, emotional, and cognitive outcomes.[39–41]

SWD is prevalent in human TBI populations: a meta-analysis of over 20 clinical studies found that post-TBI SWD occurred in approximately 50% of brain injuries.[42] Rates of post-TBI sleep disturbance may be even higher in military populations, with self-report rates as high as 97% even after a mild TBI. [43] However, for clarity, this thesis will not review studies on post-TBI sleep-wake changes in military, pediatric, or geriatric populations.

The nature of post-TBI SWDs is not necessarily consistent; there are reports of excessive daytime sleepiness (EDS) and increased sleep need,[4,33–35] but also of insomnia, sleep apnea, and circadian rhythm disturbance.[44–46] Prevalence estimates of post-TBI SWDs vary greatly between studies: insomnia prevalence may be between 5-70%[45] and EDS prevalence may be between 25-60%.[4,33,34] Variation in insomnia and EDS prevalence is likely due, at least in part, to differences between subjective and objective measures. TBI patients are poor estimators of their own symptoms of insomnia, daytime sleepiness, and total sleep times, with large differences between self-reported data and objective data in the same cohorts.[34,47,48] Estimates of post-TBI onset sleep apnea range from 0% [38] to ~35%,[49] with variation likely due to small sample sizes. There appear to be no clear estimates on the prevalence of circadian rhythm disorders after TBI.[50]

Evidence is mixed as to whether greater severity of injury results in a greater probability of developing SWD or experiencing worse symptoms of SWD. Ponsford and colleagues found greater injury severity was associated with greater sleep time in TBI patients.[36] Imbach and colleagues found that those with severe TBI had greater total sleep time than those with mild TBI, but did not have differences in objective measurements of daytime sleepiness at six months post-injury.[34] However, when this same cohort was examined again a year later, there was no association between injury severity and any sleep-wake outcome.[51] Furthermore, others have reported no correlation between TBI severity and any measures of sleep-wake disturbance.[35,48,52]

Sleep-wake disturbance begins in the acute period after TBI (within the first seven days[53]), and persists for at least three years.[35] Although patients may recover with time,[54] many groups have reported no association between SWD and amount of time since injury.[35,51,52,55]

The vast majority of studies have found that TBI patients have difficulty maintaining wakefulness during the day. Excessive daytime sleepiness (EDS) can be measured subjectively or objectively. Subjectively, questionnaires like the Epworth Sleepiness Scale (ESS) are used to assess feelings and behaviors associated with EDS. The ESS includes 8 questions, with each rated from 0-3. A score of less than 10 is considered normal, while a score greater than 10 indicates subjective EDS.[56]

EDS can also be assessed objectively using the Multiple Sleep Latency Test (MSLT), which measures how quickly subjects fall asleep when given a daytime sleep opportunity. Normal subjects generally fall asleep within 10-20 minutes; less than 10 minutes indicates abnormal sleepiness,[57,58] although some groups use 5[4,59] or 8 minutes[34,60] as the cut off.

Various studies have indicated that TBI patients have high rates of EDS when assessed objectively [34,38,51,58,59,61] or subjectively.[35,52,59] In agreement with this, TBI patients also have high rates of daytime napping.[38,62] As previously mentioned, TBI patients may misperceive their level of sleepiness; some may not have feelings of sleepiness, but their objective MSLT scores indicate that they have EDS.[34] While objective and subjective EDS have been found to be correlated in neurotypical populations,[63] ESS scores do not necessarily correlate with sleep latency on the MSLT in brain injured subjects.[48] This may lead to under-reporting of EDS if only subjective measures are used.

Time spent in various sleep-wake states is also altered after TBI. Sleep-wake state time can be measured in several ways. Sleep diaries are the most inexpensive, but also the most subject to error; TBI patients underestimated their nightly sleep by more than an hour.[38] Actigraphy measures sleep through the use of a wrist device that is highly sensitive to movement, thus providing a reasonably accurate estimate of sleep and wake time.[64] Polysomnography (PSG), the gold standard, uses electroencephalograph (EEG), electrooculograph (EOG), and electromyograph (EMG) activity to assess time spent in wake, rapid eye movement (REM) sleep, and the different stages of non-rapid eye movement (NREM) sleep. PSG is also the only technique that can be used to assess spectral

frequency of the EEG waveform. However, polysomnography is expensive, inconvenient for participants, and is highly subject to the first night effect, in which patients' sleep is perturbed by sleeping in a new setting.[65]

At chronic time points, several groups have found changes in sleep-wake time, or macroarchitecture. Studies using sleep diaries sometimes[38] but not always[52,62,66] found changes in total sleep time. However, those using actigraphy generally found an increase in total sleep time compared to controls.[34,38] Studies using PSG have revealed changes in sleep architecture as well. Notably, some groups have found increases in light NREM sleep[61] or in slow wave sleep, a type of deep NREM sleep.[37,38,67,68] Studies generally showed no differences in time spent in REM,[34,37,38,51,68,69] although a few indicate reduced REM sleep.[61,67,70]

Many studies indicate that sleep quality is poorer and that the sleep and wakefulness states are fragmented in TBI patients. Subjectively, sleep quality can be assessed with the Pittsburgh Sleep Quality Index (PSQI) and indeed PSQI scores are generally worse in those with TBI than in controls.[32,52,55,69,71–75] Objectively, sleep quality or sleep fragmentation can be assessed with actigraphy or PSG by measuring wake after sleep onset (WASO), number of nighttime awakenings, or the length of sleep and wake bouts throughout the night. Using these objective measures, many groups found that individuals with TBI had more fragmented sleep.[37,53,67,70] However, one group found the opposite: that NREM sleep was more consolidated in TBI patients.[34] Others reported no differences in sleep fragmentation or WASO.[38,51,68]

TBI patients may display differences in EEG spectral frequencies, especially in delta power, during wakefulness or NREM sleep. Delta power is the lowest frequency band, usually defined as less than 4 or 4.5 hertz.[76–78] During NREM sleep, groups have variously found a trend toward increased delta power,[34] a trend toward decreased delta power,[68] increases in beta power,[73] and no change in spectral power.[51,79] During wakefulness, different groups have reported increased delta power[69] or increased slow-wave activity.[80] Since delta power and slow waves are associated with high sleep pressure,[81,82] these increases may be related to EDS observed in TBI patients. However,

another group found no change in delta power during wakefulness,[79] so more research must be done before meaningful conclusions about TBI and changes in spectral frequency can be made.

Perhaps problematically, many studies do not differentiate between TBI patients with and without pain. This is a potential confounding factor because TBI patients with pain had significantly higher levels of beta power during all stages of sleep compared to TBI patients without pain.[83]

In general, human studies indicate a high rate of SWD after TBI. Often, this is characterized by EDS, increased sleep time, and increased sleep fragmentation, although there are many exceptions. The changes in spectral power after TBI appear to be inconsistent and may be complicated by the presence of other factors such as pain.

Traumatic brain injury and sleep: animal studies

Studies using rodent models of TBI also found sleep and wake abnormalities.[76–78,84–88] Although animal models are ideally more standardized than human TBI, there is potentially as much heterogeneity in the rodent studies as in the human studies.

As in human studies, many but not all rodent studies found changes in macroarchitecture. Some studies have found an increase in sleep at acute time points (within 7 days of injury),[87–89] while others did not.[84] Some studies found an increase in dark period NREM sleep at chronic time points (7 days post-injury or later).[76–78] However, some studies found no changes in macroarchitecture at chronic time points[89] and one study found a decrease in sleep time at chronic time points.[90] In general, REM sleep time was not altered, either at acute or chronic time points.[76–78,84,85,90] Studies that found increases in sleep time are consistent with findings in humans[34,38,51] and increased sleep during the active period may be an animal correlate of the EDS and daytime napping seen in humans.[34,38,62]

After TBI, sleep or wake bout durations may be shortened at acute[84,87] and chronic time points,[76,85,86,90] indicating sleep fragmentation. Increased fragmentation is sometimes[84,85,87] but not always[76] confined to the dark period. However, other studies have found no differences in sleep-wake fragmentation[89,91] and one study even found that sleep was more consolidated.[78] Observed sleep-

wake fragmentation is in agreement with some of the human literature[37,53,67,70] and reduction in the number of long wake bouts during the active period may indicate an inability to maintain long bouts of wakefulness, potentially analogous to daytime napping observed in human TBI patients.[38,62]

Several groups have found alterations in delta power after TBI. As previously mentioned, delta power is thought to indicate sleep pressure.[81] Some groups found an increase in NREM delta power after TBI,[77,78] others found increases in NREM delta power only after mild sleep deprivation.[76] Others found the opposite: that there was a significant shift toward higher frequencies during NREM sleep.[90] Other groups found increases in delta power only during wakefulness.[84] Brain injured mice also had significantly more EEG slow waves during wakefulness, again potentially indicating greater sleep pressure.[80] Reductions in theta power during wake have also been observed, although the significance of this remains unclear.[76]

This heterogeneity in animal models may be due to a number of factors including differences in species (mouse or rat), injury model (controlled cortical impact, weight drop, fluid percussion, acceleration-deceleration, or blast injury), use of anesthesia or not, injury location (lateral or midline), injury severity, method of sleep and wake assessment (EEG or piezoelectric) and time of day that the injury was delivered. Reasons for differences have not been conclusively established.

Post-TBI sleep: adaptive or pathological?

Some have hypothesized that excessive sleep after TBI is reparative.[38] However, studies that have attempted to elucidate the role of sleep after TBI have produced mixed effects. Six hours of sleep disruption immediately following TBI did not influence neurological or cognitive function.[92] One study even found that 24 hours of total sleep deprivation after TBI actually reduced gross morphological damage and neurological deficits.[93] Treatment with the resolvin molecule RvE1 increased sleep immediately following TBI but this did not result in improvements in neuromotor function or cognition.[94] These studies seem to indicate that excessive sleep post-TBI is not reparative. However, another study found that both sleep deprivation and pharmacological sleep induction improved cognitive outcomes and reduced axonal damage following TBI; the authors

speculated that this beneficial effect of sleep deprivation was due to the sleep rebound that followed it.[95] This would seem to provide support for the reparative effects of sleep after TBI.

Although 6 hours of sleep deprivation immediately following TBI or sham procedures differently affected gene expression in the brains of each group of mice (with TBI and SD producing changes in genes involved in GABA receptor signaling, serotonin receptor signaling, and cell death and survival), the functional consequences of these transcriptional changes remain to be examined.[84] Similarly, 18 hours of post-TBI sleep disruption reduced glymphatic clearance of solutes from the brain, although again the functional significance of this is unknown.[96] Interestingly, pre-existing sleep debt does not seem to influence neuronal susceptibility to mild traumatic brain injury either, at least in the acute phase.[97] Clearly, more research is needed to elucidate whether increased sleep after TBI is beneficial for recovery or whether it is a symptom of the disease process.

Additionally, all of the previous research only examined the effects of sleep in the acute phase of TBI. In humans, sleep disturbances do not seem to be limited to the acute post-TBI period: they may persist for 5 years or more.[98] Increased sleep need may play different roles in the acute vs. chronic stages of the disease.

Treatments for post-TBI sleep-wake difficulties

There have been many attempted treatments for post-TBI SWD, generally with mixed results. Some trials have aimed to decrease daytime sleepiness with stimulants and wake-promoting agents. The selective serotonin reuptake inhibitor sertraline did not improve daytime sleepiness.[99] One study indicated that the stimulant methylphenidate reduced daytime sleepiness in TBI patients,[99] while another found no effect on sleep-wake behavior.[100] Studies of modafinil yield similar mixed results – with some groups finding that it reduced post-TBI daytime sleepiness[101] and others finding that it had no effect.[102] Although armodafinil increased daytime sleep latency as measured on the MSLT, the average latency was still 7.2 minutes,[103] thus still falling under the 10 minute threshold that is considered an abnormally sleepy score.[104] Furthermore, subjective EDS, measured with ESS, was not improved by armodafinil.[103]

Other studies have attempted to improve nighttime sleep quality. The tricyclic antidepressant amitriptyline did not improve any metric of sleep quantity or quality in TBI patients with chronic SWD.[105] Studies of melatonin and the melatonin agonist Ramelteon have had mixed effects on sleep parameters, either improving, having no effect, or worsening sleep quality depending on the study and the metric being evaluated.[105,106]

Furthermore, great care must be taken when administering sleep aids to brain injured individuals. Although a rodent study found that acute treatment with the sleep aid sodium oxybate improved cognition at later time points,[95] chronic treatment with sleep aids may have negative effects on recovery. For example, benzodiazepines and even zolpidem (in some studies) can have detrimental affects on next-day cognition,[107] and antidepressants can reduce seizure threshold.[108] This is particularly problematic in TBI populations, who may already have cognitive difficulties[109] and be at risk for developing post-traumatic epilepsy.[110]

Non-pharmacological treatments generally have fewer detrimental side effects, but have similarly mixed efficacy. A sleep hygiene implementation program failed to produce any improvements on any metric.[111] One small study on blue light therapy indicated promising improvements in daytime sleepiness, although it has yet to be replicated.[112] A few small studies have indicated that specially tailored cognitive behavioral therapy (CBT) may improve post-TBI sleep quality.[113,114] However, CBT is costly and time consuming, and better pharmacological treatments are sorely needed. Yet the biological processes that underlie these sleep-wake disturbances have not yet been conclusively established, which makes it difficult to determine effective treatment approaches.

Mechanisms for post-TBI sleep-wake disturbance

There are many factors that seem to predispose humans to long-term post-TBI sleep disturbance. As previously mentioned, severity of injury may or may not be a factor in the development of long-term SWD. Other studies have indicated that brain morphology[115] and certain clock gene polymorphisms[116] may predict susceptibility to long-term post-TBI SWD. However, post-

TBI changes in sleep-wake regulatory neurotransmitters have received the most attention as potential mechanisms for post-TBI SWD.

Traumatic brain injury and sleep-wake regulatory neurotransmitters and hormones

Many mechanisms have been proposed to underlie post TBI sleep-wake disturbances. In particular, researchers have investigated the effects of TBI on several sleep-wake regulatory neurotransmitters and hormones. Many of these transmitters and hormones are produced in deep brain structures such as the hypothalamus, brainstem, and pineal gland. Although many groups have studied the mechanisms of cell death in cortical and hippocampal neurons after TBI, there has been very little research on mechanisms of cell death in these deep brain areas. Some have proposed that the hypothalamus, brainstem, and pineal gland may be especially vulnerable to shearing forces that occur during TBI.[115,117,118] Indeed, even in mild TBI there is significantly reduced structural and functional connectively of the hypothalamus to other brain areas, perhaps indicating axonal damage to hypothalamic neurons.[119]

Inflammatory processes likely also play a role; activated astrocytes were much more dense in the hypothalamus in humans who died of TBI than those who died of other causes.[120] Animal studies have also revealed astrocytosis and other inflammatory processes occurring in the hypothalamus[87] and brainstem[121] of animals subjected to non-fatal TBI. The exact role of astrocytosis in post-TBI neurotransmitter dysregulation has yet to be elucidated and is complicated by the fact that astrocyte activation can aid in reparative processes or trigger secondary damage following TBI.[122]

Inflammatory cytokines are also dramatically upregulated after TBI.[88,123,124] Many sleep-wake regulatory neuronal populations, such as hypocretin and MCH populations, are susceptible to neuroinflammation,[125,126] so this may be another avenue through which these neurons are damaged. Levels of sleep-wake regulatory neurotransmitters, such as hypocretin, histamine, norepinephrine, and serotonin may also be regulated by pro-inflammatory cytokines like tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β).[127–130]

One sleep-regulatory hormone that is affected by TBI is melatonin, produced by the pineal gland. Melatonin seems to be increased acutely, but at chronic time points levels of nighttime melatonin are reduced.[37,131] Melatonin receptors are also altered after TBI in humans.[132] However, as previously discussed, melatonin agonists do not seem to have particularly robust effects on sleep-wake behavior post-TBI,[105,106] indicating that melatonin dysregulation is not the main mechanism underlying post-TBI SWD.

The brainstem may also be damaged in human TBI.[120,133] In the brainstem of human TBI patients, the serotonergic neurons of the dorsal raphe nucleus and noradrenergic neurons in the locus coeruleus were reduced, while the cholinergic neurons of the pedunculopontine and laterodorsal tegmental nuclei were not affected.[134] However, authors of this study indicated that damage to the brainstem wake-promoting neurons was not as great as other studies had shown hypothalamic damage to be, and thus was probably not the main cause of impaired wakefulness.[134]

Neuronal populations in the hypothalamus may also be affected. Melanin-concentrating hormone (MCH) is a neuropeptide produced exclusively in the hypothalamus that is involved in sleep-wake state regulation.[135] Studies in fatal TBI indicate that the number of MCH neurons may[120] or may not[118] be reduced. However, studies in rodent models of non-fatal TBI have consistently not found changes in the number of MCH neurons at acute or chronic time points.[78,87,136]

Histamine is another hypothalamic neurotransmitter that is important in sleep-wake behavior.[137] Histaminergic neurons of the tuberomammillary nucleus are reduced in fatal human TBI.[120] In a weight-drop rodent model of TBI, histaminergic neurons were reduced at 28 days after injury,[78] but in a controlled cortical impact model, histaminergic neurons were not different from controls at 15 days after injury.[77] Thus, more work must be done to elucidate the effects of TBI on histaminergic neurons. Importantly, while mice lacking brain histamine display some alterations in sleep-wake behavior, they do not have significantly different amounts of spontaneous sleep over a 24 hour period, suggesting that other neurotransmitter systems may be able to compensate for a lack of histamine.[138] Indeed, histaminergic neurons are increased by 64% in narcoleptic humans.[139] Given

that these patients still displayed classic symptoms of narcolepsy including inability to maintain wakefulness, this suggests that even a dramatic upregulation of histaminergic neurons is not enough to compensate for a reduction in hypocretin and thus that hypocretin potentially has a larger influence on sleep-wake behavior than histamine.

Traumatic Brain Injury and Hypocretin

A myriad of studies in humans and in animal models have shown that the hypocretinergic system is affected by traumatic brain injury. Hypocretin (also called orexin) is a neuropeptide that has two varieties: hypocretin-1 (orexin-A) and hypocretin-2 (orexin-B).[140] There are also two types of hypocretin receptors, which are G-protein coupled receptors (GPCRs): hypocretin receptor 1, which preferentially binds hypocretin-1, and hypocretin receptor 2, which binds hypocretins 1 and 2.[140] The vast majority of studies on the interactions of hypocretin and TBI have focused on hypocretin-1. Thus, for the purposes of this thesis, the term hypocretin will refer to hypocretin-1 unless otherwise specified.

In cases of fatal TBI in humans, hypocretin neurons are reduced by 21 - 27% compared to individuals who died from other causes.[118,120] In the acute phase of TBI (within 4 days), 95% of patients with moderate to severe TBI had abnormally low levels of hypocretin in the cerebrospinal fluid (CSF).[141] At 6 months post-TBI, CSF hypocretin levels were significantly lower in humans with excessive daytime sleepiness (EDS) than in the CSF of those without EDS.[4]

Hypocretinergic dysfunction is also observed in animal models of TBI. Within the first three days after TBI, hypocretin dynamics were significantly altered. Microdialysis revealed significantly lower levels of hypocretin protein in the hypothalamus and hippocampus in the acute period after TBI during the light and dark phases.[87] The diurnal rhythm of hypocretin levels was also significantly blunted in brain injured animals, with much less of a difference between hypocretin levels during the light and dark phases.[87] Somewhat paradoxically, this study also found that despite lower levels of hypocretin protein in brain injured animals, these animals also had slightly greater numbers of

hypocretin-producing neurons in the hypothalamus at 3 days post-injury compared to sham-injured animals.[87]

At chronic time points (15 days post-injury and later), some groups observed a significant decrease in the number of hypocretin-producing cells in the hypothalamus.[77,85] Others did not observe a decrease in number of hypocretin neurons, but did observe a decrease in the activation of these neurons as measured by C-fos reactivity.[76] Another study found no reduction in hypocretin neurons but did not measure neuronal activity.[78] Several factors may affect the number of hypocretin neurons observed in these studies. In rodents, the number of hypocretin neurons is affected by age,[142] gender,[143] diet,[144] and inflammation.[126] Furthermore, even in uninjured animals, the number of hypocretin immunopositive neurons varies by approximately 25% depending on circadian phase, with significantly more neurons observed during the dark phase.[145] Although studies have shown that normal hypocretin protein circadian fluctuations are blunted by TBI,[87] no studies have yet examined whether TBI affects circadian differences in hypocretin neuron number.

To our knowledge, only one study has examined the effect of TBI on hypocretin receptors. This study found that hypocretin receptor 1 was upregulated beginning 6 hours after TBI and peaking at 1 day post injury in the penumbra of the injury.[146] In the penumbra, these receptors were found on neurons and microglia but not astrocytes.[146] No behavioral measurements were obtained in this study, so the implication of this transient upregulation of receptors remains unexplored.

Thus far, to our knowledge, no direct pharmacological or genetic modulations of hypocretin have been attempted in conjunction with TBI models, so this area is ripe for investigation. Promisingly, branched chain amino acid (BCAA) therapy increased hypocretinergic neuron activity after TBI as well as ameliorating some of the sleep-wake abnormalities observed after TBI.[76] However, it is unclear whether BCAA therapy may have acted through other channels as well, so a more controlled examination of the role of hypocretin in post-TBI behavioral changes is required. It is particularly important to examine the exact role of hypocretin, rather than just hypocretin-positive

neurons, as hypocretin neurons also contain dynorphin which may act synergistically or in opposition to the effects of hypocretin.[147–149]

Hypocretinergic neurons are a relatively small population of neurons localized in the hypothalamus, yet their projections extend to many brain regions and many levels of the spinal cord.[150,151] As such, even small changes in this neuronal population may have far-reaching effects.

Hypocretin and sleep-wake behavior

As stated above, hypocretin neurons make up an extremely small subset of neurons: neurotypical humans have as few as 20,000-50,000.[140] In spite of this, hypocretin plays an essential role in a vast array of behaviors. Although this thesis will focus mainly on the role of hypocretin in sleep-wake behavior, hypocretin also plays a role in pain,[152–154] olfaction,[155] cognition and memory,[156,157] reward and addiction,[158] hunger and energy homeostasis,[159,160] depression,[161,162] anxiety and modulation of the hypothalamic-pituitary-adrenal axis,[163] and modulation of several other endocrine systems.[164,165]

A loss of hypocretinergic neurons (or a decrease in their functionality) is important in the context of TBI because of the essential role that hypocretin plays in the regulation of sleep-wake behavior. Hypocretin is essential for promoting wakefulness and for stabilizing the wakefulness state.[166]

In animals, hypocretin levels in brain[167] and CSF[168] are highest at the end of the active period. They are also elevated during sleep deprivation.[168] Although studies in humans found that CSF hypocretin levels were higher during the inactive period, this likely indicates a delay between the time hypocretin is produced in the hypothalamus and the time it reaches the lumbar spine.[169,170] Elevation at the end of the active period and during sleep deprivation has led some to conclude that hypocretin acts to oppose the natural sleep drive, helping to keep individuals awake when homeostatic sleep drive is high.[168]

Hypocretin neurons display similar activity patterns: they are most active during natural wakefulness and sleep deprivation, indicating that they are important in the maintenance of

wakefulness.[171–173] Conversely, firing is very low or absent during both NREM and REM sleep,[172,174] except that neurons are active just preceding a REM sleep to wake transition.[172,173]

Intracerebroventricular (ICV) injection of hypocretin increases wakefulness,[175–177] decreases delta power and increases beta power.[178] Intranasal administration of hypocretin increases alertness and activity in animal models[179,180] and may help alleviate some symptoms of narcolepsy in humans.[181] Local injections of hypocretin into the locus coeruleus,[182] lateral preoptic area,[183] laterodorsal tegmental nucleus,[184] and basal forebrain,[185] also reduce sleep and promote wakefulness.

Wakefulness can also be increased by pharmacogenetic or optogenetic activation of hypocretin neurons.[186,187] Optogenetic activation of hypocretin neurons increased transitions from sleep (either NREM or REM sleep) into a state of wakefulness.[187] Pharmacogenetic activation of hypocretin neurons increased wakefulness at the expense of NREM and REM sleep.[186]

Conversely, the role of hypocretin in sleep and wake has also been explored using optogenic silencing or pharmacological antagonism. Optogenetic silencing produced decreases in wakefulness and increases in slow-wave (NREM) sleep with no change in REM sleep.[188,189] This silencing also increased sleep/wake state fragmentation.[188] Similarly, pharmacogenetic suppression of hypocretin neuron activity during the dark period produces decreases in wakefulness, increases in NREM sleep, and no change in REM sleep time.[186] Studies of hypocretin antagonists in humans and animals consistently find decreases in wakefulness and increases in NREM sleep, although some find increases in REM[190–192] and others do not.[193–195].

The role of hypocretin in sleep and wakefulness has also been explored through genetic ablation of hypocretin in animal models and in naturally occurring hypocretin deficiency in humans. In humans, a total loss of hypocretinergic neurons results in type 1 narcolepsy.[196,197] Behaviorally, type 1 narcolepsy is characterized by excessive daytime sleepiness (both objective and subjective), sleep-onset REM, cataplexy, and fragmented sleep.[104,198–200]

Similar behaviors have been reported in hypocretin knockout mice.[201] More specifically, hypocretin knockout mice display less wakefulness,[201,202] more NREM sleep,[201] more REM sleep,[201,202] fragmented sleep and wake bouts,[203–205] sleep-onset REM periods during the dark period,[201] and behavioral arrests which are the animal correlate of cataplexy.[201,205,206] However, one group found somewhat contradictory results, with no changes in wakefulness during the dark period and an increase in wakefulness in knockout mice during the light period.[207]

What explains these disparate findings about hypocretin and REM such that reducing hypocretinergic activity sometimes increases REM sleep and sometimes not? It seems likely that the effects of hypocretin antagonism/absence on REM sleep are dose dependent. Some studies indicate that at low doses, hypocretin antagonists increase NREM sleep, but not REM sleep, whereas a high dose of the same antagonist increased both NREM and REM sleep.[208] Even at high doses, hypocretin antagonists generally do not induce sleep-onset REM periods, which are a common feature of narcolepsy, which is a state of complete hypocretin absence.[209,210] In support of this, homozygous hypocretin knockout mice displayed an animal form a cataplexy, whereas heterozygous mice did not. [201]

Hypocretinergic neurons modulate the activity of several other sleep-wake regulatory brain centers: they have excitatory projections to the noradrenergic neurons of the locus coeruleus, dopaminergic neurons of the ventral tegmental area, cholinergic neurons of the basal forebrain, serotonergic neurons of the dorsal raphe nucleus, and the histaminergic cells of the tuberomammillary nucleus.[151,176,211–214] There is also evidence that hypocretin inhibits the firing of sleep-promoting MCH neurons through GABAergic microcircuits.[215]

Hypocretinergic neurons also form a positive feedback loop: hypocretin itself produces an excitatory response in hypocretinergic neurons.[216] Hypocretinergic neurons also project directly to the cortex, where they may directly contribute to the low-amplitude, high-frequency EEG pattern characteristic of wakefulness.[217] Humans with narcolepsy (i.e. deficient in hypocretin), displayed

altered rhythmicity of melatonin secretion, suggesting hypocretin may control melatonin release as well.[218]

Interestingly, since hypocretin deficits are also implicated in pain,[154], especially headache pain,[153] poor cognitive performance,[156,157] and depression,[161,162] hypocretinergic dysfunction may not only be involved in post-TBI SWD, but also in the pain,[219] cognitive deficits,[220] and depression[221] that occur after TBI.

Specific aims

Overall, the objective of the experiments described herein was to determine the role of the neuropeptide hypocretin in sleep-wake behavior in the context of TBI. The central hypothesis is that hypocretin is a necessary mediator of changes in sleep behavior that occur after TBI.

Aim 1 (described in detail in Chapter 2) was to determine the time course of changes in sleep-wake behavior and sleep-wake associated neuronal populations. EEG analysis of sleep-wake behavior was performed at baseline, 7 days post-surgery, and 15 days post-surgery in animals subjected to sham, mild, or moderate controlled cortical impact (CCI). Populations of hypocretinergic, MCH-ergic, and histaminergic neurons in the hypothalamus as well as cholinergic neurons in the basal forebrain were identified with immunohistochemistry and counted using unbiased stereology.

Aim 2 (described in Chapter 3) was to compare sleep-wake responses to TBI in wild type and hypocretin knockout mice. To attain a more comprehensive trajectory of behavioral response to TBI, sleep-wake behavior was assessed at baseline and 3, 7, 15, and 30 days post-surgery. As mild injury did not produce significant changes in sleep-wake behavior in Aim 1, animals in Aim 2 received either sham or moderate injury. Hypocretin cell counts were also performed to confirm the hypocretin cell loss found in Aim 1.

The work presented here has contributed to our understanding of the mechanisms that underlie post-TBI sleep-wake disturbance and will hopefully contribute to more effective therapies in the future.

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Chapter 2: Hypocretinergic and Cholinergic Contributions to Sleep-Wake Disturbances in a Mouse Model of Traumatic Brain Injury[1]

Abstract

Disorders of sleep and wakefulness occur in the majority of individuals who have experienced traumatic brain injury (TBI), with increased sleep need and excessive daytime sleepiness often reported. Behavioral and pharmacological therapies have limited efficacy, in part, because the etiology of post-TBI sleep disturbances is not well understood. Severity of injuries resulting from head trauma in humans is highly variable, and as a consequence so are their sequelae. Here, we use a controlled laboratory model to investigate the effects of TBI on sleep-wake behavior and on candidate neurotransmitter systems as potential mediators. We focus on hypocretin and melanin-concentrating hormone (MCH), hypothalamic neuropeptides important for regulating sleep and wakefulness, and two potential downstream effectors of hypocretin actions, histamine and acetylcholine. Adult male C57BL/6 mice (n=6-10/group) were implanted with EEG recording electrodes and baseline recordings were obtained. After baseline recordings, controlled cortical impact was used to induce mild or moderate TBI. EEG recordings were obtained from the same animals at 7 and 15 days post-surgery. Separate groups of animals (n=6-8/group) were used to determine effects of TBI on the numbers of hypocretin and MCH-producing neurons in the hypothalamus, histaminergic neurons in the tuberomammillary nucleus, and cholinergic neurons in the basal forebrain. At 15 days post-TBI, wakefulness was decreased and NREM sleep was increased during the dark period in moderately injured animals. There were no differences between groups in REM sleep time, nor were there differences between groups in sleep during the light period. TBI effects on hypocretin and cholinergic neurons were such that more severe injury resulted in fewer cells. Numbers of MCH neurons and histaminergic neurons were not altered under the conditions of this study. Thus, we conclude that moderate TBI in mice reduces wakefulness and increases NREM sleep during the dark period,

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effects that may be mediated by hypocretin-producing neurons and/or downstream cholinergic effectors in the basal forebrain.

Introduction

Traumatic brain injury (TBI) is a major public health problem that is considered a silent epidemic because of the long-term cognitive deficits and behavioral and medical complications experienced by survivors. In the United States alone, more than 5.3 million individuals currently suffer from a TBI-related disability (Chew and Zafonte, 2009). The neuropsychiatric consequences of TBI may include sleep disorders, mood disorders, personality changes, and cognitive impairment (Bhalerao et al., 2013). Chronic sleep-wake disturbance is highly prevalent, affecting the majority of individuals who have sustained a TBI (Kempf et al., 2010; Rao and Rollings, 2002). Many TBI patients report regular daytime napping and increased sleep need (Ponsford et al., 2013; Ponsford and Sinclair, 2014), and excessive daytime sleepiness (EDS) occurs in approximately 25-42% of individuals who have suffered TBI (Ponsford and Sinclair, 2014; Sommerauer et al., 2013). The alterations in sleep-wake behavior after TBI may be prolonged, and evident for years after the trauma (Kempf et al., 2010). Despite the debilitating effects of post-TBI sleep-wake disturbances, their etiology is not well understood. Furthermore, current behavioral and pharmacological therapies targeting post-TBI sleep-wake disturbances have only limited efficacy (Chew and Zafonte, 2009; Ouellet et al., 2015; Ponsford and Sinclair, 2014; Rue-Evans et al., 2013; Sheng et al., 2013).

Post-TBI sleep-wake disturbances may be due, in part, to altered neurotransmitter systems that regulate sleep and wakefulness. Neurotransmitter systems implicated in arousal include, among others, hypocretin (a.k.a. orexin) neurons of the lateral hypothalamus (Adamantidis et al., 2007; Brisbare-Roch et al., 2007), cholinergic neurons of the basal forebrain (Arrigoni et al., 2010; Irmak and de Lecea, 2014), and histaminergic neurons in the tuberomammillary nucleus (Brown et al., 2001; Brown et al., 2012; Parmentier et al., 2002). Of importance to the etiology of post-TBI disturbances, hypocretin promotes wakefulness and stabilizes the sleep-wake cycle (Kilduff and

Peyron, 2000; Krystal et al., 2013; Mochizuki et al., 2004; Taheri et al., 2002; Zeitzer et al., 2006). Activation of hypocretin neurons increases transitions from sleep to wakefulness (Adamantidis et al., 2007) and antagonizing hypocretin induces somnolence (Brisbare-Roch et al., 2007; Hoever et al., 2012; Morairty et al., 2014).

In contrast, melanin-concentrating hormone (MCH) neurons are sleep-promoting (Peyron et al., 2009): intracerebroventricular injection of MCH (Verret et al., 2003) or optogenetic stimulation of MCH neurons increases NREM sleep and REM sleep (Jego et al., 2013; Konadhode et al., 2013), whereas MCH deficient mice sleep less (Tsunematsu et al., 2014; Willie et al., 2008). MCH neurons are intermingled with hypocretin neurons in the lateral hypothalamus, and as such, damage to the hypocretin and/or MCH neurons of the lateral hypothalamus could alter sleep-wake behavior after TBI. Indeed, hypocretin is reduced in the hypothalamus of mice (Willie et al., 2012) and in cerebrospinal fluid of human patients (Baumann et al., 2005) after TBI. Furthermore, the number of hypocretin neurons are reduced in post mortem brains of patients who died from TBI (Baumann et al., 2009). In cases of fatal TBI in humans, one study found a significant reduction in MCH neurons (Valko et al., 2015), whereas another found that MCH neurons were not affected (Baumann et al., 2009). To our knowledge, numbers of hypocretin or MCH neurons have not been investigated in cases of nonfatal TBI in humans.

Although post-TBI alterations in sleep may be mediated, in part, by direct actions of hypocretin, these changes in arousal state could also be due to actions of modulatory systems downstream of hypocretin. Hypocretinergic neurons project to many brain regions. For example, the histaminergic neurons of the tuberomammillary nucleus (TMN) and the cholinergic neurons of the basal forebrain are both strong promoters of wakefulness (Haas et al., 2008; Han et al., 2014), and these brain regions are densely innervated by hypocretinergic projections [reviewed in (Arrigoni et al., 2010; Sundvik and Panula, 2015)]. Importantly, these systems may also be perturbed by TBI. Fatal TBI in humans causes a dramatic reduction in numbers of histaminergic neurons (Valko et al., 2015) and a reduction in activity and immunoreactivity of choline acetyltransferase (ChAT), an enzyme essential

for acetylcholine synthesis (Dewar and Graham, 1996; Murdoch et al., 1998; Murdoch et al., 2002). To our knowledge, histaminergic and cholinergic neuronal populations have not been studied within the context of sleep-wake disturbance after experimental TBI.

The primary goal of the present study was to determine the effects of TBI on sleep-wake behavior and hypocretin/MCH cell numbers and their downstream targets in mice. We used the controlled cortical impact (CCI) model to induce mild or moderate TBI and determined the time course of effects on these and other outcome measures. We report that sleep is altered and hypocretin and basal forebrain cholinergic cell numbers are reduced in an injury severity-dependent manner. Cell counts for MCH and histamine neurons were not altered by TBI under the conditions of this study. Collectively, these data suggest that the effects of TBI on sleep may be mediated by hypocretinergic and cholinergic mechanisms.

Methods

Animals

Adult male C57BL/6J mice (~3-4 months old at time of use; Jackson Laboratory, Bar Harbor, ME) were group housed until baseline testing or surgery, after which they were single housed. Mice were housed under a 12:12 light:dark cycle at 29 ± 1º C with food and water provided ad libitum. All procedures involving the use of animals were approved by the University of Washington IACUC in accordance with the US Department of Agriculture Animal Welfare Act and the National Institutes of Health policy on Humane Care and Use of Laboratory Animals.

Recording Apparatus

Sleep-wake behavior of mice was determined based on the electroencephalogram (EEG) and cage activity patterns. EEG signals were amplified, filtered, and recorded for offline processing using custom software written in LabView for Windows (ICELUS, M. Opp, University of Washington; National Instruments, Austin, TX) as previously described (Baracchi and Opp, 2008; Ingiosi et al., 2015). EEG and cage activity records were visually scored in 10-second epochs. Raw EEG signals

were subjected to fast Fourier transformation, yielding power spectra between 0.5 and 30 Hz in 0.5-Hz frequency bins. Arousal states were determined as previously described and classified as non-rapid eye movement (NREM) sleep, rapid eye movement (REM) sleep, or wakefulness (WAKE) based upon published criteria [e.g., (Baracchi and Opp, 2008; Ingiosi et al., 2015; Sutton and Opp, 2014)].

Experimental Design

A schematic of the protocols used in Experiments 1 – 3 is presented in **Fig. 1.**

Experiment 1: Effects of TBI on Mouse Sleep-wake Behavior

For Experiment 1, EEG electrodes were surgically implanted into the skull under isoflurane anesthesia. The leads from the screw electrodes were soldered to the pins of a plastic connector (Digi-Key, ED85100-ND) to allow coupling to the recording system. Dental acrylic (Integrity Caulk, Dentsply) covered the electrodes and formed a headpiece to which the flexible recording tether could be connected. The section of the skull over the left parietal cortex was not covered with dental acrylic at this time. The incision was closed with sutures, and a subcutaneous injection of an analgesic (0.5mg/kg buprenorphine) was given at the end of the surgery. Mice were allowed 7 days to recover before they were attached to a flexible tether for habituation to the recording system. After 3 days of habituation to the tether and recording environment, 48-hour undisturbed baseline recordings were obtained.

After the 48-hour baseline recordings, mice were randomized to sham surgeries (n=7; control mice), or to controlled cortical impact (CCI; n=16) to induce TBI as previously described (Febinger et al., 2015). In both groups, a 5-mm diameter craniotomy using a trephine was made over the left parietal cortex, approximately -2mm relative to bregma and 2.5mm lateral to the midline. A unilateral impact between lambda and bregma is routinely used in protocols using CCI to induce TBI (Boulet et al., 2013; Boychuk et al., 2016; Febinger et al., 2015; Miller et al., 2014). The skull fragment was removed without disrupting the underlying dura, and TBI was induced in the experimental group. Mice in the experimental group were subjected to CCI using the Leica Impact One system (Richmond, IL)

equipped with an electrically-driven 3-mm diameter metal piston controlled by a linear velocity displacement transducer. CCI parameters were: 5.0 m/s impact velocity; 100 msec dwell time; and impact depth of 0.5 mm (mild TBI; n=10) or 1.0 mm (moderate TBI; n=6). Sham (control) animals received identical anesthesia and craniotomy without the CCI injury. A sterilized disc created from a polystyrene weighing boat was placed over the craniotomy and covered with dental acrylic. We (Febinger et al., 2015) and others (Miller et al., 2014) have used this or a similar technique to protect the brain after craniotomy. The incision was closed with sutures and mice were returned to their home cages. All mice received a subcutaneous injection of analgesic (0.5mg/kg buprenorphine) at the end of the surgery. Animals were closely monitored after surgery and none displayed overt signs of infection.

Additional 48 h recordings were obtained from all mice on days 6 – 7, and 14 – 15 post-surgery. As such, a within-subjects protocol was used in which pre- and post-surgery recordings were obtained from each animal. Sleep-wake state was determined and the EEG subjected to fast Fourier transformation to produce power spectra between 0.5 and 30 Hz in 0.5 Hz bins as described previously (Baracchi and Opp, 2008). Power in the delta (0.5-4.5 Hz) frequency band was normalized to the total state-specific power (NREM sleep) summed across all frequency bins from 0.5 to 30 Hz for the light and dark periods and this value was expressed as a percent of total power [see (Ingiosi et al., 2015)].

Experiment 2: Effects of TBI on Neuromotor Function

A separate cohort of mice was used to to determine effects of TBI on neuromotor function or neuronal populations. Neuromotor testing was performed during the light period, and therefore disrupts the normal sleep-wake patterns of mice. Because we wanted to determine the effect of TBI on sleep-wake behavior and neuromotor performance at the same time points (i.e. 7 and 15 days post injury), it was necessary to use different groups of animals. We, and others (Rowe et al., 2014b; Sabir et al., 2015) have used this approach of separate cohorts manipulated in parallel.

The impact of TBI on neuromotor function was determined by calculating a composite

neuroscore from neuromotor tests performed during baseline evaluations prior to surgery, and at 7 and 15 days post-surgery. The composite neuroscore was calculated for each mouse, and was derived from measures of forelimb and hindlimb flexion, lateral pulsion reaction, and inclined plane strength/coordination (Fujimoto et al., 2004). Briefly, measures of flexion and lateral pulsion reaction are derived from rodent's reflexes to reach and grasp when lifted or to resist lateral pressure by coordinating movements of all limbs. For the flexion and lateral pulsion reaction tasks, assessments were performed on right and left sides and the ability of the mouse was rated on a scale of 0 (severely impaired) to 4 (no impairment). The inclined angle board consists of a flat acrylic plane that is adjustable from 0° - 90°. The surface is covered with a mat with grooves oriented in the vertical plane so there is traction for the mouse. A mouse must freely stand on the plane for 5 sec to successfully complete the assessment at that angle. Baseline testing starts with the plane at an angle of 40°. The angle of the board increases in 2.5° increments until the mouse can no longer stand unassisted. After TBI or sham surgery, assessment of each mouse starts 10º below the lowest baseline angle value for that animal. The maximum angle at which the mouse remains on the angle board is recorded. The post-surgical maximum angle is subtracted from the baseline maximum angle, and a score of 4 recorded if there is no change, 3 for a 2.5º decrease from baseline, 2 for a 5º decrease from the baseline, a 1 for a 7.5º decrease, and 0 for a 10º or more difference from baseline. Larger differences in angle indicate reduced strength and/or motor coordination.

Scores on all components (forelimb and hindlimb flexion; lateral pulsion reaction; angle board) were summed to calculate the composite neuroscore, with the maximum possible score being 28 points. Larger composite neuroscores indicate better performance / less impaired neuromotor skills. The investigator assessing neuromotor function was unaware of the surgical manipulation (sham, CCI) of the animal being tested.

Five days prior to surgery, baseline neuromotor testing was performed. Sham surgeries (n=18) and mild (n=17) or moderate (n=17) CCI surgeries were performed on mice as described in Experiment 1, and animals were placed back into their home cages for recovery. On post-surgical

days 6 or 14, mice were again evaluated for CN measures. After the completion of neuromotor testing, animals were returned to their home cages and were sacrificed the next day (7- or 15 days post-TBI). At approximately 6 hours after light onset, animals were deeply anesthetized with isoflurane and transcardially perfused with 20 mL chilled phosphate buffered saline, followed by 15 mL chilled 4% paraformaldehyde. The brains were removed and post-fixed in 4% paraformaldehyde for 24 h at 4º C, and then transferred to a 30% sucrose solution until sectioning and staining. Neuromotor behavior was analyzed in all animals at baseline, at 7 days post-surgery [sham (n=8), mild TBI (n=8), moderate TBI (n=8)], and at 15 days post-surgery [sham (n=10), mild TBI (n=9), moderate TBI (n=9)].

Experiment 3: Effects of TBI on Numbers of Hypocretin and MCH Neurons

A subset of mice used in Experiment 2 was randomly selected for use in Experiment 3. Mice were perfused and brains removed for immunohistochemical assessment of TBI effects on selected neurotransmitter systems. Some mice were perfused 7 days post-surgery [sham (n=7), mild TBI (n=8), moderate TBI (n=8)] and some 15 days post-surgery [sham (n=6), mild TBI (n=8), moderate TBI (n=8)].

Brains were sectioned on a Leica cryostat at 40 μm. Sections were stored in cryoprotectant until immunohistochemical staining for hypocretin or MCH. Free-floating sections in a 1:3 series were processed for hypocretin-1 (rabbit anti-mouse orexin-A; H-003-30; Phoenix Pharmaceuticals, Inc.; 1:10,000 dilution) and MCH (rabbit anti-mouse MCH; H-070-47; Phoenix Pharmaceuticals, Inc.; 1:20,000 dilution) as described in (Willie et al., 2012).

Experiment 4: Impact of TBI on Tuberomammillary Histaminergic Neurons and Basal Forebrain Cholinergic Neurons

Two additional groups of mice were used to determine effects of TBI on histaminergic neurons in the tuberomammillary nucleus and cholinergic neurons in the basal forebrain. Based upon the injury severity and time course of TBI effects on hypocretin neurons as determined in Experiment 3, mice (n=7/condition) were subjected to either sham surgery or moderate TBI (1.0 mm controlled

cortical impact depth) as previously described. Animals were perfused at 15 days post-surgery and brains removed and sectioned as previously described. IHC for histidine decarboxylase (HDC) and choline acetyltransferase (ChAT) was used to identify histaminergic and cholinergic neurons, respectively. The protocol for HDC was that used in the laboratory of Dr. Thomas Scammell (Beth Israel Deaconess Medical Center/Harvard Medical School). Briefly, free-floating sections in a 1:2 series were processed with rabbit anti-HDC (1:5,000, American Research Products, 03-16045), then incubated with donkey anti-rabbit conjugated to AlexaFluor 555 (Invitrogen; A31572; 1:500 dilution).

IHC for ChAT was performed in a similar fashion as the stains for hypocretin and MCH and similar to previously published protocols (Schmidt and Grady, 1995): free-floating sections in a 1:3 series were processed for ChAT (Abcam; ab18736; 1:2,000 dilution). Sections were incubated in biotinylated secondary antibody (Abcam; ab97123; 1:500 dilution), then an avidin-biotin complex, and developed with diaminobenzidine.

Estimating Cell Numbers

Cell numbers were estimated using quantitative methods for unbiased stereology (West et al., 1991). Briefly, positively stained cells were visualized on an Olympus BX-51 fluorescent stereoscope using Stereo Investigator 10 (MBF Biosciences, Williston, VT). Colorimetric IHC-processed tissue (stains for hypocretin, MCH, and ChAT) was visualized using brightfield microscopy, whereas fluorescent IHC-processed tissue (stain for HDC) was visualized with fluorescent microscopy.

Hypocretin cell number estimates were obtained from 7 sections spanning approximately -1.20 mm to -2.10 mm from bregma (Paxinos and Franklin, 2001). Estimates of MCH cell numbers were obtained from 11 sections spanning approximately -1.00 mm to -2.30 mm relative to bregma. The contour for the perifornical-lateral hypothalamic region was outlined using a 4x objective. Cells were then counted using the 60x objective and optical fractionator, with a counting frame of 50x50 microns and a grid size of 100x100 microns. ChAT-positive cells were counted in two basal forebrain nuclei using the alternative nomenclature (areas Ch1&2 and Ch3&4) as described by others (Boutros et al., 2015; Mesulam et al., 1983). Areas Ch1&2 and Ch3&4 were outlined using a 4x objective, and then

cells were counted using the 60x objective and optical fractionator, with a counting frame of 50x50 microns and a grid size of 100x100 microns. Acetylcholine cell number estimates for Ch1&2 were obtained from 5 sections spanning approximately 1.00 mm to 0.3 mm relative to bregma. Acetylcholine cell number estimates for Ch3&4 were obtained from 11 sections spanning approximately 1.00 mm to -0.70 mm relative to bregma. For HDC-positive cells, the tuberomammillary nucleus was outlined using a 4x objective, then cells were counted as described above. HDC cell number estimates were obtained from 7 sections spanning approximately -2.20 mm to -2.80 mm relative to bregma. All cell counts were obtained from the hemisphere ipsilateral to injury. TBI-induced changes in cellular and tissue outcomes (cell death, inflammatory cytokine expression, presence of immune cells, etc.) are typically most severe on the side ipsilateral to injury (Hall et al., 2005; Timaru-Kast et al., 2012), and previous studies using unilateral CCI have examined hypocretin neuron number and function (using *in vivo* microdialysis) in the hypothalamus ipsilateral to injury (Willie et al., 2012).

Statistical Analyses

Two types of statistical analyses were used in this study. We first determined the impact of TBI on outcome measures across time (baseline, 7 days, 15 days), and as such these analyses were designed to reveal differences within each group relative to pre-surgery baseline values. The second type of statistical analysis was used to determine differences between outcome measures with respect to the impact of injury severity (sham, mild TBI, moderate TBI). All analyses were performed using SPSS for Windows (IBM Corporation, Armonk, NY). Data are presented as mean ± SEM, unless otherwise indicated. An alpha value of $p < 0.05$ was accepted as indicating a significant difference between or among groups, whereas an alpha value of $0.05 < p < 0.1$ was considered a trend.

Percent time spent in WAKE, NREM sleep, and REM sleep was evaluated within manipulation (injury severity) in 4 hour blocks using a repeated measures ANOVA across three time points (baseline, 7 days, 15 days). Sphericity (an assumption of a repeated measures ANOVA) was tested

with Mauchly's test of sphericity. If the assumption of sphericity was violated, the Greenhouse-Geisser correction was used. If significant time effects were detected, post-hoc tests using the Bonferroni correction were used to determine differences between timepoints.

To determine the impact of injury severity on sleep-wake behavior, difference scores were calculated for each parameter by subtracting baseline values from those obtained 7- or 15 days post-surgery. These difference scores were evaluated independently for the 12 h light and dark periods within timepoint (7 days, 15 days) using a one-way ANOVA with injury severity (sham, mild, or moderate) as the between-subjects factor. If significant effects of injury severity were detected, post-hoc comparisons were made using Tukey's HSD to determine differences between injury severity groups.

Normalized NREM delta power was evaluated separately for the 12h light and dark periods within injury severity group (sham, mild TBI, moderate TBI) using a repeated measures ANOVA across the three time points (baseline, 7 days, 15 days). Assumptions of sphericity were evaluated with Mauchly's test, and the Greenhouse-Geisser correction used if necessary. If significant time effects were detected, post-hoc tests using the Bonferroni correction were used to determine differences between timepoints.

Composite neuroscores (CN) were evaluated using a one-way ANOVA within timepoint (7 days, 15 days) with injury severity (sham, mild TBI, moderate TBI) as the independent variable. If significant effects of injury severity were detected, post-hoc comparisons were made using Tukey's HSD to determine differences between injury severity groups.

Estimated numbers of hypocretin- or MCH-positive cells were evaluated with a two way ANOVA with timepoint (7 days, 15 days) and injury severity (sham, mild TBI, moderate TBI) as factors. If significant effects of timepoint or injury severity were detected, post-hoc comparisons by Tukey's HSD were used to determine differences between groups. Statistical evaluations of estimated numbers of histamine and acetylcholine neurons were made using one-way ANOVA with injury severity (sham, moderate) as the independent variable.

Results

Experiment 1: Effects of TBI on Mouse Sleep-wake Behavior

To determine the time course of responses to TBI, sleep-wake behavior was evaluated in 4h time blocks across 24 h recording periods with a repeated measures ANOVA. Time spent in NREM sleep, REM sleep, and wakefulness was not altered in control mice subjected to sham surgery under the conditions of this study (**Fig. 2**). Sleep-wake behavior was not subtantively altered in mice subjected to mild TBI (0.5 mm controlled cortical impact depth; **Fig. 2**), although there were some modest but statistically significant differences in REM sleep [hours 9-12 [$F(2,78) = 7.248$, $p = .001$] and 13-16 [$F(2,78) = 3.839$, $p=0.026$] after mild TBI (**Fig. 2**). In contrast, sleep-wake behavior of mice subjected to moderate TBI (1.0 mm controlled cortical impact depth) was dramatically altered (**Fig. 2**). Mice subjected to moderate TBI spent less time in wakefulness during the dark period [hours 17-20: $F(2,46) = 3.384$, $p = .043$; hours 21-24: $F(2,46) = 6.497$, $p =0.003$]. For both of these 4-h time blocks, the reduction in wakefulness at 15 days post-surgery differed significantly from the same time blocks during baseline (hours 17-20, $p=0.034$; hours 21-24, and 0.004). These mice had corresponding increases in NREM sleep during the same periods [hours 17-20: $F(2,46) = 4.142$, $p =0.022$; hours 21-24: $F(2,46) = 6.194$, $p =0.004$], which was due to significant differences between baseline and fifteen days post-surgery (hours 17-20, $p=.019$; hours 21-24, $p=0.005$). REM sleep of mice subjected to moderate TBI increased during one 4-h time block [hours 9-12: $F(2,46) =6.361$, $p =0.004$], and post hoc tests revealed these differences were due to values obtained 7 days post-surgery.

To determine the impact of increasing injury severity on sleep-wake behavior, we directly compared differences between animals subjected to mild TBI and those subjected to moderate TBI. We first calcluated hourly difference scores for each animal by subtracting values obtained after surgery (sham, mild TBI, moderate TBI) from corresponding baseline (pre-surgery) values. These hourly difference scores were compared among conditions independently for the 12h light periods and 12h dark periods (**Fig. 3**). No differences with respect to the impact of TBI on sleep were

revealed among any of the conditions during the light period. At 7 days post-surgery, there were significant differences among groups during the dark period in time spent awake [$F(2, 273) = 3.389$, p=0.035] and in NREM sleep [$F(2, 273) = 3.275$, p=0.039] (**Fig 3**). Post hoc tests indicated that mice subjected to moderate TBI spent less time in wakefulness than did mice subjected to mild TBI (p=0.041), and they tended to spend less time awake than did mice that had sham surgeries (p=0.079). Similarly, mice subjected to moderate TBI spent more time in NREM sleep than mice subjected to mild TBI (p=0.044). Significant differences in sleep-wake behavior among groups persisted for at least 15 days, specifically in time spent awake [$F(2, 273) = 3.831$, p =0.023] and in NREM sleep [$F(2, 273) = 4.739$, p=0.009] during the dark period. Post hoc tests revealed that moderately injured animals spent less time awake than did mildly injured animals (p=0.029), and there was a trend less time awake compared to sham animals (p=0.054). Mice subjected to moderate TBI spent more time in NREM sleep compared to those subjected to mild TBI (p=0.011) or to sham surgeries (p=0.036). No significant differences among groups were observed in number of transitions between sleep-wake states at either of the post-surgical time points (data not shown).

NREM delta power (0.5-4.5 Hz) was normalized by expressing each 0.5 Hz frequency bin as a percentage of total power. These values were then evaluated during the 12h light and dark periods (**Fig. 4**). There were no differences in this measure of spectral characteristics during the light period in EEGs obtained from mice subjected to sham surgery or mild TBI. However, NREM delta power during the light period significantly increased in animals subjected to moderate TBI (1.0 mm cortical impact depth) [$F(1.572, 99.058) = 14.15$, p0<.001]. Post hoc tests revealed that NREM delta power significantly increased 7 days (p =0.002) and 15 days (p<0.001) post-surgery compared to baseline. During the dark period, NREM delta power was significantly reduced in mice subjected to sham surgeries [$F(2,106) = 8.955$, p <.001) due to differences between baseline and 7 days (p=.003). Spectral analysis of the EEG of mice subjected to mild TBI indicated increased NREM delta power [$F(1.723, 91.3) = 16.204$, p<.001], due to changes that occurred 7 days post surgery (p=0.01, **Fig. 4**). NREM delta power increased in mice subjected to moderate TBI [$F(2, 96) = 4.943$, p =0.009], an

effect due to changes 15 days post surgery (p=0.015).

Experiment 2: Effects of TBI on Neuromotor Function

Neuromotor function did not differ, based on composite neuroscores, among groups 7 days post-injury (**Fig. 5**). However, by 15 days post-injury, one-way ANOVA revealed a small but significant difference in composite neuroscore values among groups [$F(2,25) = 9.266$, p=.001], with neuromotor function of animals subjected to moderate TBI being worse than that of mice subjected to sham surgeries (Tukey's post-hoc comparison; p=.001).

Experiment 3: Effects of TBI on Numbers of Hypocretin and MCH Neurons

Hypocretin

Hypocretin immunoreactivity differed among groups of mice in an injury-dependent manner (**Fig. 6**), as revealed by two-way ANOVA [$F(5,39)=9.911$, p<0.001]. There was a main effect of injury severity on number of hypocretin-positive cells [$F(2,39)=20.653$, p<0.001; **Fig. 6A & B**], a trend towards a main effect of time since surgery [$F(1,39)=3.05$, p=0.089], but no interaction effect [$F(2,39)=2.33$, p=0.11]. Post hoc tests indicated that relative to sham tissue, tissue obtained after mild and moderate TBI contained incrementally fewer hypocretin-positive cells. Even though a two way ANOVA showed only a trend toward a main effect for time, when analyses are restricted to tissue obtained from mice subjected to moderate TBI, an independent t-test revealed there were significantly fewer hypocretin-positive cells present at 15 days than at 7 days [$t(14)=2.788$, p=0.015]. Numbers of hypocretin-producing neurons were fewest in hypothalamus of mice subjected to moderate TBI and sacrificed 15 days post-surgery.

MCH

Numbers of MCH-positive cells did not differ among groups irrespective of injury severity or time post manipulation as revealed by a two-way ANOVA [$F(5,39)=0.312$, p=0.903]; (**Fig. 6C & D**).

Experiment 4: Impact of TBI on Tuberomammillary Histamine Neurons and Basal Forebrain Cholinergic Neurons

There was no significant difference between numbers of histamine neurons in the ventral TMN between the sham and 1mm depth impact groups at 15 days post injury [$F(1,12)=0.231$, $p=0.639$; **Fig. 7A & B**]. However, there were significantly fewer ChAT-positive neurons in both Ch1&2 [$F(1,12)=6.089$, $p=0.03$; **Fig. 7C & D**] and Ch3&4 [$F(1,12)=7.12$, $p=0.02$; **Fig. 7E & F**] in tissue obtained from mice subjected to moderate TBI.

Discussion

Sleep-wake disturbances are frequently reported in individuals suffering from TBI (Baumann et al., 2007; Ponsford et al., 2013; Ponsford and Sinclair, 2014; Sommerauer et al., 2013). Post-TBI sleep-wake disturbances may negatively impact functional and cognitive recovery and are associated with increased anxiety, depression, and pain (Cantor et al., 2008; Chan and Feinstein, 2015; Chaput et al., 2009; Chiu et al., 2014; Rao et al., 2014). However, injuries resulting from trauma are highly variable, and as a consequence so are their sequelae. Controlled laboratory studies provide a means of standardizing injury severity, and although variability exists between and among animals, results may more readily provide insightful information with respect to potential mechanisms by which TBI alters sleep. The current study aimed to characterize the effects of injury severity and time course on aspects of sleep after mild or moderate TBI. By determining the impact on candidate neurotransmitter systems, we sought to elucidate potential mechanistic substrates that may be therapeutic targets for intervention after TBI.

Effects of TBI on Mouse Sleep-wake Behavior

Our data indicate that in this TBI model wakefulness is reduced and NREM sleep is increased during the period comparable to daytime in humans, i.e., the mouse dark period. We did not observe changes in REM sleep of mice after TBI under the conditions of this study when sleep-wake behavior was evaluated for the full (12 h) light or dark period (**Fig. 3**). Collectively, these results are consistent with those of human studies that report high rates of excessive daytime sleepiness (EDS) and

daytime napping post-TBI (Castriotta et al., 2007; Imbach et al., 2015; Kempf et al., 2010; Ponsford et al., 2013; Ponsford and Sinclair, 2014; Sommerauer et al., 2013). Just as our study found no consistent effects, human polysomnographic studies often (although not always) fail to reveal changes in REM sleep after TBI (Baumann et al., 2007; Imbach et al., 2015; Sommerauer et al., 2013).

Results of pre-clinical studies using rodents also demonstrate acute (Rowe et al., 2014b; Sabir et al., 2015; Willie et al., 2012) and chronic (Lim et al., 2013; Skopin et al., 2015) changes in sleep. Deficits in wakefulness or difficulty maintaining long periods of wakefulness in rodents after experimental TBI are most robust during the dark (active) period (Lim et al., 2013; Skopin et al., 2015). We are aware of two studies that did not demonstrate chronic changes in rodent sleep-wake behavior after TBI. Noain and colleagues (Buchele et al., 2015) restricted their determination of sleep-wake behavior to the light period, the period during which sleep was not consistently altered after TBI in our present study. One study by Lifshitz and colleagues (Rowe et al., 2014a) did not find any persistent sleep-wake changes during the light or dark periods. Several factors may account for differences between our study and that of Lifshitz, including the manner in which sleep-wake behavior was inferred (EEG recordings in the present study vs. piezoelectric detection of movements), the method to induce TBI (CCI in our study vs. fluid percussion), and brain injury location (lateral in our study vs. midline).

Some pre-clinical and clinical studies demonstrate fragmented sleep after TBI (Hazra et al., 2014; Lim et al., 2013; Shekleton et al., 2010) whereas others do not (Baumann et al., 2007; Imbach et al., 2015; Sommerauer et al., 2013). Sleep of mice in our study using the CCI model to induce mild to moderate TBI is not fragmented. Reasons for differences in the literature with respect to this aspect of sleep are not clear, but could be due to aforementioned differences in the model used and severity and location of injury in animal studies. Clinical studies report effects on patients who have been subjected to head trauma from a variety of sources, and the extent of damage based upon assessment of neurocognitive function is highly variable. This diversity in injury and patient

characteristics is important because severity of injury, presence of co-morbid factors such as intracranial hemorrhage, certain polymorphisms, and patient brain characteristics appear to impact the development of sleep-wake disturbance after TBI (Hong et al., 2015; Imbach et al., 2015; Yaeger et al., 2014).

In addition to changes in time spent in wakefulness and NREM sleep, spectral characteristics of the EEG (particularly delta power) are often altered after TBI. In humans, NREM delta power may increase (Imbach et al., 2015) or decrease (Rao et al., 2011) after TBI. At least one rodent study demonstrates increases in EEG delta power during wakefulness after TBI (Sabir et al., 2015), which may be a correlate of subjective daytime sleepiness or fatigue in humans (D'Rozario et al., 2013; Lal and Craig, 2002). Data in this present study demonstrate that moderate TBI increases NREM delta power during the light period at all post-injury time points determined, and during the dark period 15 days post-injury. Because NREM delta power is accepted as an indication of the depth or intensity of sleep (Borbely, 1982; Dijk et al., 1990), these data suggest that mice subjected to moderate TBI sleep more deeply, which may indicate that this pathology causes sleep pressure to build more quickly during wakefulness. Definitive experiments to test this hypothesis remain to be conducted.

Impact of TBI on Arousal-promoting Neurotransmitter Systems

Changes in sleep-wake behavior after TBI are likely due, at least in part, to changes in neuronal systems implicated in regulating this complex behavior. Although multiple neurochemical systems are involved in regulating arousal state (Brown et al., 2012; Jones, 2008), in this study we focus on the hypocretinergic system and downstream projection targets, specifically the histaminergic tuberomammillary nucleus and the cholinergic basal forebrain. Hypocretin is essential for the maintenance of wakefulness. Hypocretin neurons discharge at their maximum during active wakefulness, especially during exploration (Estabrooke et al., 2001; Lee et al., 2005b; Mileykovskiy et al., 2005); intracerebroventricular injection of hypocretin increases wakefulness (Piper et al., 2000); and optogenetic stimulation of hypocretin neurons increases transitions from sleep to wake

(Adamantidis et al., 2007). Conversely, antagonizing the hypocretinergic system promotes sleep (Brisbare-Roch et al., 2007; Hoever et al., 2012; Morairty et al., 2014) and loss of hypocretinergic signaling results in narcolepsy (Liblau et al., 2015). Hypocretin neurons are few in number, tightly clustered in the lateral hypothalamus, and project diffusely to multiple brain regions (Date et al., 1999; Peyron et al., 1998). As such, damage to hypocretin neurons could have far-ranging effects on sleep-wake behavior either directly or by affecting downstream mediators.

Hypocretin signaling is altered during acute and chronic phases of TBI. *In vivo* microdialysis studies in mice demonstrate reduced extracellular hypocretin three days after injury (Willie et al., 2012), and hypocretin is reduced in cerebrospinal fluid one to four days after injury in humans (Baumann et al., 2005). In cases of fatal TBI in humans, hypocretin cell numbers are reduced (Baumann et al., 2009), and human survivors of TBI with excessive daytime sleepiness have low levels of hypocretin in cerebrospinal fluid for at least six months after injury (Baumann et al., 2007).

Two studies in mice found that TBI impairs hypocretin cell function, but does not alter the number of hypocretin-producing neurons (Lim et al., 2013; Willie et al., 2012). There are several potential reasons for the apparent discrepancy in these previous studies and our current one. Willie and colleagues determined hypocretin cell numbers at only one time point, which was 3 days after injury (Willie et al., 2012). Our data are consistent with those of Willie et al., in that hypocretin cell numbers after TBI do not differ from control until 7 – 15 days post injury. Collectively, these data suggest that the impact of mild to moderate TBI on hypocretin cell numbers takes longer than 3 days to develop. Similarly, Lim and colleagues did not observe decreased hypocretin cell numbers after TBI (Lim et al., 2013), but they used a midline fluid percussion model and random sampling of cells rather than unbiased stereology. Lim and colleagues also used 5-7 week old mice, which are considered adolescents in some models of TBI (Lopez-Rodriguez et al., 2015); inflammatory and cellular responses in brain to injury are highly affected by age (Kumar et al., 2013; McPherson et al., 2011; Timaru-Kast et al., 2012).

Nevertheless, our results demonstrating reduced numbers of hypocretin-producing neurons

are in agreement with the majority of pre-clinical and clinical observations of persistent hypocretin dysfunction after TBI. While the present study found a loss of hypocretin neurons, others have also observed impairments in hypocretin neuron activity (Lim et al., 2013; Willie et al., 2012); thus neuronal loss and functional impairment both may play a role in post-TBI sleep-wake disturbance. The reduction and/or dysfunction of hypocretin-producing neurons is also consistent with altered sleep-wake behavior observed in this study. Hypocretin neurons discharge at their highest rates during an animal's active period (Taheri et al., 2002), and hypocretin peaks during the latter part of the night in nocturnal rodents (Fujiki et al., 2001) and the latter part of the day in diurnal monkeys (Zeitzer et al., 2003) and humans (Salomon et al., 2003). It has been hypothesized that hypocretin is a reactive homeostatic signal needed to maintain wakefulness when sleep pressure increases (Zeitzer et al., 2003), which in humans is highest during the latter part of the day. Thus, in our present study increased sleep during the latter part of the mouse active period is consistent with decreased hypocretin signaling.

Melanin-concentrating hormone (MCH) neurons are intermingled with hypocretin neurons in the lateral hypothalamus (Hassani et al., 2009; Jones and Hassani, 2013; Torterolo et al., 2011). MCH neurons are implicated in the regulation of REM sleep and under some conditions NREM sleep. For example, MCH knockout mice spend significantly more time awake than their wild type counterparts (Willie et al., 2008). MCH neurons fire at a slow rate during NREM sleep and maximally during REM sleep (Hassani et al., 2009). Persistent optogenetic stimulation of MCH neurons increases NREM and REM sleep (Konadhode et al., 2013), but selective stimulation during NREM sleep increases transitions from NREM to REM (Jego et al., 2013; Tsunematsu et al., 2014). Although MCH promotes REM, ablation of MCH neurons does not appear to affect REM sleep, indicating that this peptide may not be necessary for REM to occur (Tsunematsu et al., 2014).

Because MCH neurons are intermingled with hypocretin neurons and generally act in a reciprocal manner with respect to arousal state, we also determined effects of TBI on this neuronal population. MCH neurons are variably reported to be affected by TBI. Some human postmortem

studies report that MCH neuron numbers are reduced (Valko et al., 2015) or not significantly affected (Baumann et al., 2009) in cases of fatal TBI. Little pre-clinical research has focused on a role for MCH neurons in response to TBI; the only animal study of which we are aware that quantified changes in this cell type did not reveal changes in cell numbers after TBI (Willie et al., 2012). In the present study, we also found that TBI does not affect MCH cell number.

Hypocretin likely promotes wakefulness through several mechanisms. Although hypocretin-producing neurons are found only in the hypothalamus, they have diffuse projections with terminals in nuclei/brain regions characterized by diverse transmitter systems. For example, hypocretin neurons project to dopaminergic cells in the ventral tegmental area, noradrenergic cells in the locus coeruleus, and serotonergic cells in the dorsal raphe nucleus, in addition to direct excitatory projections to the cortex; each of these populations are important for the promotion of wakefulness [reviewed in (Krystal et al., 2013; Peyron et al., 1998)]. In the present study, we restricted our focus to the effects of TBI on two neuronal populations downstream of hypocretin: the cholinergic neurons of the basal forebrain and the histaminergic neurons of the tuberomammillary nucleus.

Cholinergic neurons fire maximally during wakefulness and REM sleep (Lee et al., 2005a) and promote cortical activation during these states (Jones, 2008). Enhancement of cholinergic signaling with acetylcholinesterase inhibitors increases wakefulness at the expense of NREM sleep and REM sleep (Jung et al., 2012). Cholinergic neurons in the basal forebrain project to the cortex and hippocampus where they promote low-voltage, high frequency EEG activity, which is characteristic of wakefulness and REM sleep (Brown et al., 2012; Shin and Dixon, 2015). As briefly mentioned, cholinergic neurons of the basal forebrain receive direct excitatory projections from hypocretin neurons in the hypothalamus (Fadel and Burk, 2010) and hypocretinergic signaling to the basal forebrain is an important modulator of sleep-wake behavior (Vazquez-DeRose et al., 2016). Cholinergic and hypocretinergic neurons project directly to the cortex, where they may work together to promote wakefulness and a state of attention, although in some cases cortical hypocretin can compensate for deficiencies in cholinergic signaling (Zajo et al., 2015). Although some research has

focused on basal forebrain cholinergic neurons as mediators of hypocretin signaling to the cortex, cholinergic neurons do have sparse projections to the hypothalamus (Henny and Jones, 2006), and a subset of hypocretinergic neurons increase their firing rate in response to acetylcholine administration (Zhou et al., 2015). Thus, reciprocal interactions between these two neurotransmitter populations may play a role in consolidating wakefulness, although the functional significance of this reciprocity is not fully understood.

Interactions between hypocretinergic and cholinergic systems may play an important role in post-TBI sleep-wake disturbance. Just as the hypocretinergic system undergoes changes after TBI, the cholinergic system is also altered. During the acute phase after TBI there is a significant upregulation of activity in the cholinergic system, which may contribute to acute excitotoxic processes (Saija et al., 1988; Shin and Dixon, 2015). However, during chronic post-TBI periods, the cholinergic system is hypoactive (Shin and Dixon, 2015) and ChAT enzyme activity (Dewar and Graham, 1996; Murdoch et al., 1998) and immunoreactivity (Murdoch et al., 2002) are reduced in humans. Our results are consistent with these and other (Schmidt and Grady, 1995) observations insofar as we report reduced numbers of ChAT positive neurons in two areas of the basal forebrain. Thus, deficiencies in cholinergic signaling may also contribute to post-TBI sleep-wake disturbances.

Finally, we examined the effects of TBI on tuberomamillary histaminergic neurons. Histamine neurons fire maximally during wakefulness, and antagonizing histamine receptors increases sleep (Brown et al., 2001). Mice lacking brain histamine are unable to stay awake in response to behavioral challenge or environmental stimuli (Parmentier et al., 2002). Although hypocretin neurons project to the histaminergic cells of the tuberomammillary nucleus, the relationship between these two neuronal populations during pathology is not completely understood. For example, although histaminergic neurons receive substantial excitatory input from hypocretin neurons (Sundvik and Panula, 2015), a decrease in hypocretin is not always paralleled by a decrease in histamine: narcoleptics have little to no hypocretin, but greater numbers of histaminergic neurons (John et al., 2013; Valko et al., 2013). We are aware of only one study that has examined numbers of histaminergic neurons after TBI;

histaminergic neurons in the tuberomammillary nucleus are reduced in post mortem tissue after fatal TBI in humans (Valko et al., 2015). Our mouse model induces moderate injury with no mortality. Whether histaminergic neuron numbers would be reduced in pre-clinical models that result in more severe injury is not known. Histaminergic processes may still be important for decreases in wakefulness after TBI, but they may be mediated by changes in receptor density rather than by changes in neuron number (Shimada et al., 2012).

Inflammation as a Mechanism Underlying Neuronal Loss

The mechanisms by which TBI causes the semi-selective loss of hypocretinergic and cholinergic neurons are not known. TBI induces a state of robust neuroinflammation, which includes activation of microglia (Febinger et al., 2015; Harish et al., 2015; Hernandez-Ontiveros et al., 2013), astrogliosis (Harish et al., 2015; Hazra et al., 2014), and upregulation of inflammatory cytokines (Senol et al., 2014; Ziebell and Morganti-Kossmann, 2010). Inflammation is a key factor contributing to secondary injury after TBI (Corps et al., 2015; Ziebell and Morganti-Kossmann, 2010) and may cause hypocretinergic and cholinergic dysfunction.

Hypocretinergic and cholinergic neurons are sensitive to inflammation. For example, sterile inflammation induced by bolus injections of lipopolysaccharide, which also activates microglia and induces an inflammatory response (Camara et al., 2015; Thomson et al., 2014), reduces hypocretin in cerebrospinal fluid (Grossberg et al., 2011; Qin et al., 2005; Vasconcelos et al., 2014). Of relevance to this present study, chronic inflammation induced by repetitive doses of lipopolysaccharide reduces numbers of hypocretin-producing cells, but not MCH cells, indicating that hypocretin neurons are more sensitive to chronic inflammation than are MCH neurons (Palomba et al., 2014). As such, effects of inflammation induced by administration of lipopolysaccharide in the absence of injury on hypocretin and MCH producing cells of the hypothalamus are similar in some respects to those of TBI. Cholinergic neurons in the basal forebrain also are reduced during inflammation induced by chronic administration of lipopolysaccharide (Willard et al., 2000) or the inflammatory cytokine tumor necrosis factor α (Zassler et al., 2003). Therefore, although additional experiments must be

conducted to determine if this is indeed the case, one potential mechanism mediating the semi-selective loss of hypocretin and acetylcholine neurons, and subsequent effects on sleep-wake behavior after TBI, is the neuroinflammatory response to this insult.

Conclusions

The present findings demonstrate that moderate CCI can be used in mice to effectively model some aspects of human sleep-wake disturbance after TBI. Furthermore, results reveal reduced numbers of hypocretinergic neurons in the hypothalamus and cholinergic basal forebrain neurons after TBI, suggesting a role for deficiency in these transmitter systems as contributing factors to post-TBI changes in sleep-wake behavior. If low levels of hypocretin or acetylcholine indeed cause somnolence and excessive daytime sleepiness, clinical research could perhaps lead to new hypocretin- or acetylcholine-based pharmacotherapies to the benefit of the millions affected by chronic TBI.

Acknowledgments

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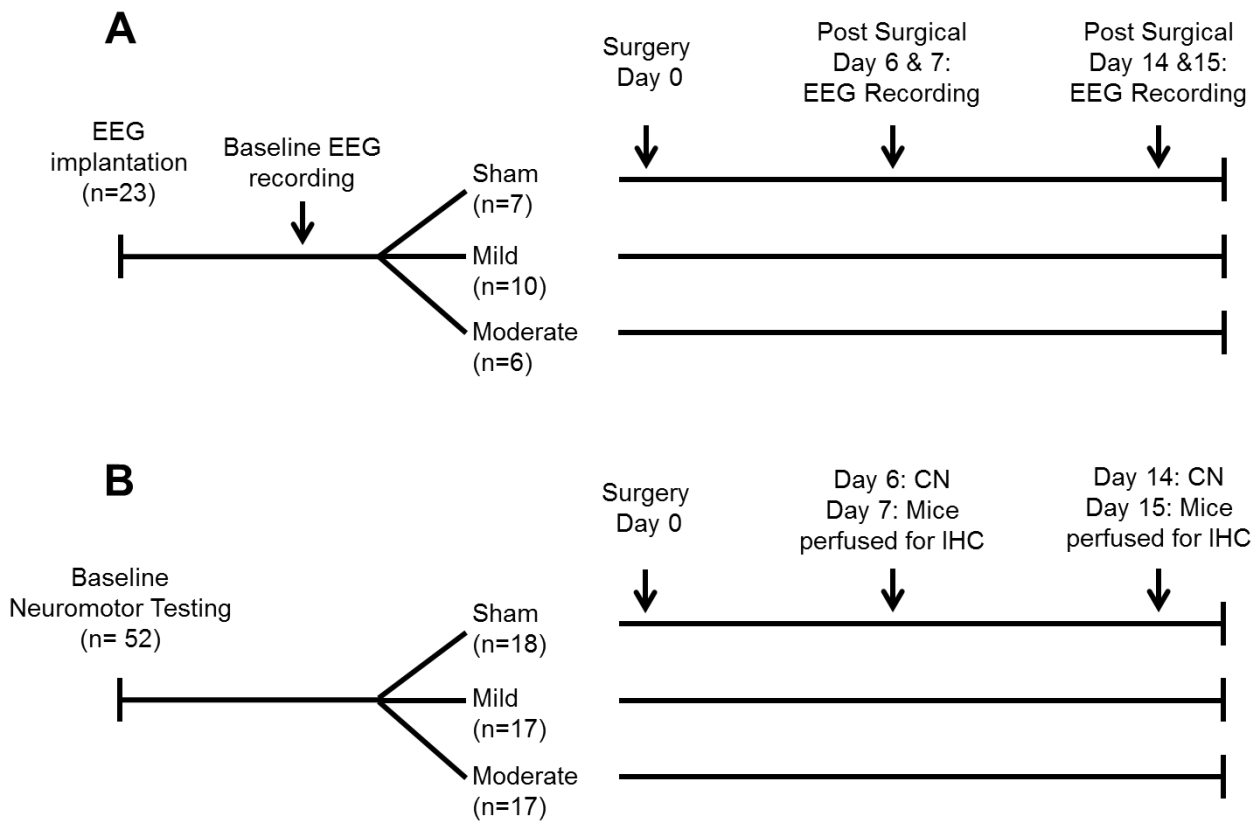


Fig. 1: Schematic representation of the protocols used in Experiments 1, 2 and 3. A) In Experiment 1, 48 h baseline electroencephalogram (EEG) recordings were obtained from undisturbed mice. Mice were then randomized into one of three surgical conditions: control (sham) surgeries; mild traumatic brain injury (TBI; 0.5 mm controlled cortical impact depth); moderate TBI (1.0 mm controlled cortical impact depth). Recordings of the EEG were obtained from the same animals one and two weeks post-surgery. **B**) Animals in Experiments 2 and 3 were used to determine the impact of TBI on neuromotor function (CN: composite neuroscore; Experiment 2) and to provide brain tissue for immunohistochemical assessment of TBI effects on selected neurotransmitters (Experiment 3). After baseline neuromotor testing, animals were randomized into either a sham surgical group (control), mild TBI group or moderate TBI group as in Experiment 1. Composite neuroscores were obtained one and two weeks post-surgery. After each neuromotor testing session, a subset of mice was sacrificed and brains removed for immunohistochemistry (IHC).

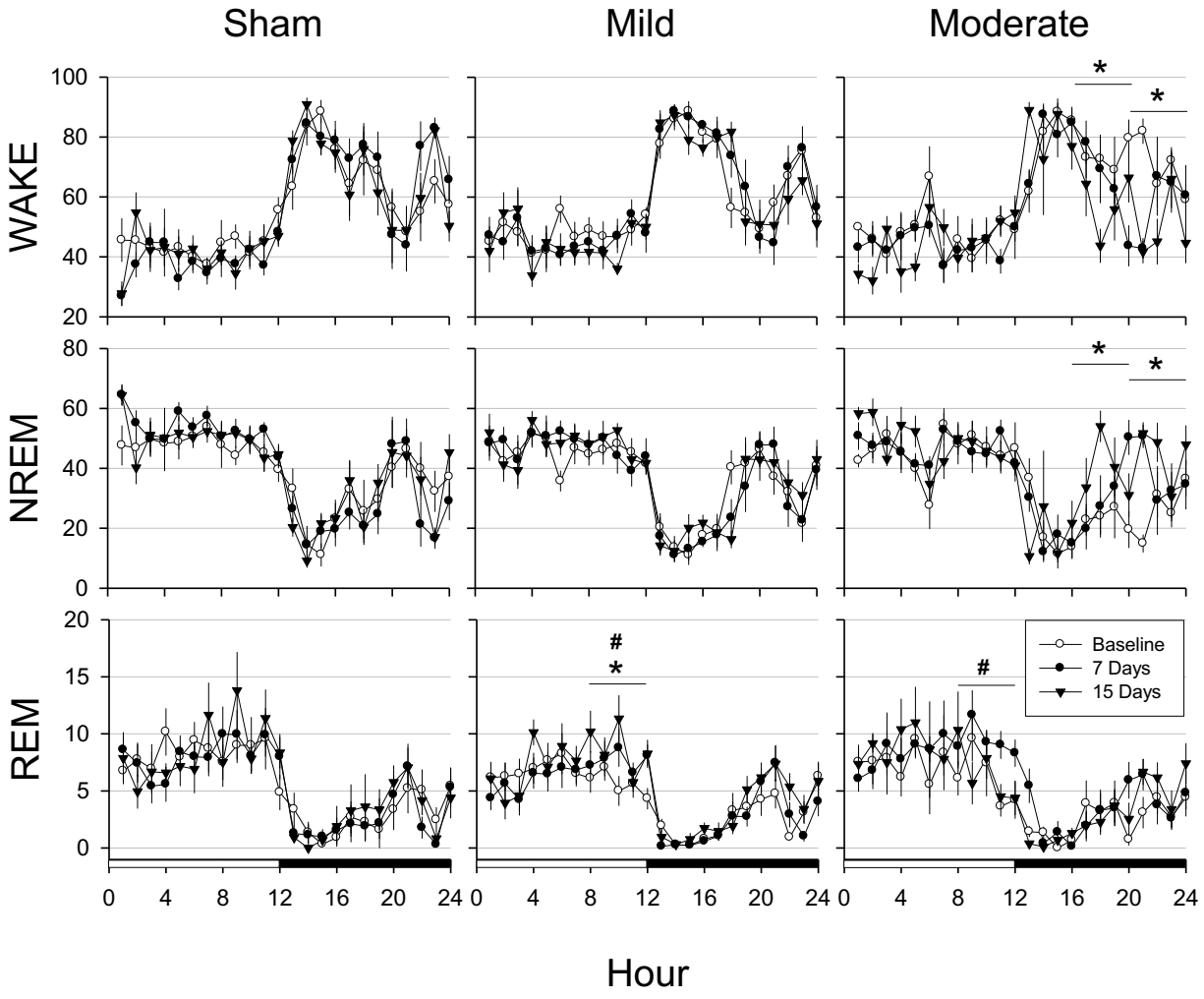


Figure 2: Traumatic brain injury alters sleep in an injury-dependent manner. Values are mean (± SEM) percent recording time spent in wakefulness (WAKE), non-rapid eye movement (NREM) sleep, or rapid eye movement (REM) sleep by control mice (sham surgeries, n=7), or those subjected to mild (0.5 mm controlled cortical impact depth, n=10) or moderate (1.0 mm controlled cortical impact depth, n=6) traumatic brain injury. Baseline recordings were obtained from undisturbed animals prior to sham surgeries and surgical procedures to induce injury. The open and filled bars on the X-axis indicate light and dark periods, respectively. Statistical analyses were performed on 4 hour time blocks. Statistically significant differences are indicated as: # = $p < 0.05$ baseline vs. 7 days post-surgery; * = $p < 0.05$ baseline vs. 15 d days post-surgery.

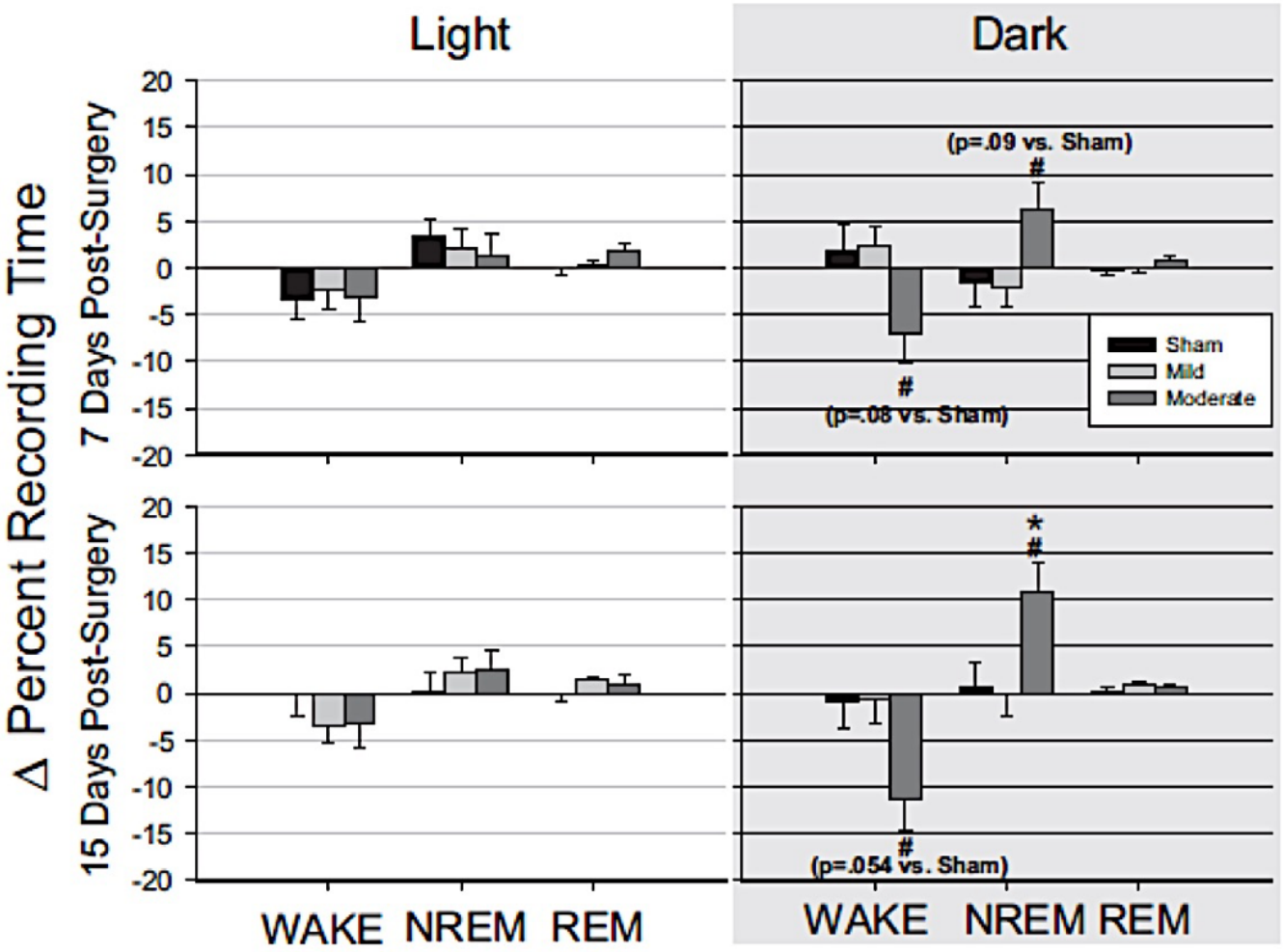


Figure 3: Traumatic brain injury decreases wakefulness and increases non-rapid eye movement sleep. Percentage of time spent in wakefulness (WAKE), non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) were determined during the 12 h light and 12 h dark period 7 days and 15 days after control surgeries (sham, n = 7), or after mild (0.5 mm controlled cortical impact depth, n = 10), and moderate (1 mm controlled cortical impact depth, n = 6) traumatic brain injury. Values are means (± SEM) expressed as change in percent recording time relative to undisturbed baseline (represented as the zero line). Statistically significant differences are indicated as: * = $p < 0.05$ vs. sham (control); # = $p < 0.05$ vs. mild TBI. Statistical trends for differences between moderate TBI and sham (control) are given in parentheses.

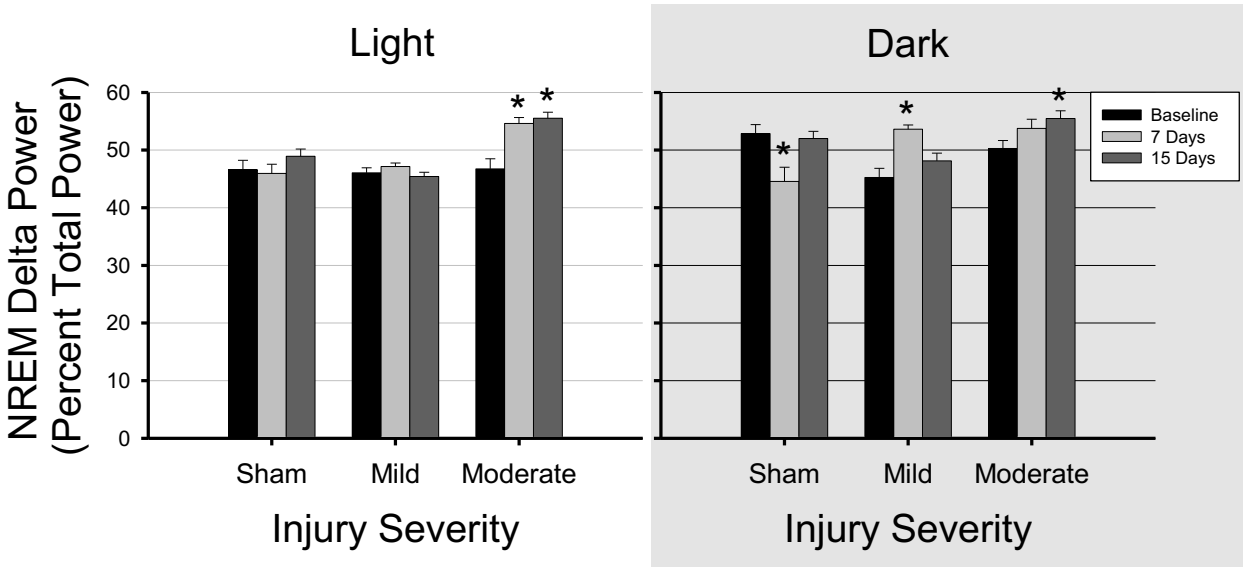


Figure 4: Traumatic brain injury increases delta power during non-rapid eye movement sleep. Power in the electroencephalogram (EEG) delta frequency band (0.5 – 4.5 Hz) was determined during the light period and dark period from artifact-free state-specific epochs. Recordings were obtained during pre-surgery baseline conditions, and 7 days and 15 days after control surgeries (sham, n = 7), mild (0.5 mm controlled cortical impact depth, n = 10), and moderate (1.0 mm controlled cortical impact depth, n = 6) traumatic brain injury. Delta power was normalized as the percent of the total power and is plotted as mean ± SEM. Statistically significant differences are depicted as: * $p < 0.05$ vs. baseline within the same condition.

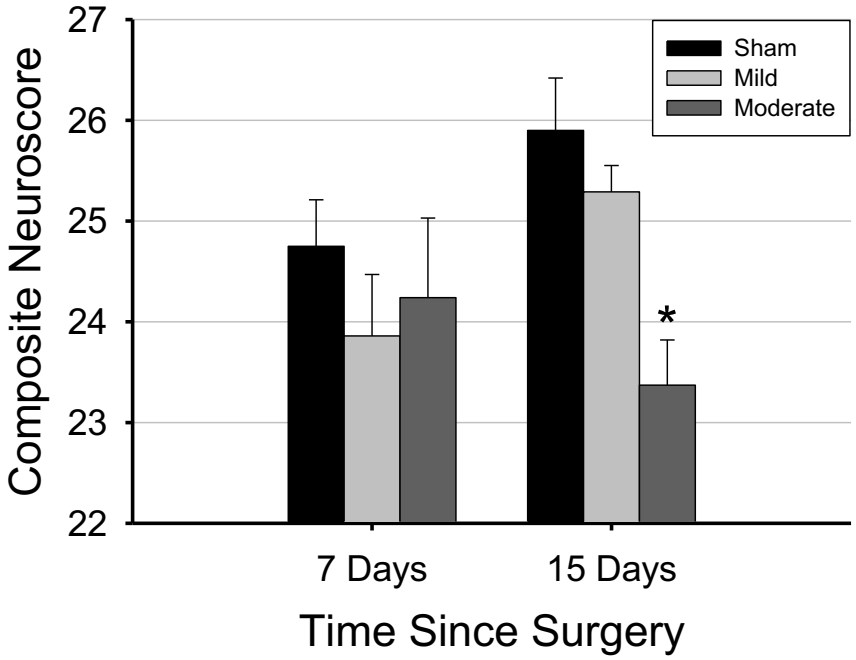
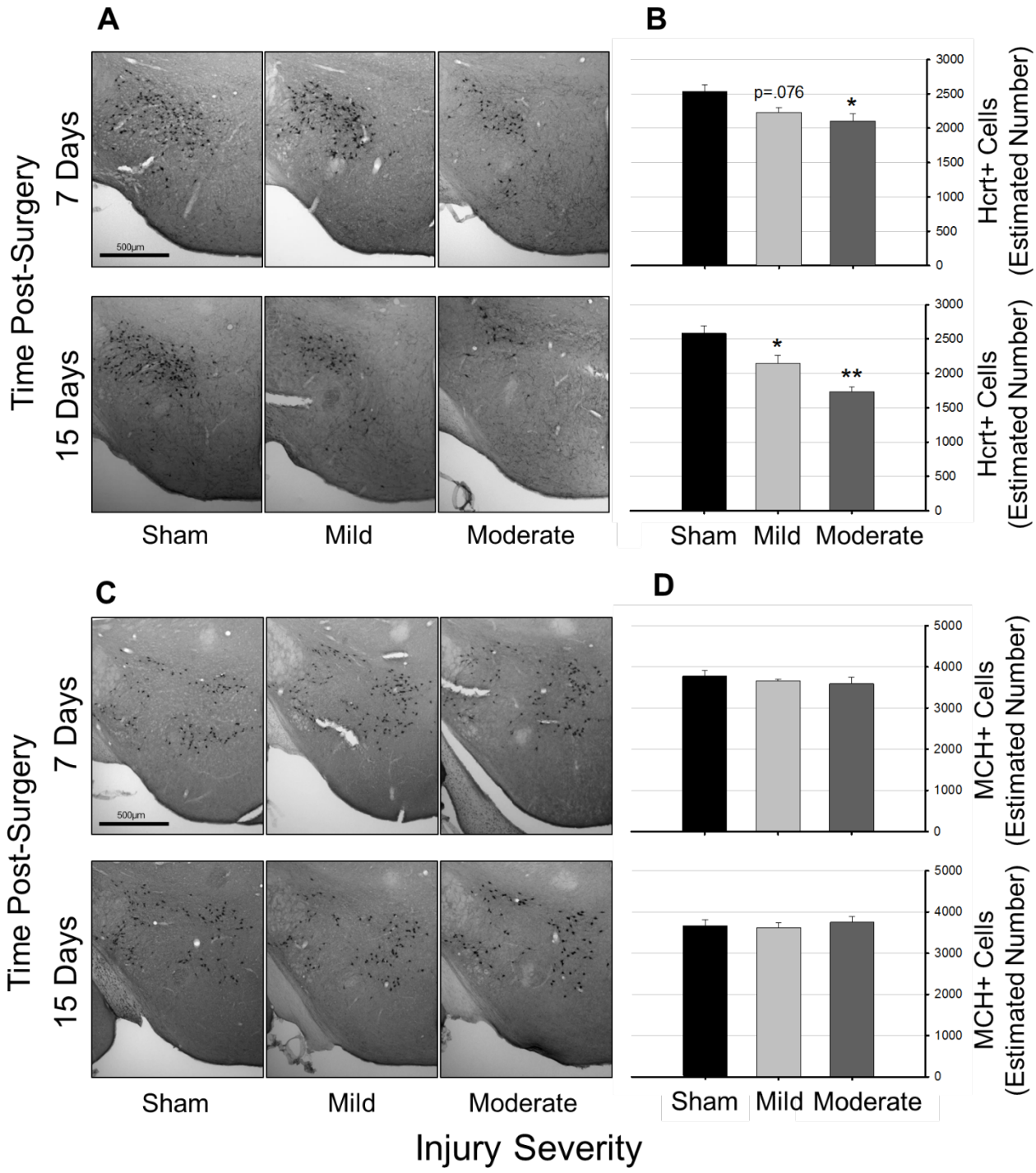


Figure 5: Traumatic brain injury impairs neuromotor function. Neuromotor function, as quantified by composite neuroscores, is impaired in animals subjected to moderate traumatic brain injury during the chronic post-injury phase. Values are means ± SEM obtained from control mice (sham surgeries, 7 days, n = 8, 15 days, n = 10), or mice subjected to mild (0.5 mm controlled cortical impact depth, 7 days, n = 8, 15 days, n = 9) or moderate (1.0 mm controlled cortical impact depth, 7 days, n = 8, 15 days, n =9) traumatic brain injury. Statistically significant difference are indicated as: * = $p < 0.05$ vs. values from control (sham) mice at same time point.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Injury Severity

Figure 6: Traumatic brain injury reduces numbers of hypocretin neurons, but not melanin-concentrating hormone neurons. Representative photomicrographs of hypocretin- (Panel A) and melanin-concentrating hormone (MCH) positive cells (Panel C) in tissue obtained from control (sham) mice, and mice subjected to mild (0.5 mm controlled cortical impact depth) or moderate (1.0 mm controlled cortical impact depth) traumatic brain injury. Numbers of hypocretin (Panel B) and MCH (Panel D) cells in the perifornical-lateral region of the hypothalamus ipsilateral to the injury site were estimated using unbiased stereology and the optical fractionator method. Values in panels B and D are means ± SEM obtained from control mice (sham surgeries, 7 days, n = 7, 15 days, n = 6), or mice subjected to mild (0.5 mm controlled cortical impact depth, 7 days, n = 8, 15 days, n = 8) or moderate (1.0 mm controlled cortical impact depth, 7 days, n = 8, 15 days, n =8) traumatic brain injury. Statistical differences relative to sham (control) animals are depicted by: * = $p < 0.05$, ** = $p < 0.01$.

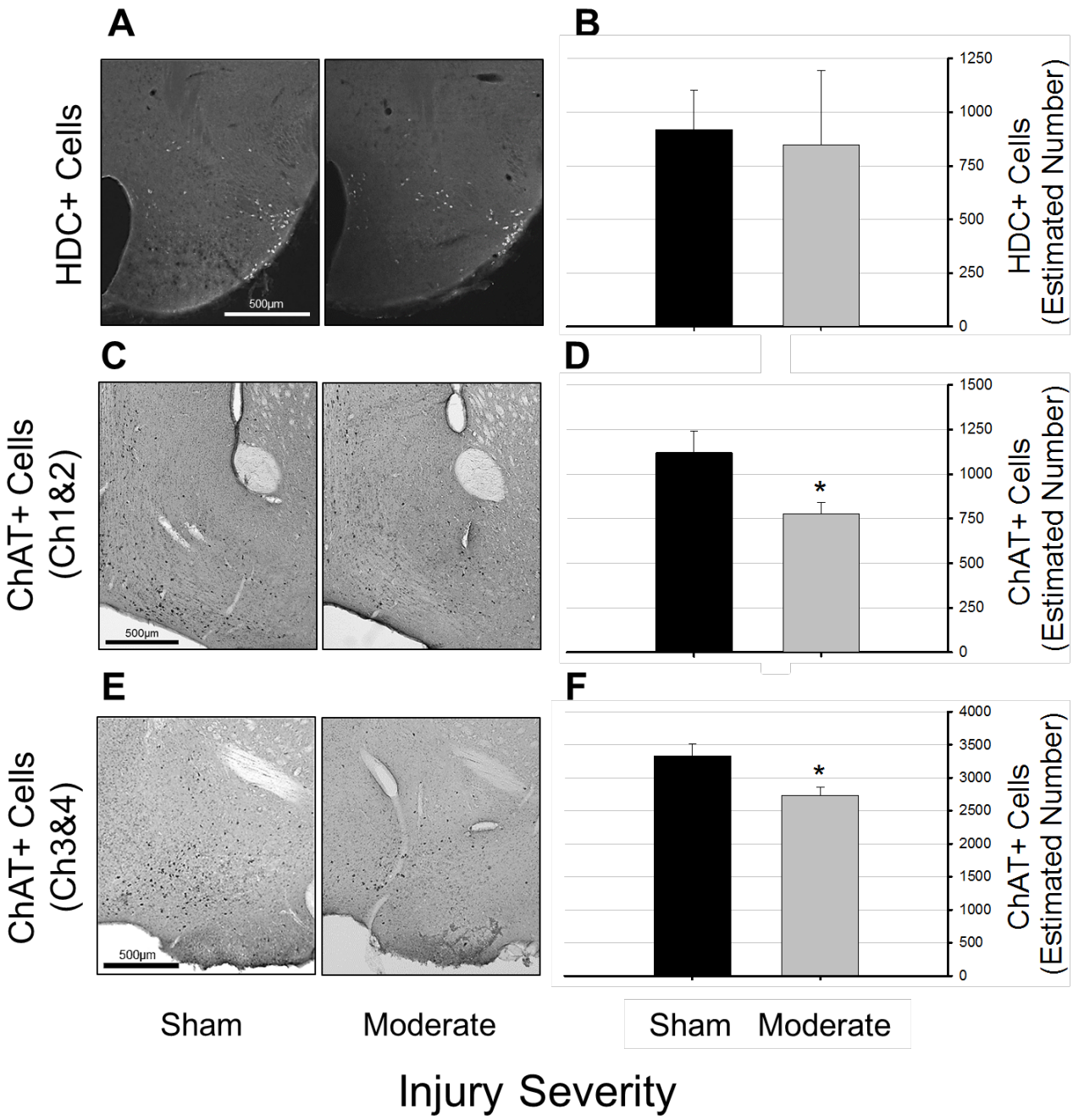

Figure 7: Traumatic brain injury reduces numbers of cholinergic neurons in the basal forebrain, but not histaminergic neurons in the tuberomammillary nucleus. Representative photomicrographs of histidine decarboxylase (HDC) (Panel A) and choline acetyltransferase (ChAT) (Panels C and E) positive cells in tissue obtained from control (sham, n=7) mice, and from mice subjected to moderate (1.0 mm controlled cortical impact depth, n=7) traumatic brain injury. Tissue was obtained at 15 days post-surgery. Numbers of HDC- (Panel B) and ChAT-positive cells (Panels D and F) ipsilateral to the injury site were estimated using unbiased stereology and the optional fractionator method. Statistically significant differences relative to cell numbers determined from control (sham) mice are indicated as * = $p < 0.05$. Data in panels B, D, and F are presented as means ± SEM.

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Chapter 3: Hypocretin mediates sleep-wake disturbances in a mouse model of traumatic brain injury[2]

Abstract

Traumatic brain injury (TBI) is a major cause of disability worldwide. Post-TBI sleep and wake disturbances are extremely common and difficult for patients to manage. Sleep and wake disturbances contribute to poor functional and emotional outcomes from TBI, yet effective therapies remain elusive. A more comprehensive understanding of mechanisms underlying post-TBI sleep and wake disturbance will facilitate development of effective pharmacotherapies. Previous research in human patients and animal models indicates that altered hypocretinergic function may be a major contributor to sleep-wake disturbance after TBI. In this study, we further elucidate the role of hypocretin by determining the impact of TBI on sleep-wake behavior of hypocretin knockout (HCRT KO) mice. Adult male HCRT KO mice were implanted with EEG recording electrodes, and pre-injury baseline recordings obtained. Mice were then subjected to either moderate TBI or sham surgery. Additional recordings were obtained and sleep-wake behavior determined at 3-, 7-, 15-, and 30 days after TBI or sham procedures. At baseline, HCRT KO mice had a significantly different sleep-wake phenotype than control C57BL/6J mice. Post-TBI sleep-wake behavior was altered in a genotype-dependent manner: sleep of HCRT KO mice was not altered by TBI, whereas C57BL/6J mice had more non-rapid eye movement (NREM) sleep, less wakefulness, and more short wake bouts and fewer long wake bouts. Numbers of hypocretin-positive cells were reduced in C57BL/6J mice by TBI. Collectively, these data indicate that the hypocretinergic system mediates the alterations in sleep-wake behavior that develop after TBI in this model, and suggest potential therapeutic interventions.

[2] This chapter will be submitted to *The Journal of Neurotrauma*.

Introduction

Traumatic brain injury (TBI) is a dramatic public health issue worldwide, with major implications for human health and healthcare cost. In the United States alone, as many as 5.3 million individuals may suffer from long-term disability related to TBI.[1] The direct cost of TBI in the United States is estimated at $9.2 billion, with more than $50 billion additionally lost due to lost productivity.[2]

Disorders of sleep and wakefulness are among the most common consequences of TBI, affecting up to 70% of people who suffer a brain injury.[3] Sleep disturbances may begin within days after injury [4,5] and persist for years.[6,7] Sleep and wake disturbance affects other aspects of quality of life: post-TBI sleep disturbance predicts poorer cognitive, social, and emotional outcomes.[8–10] In general, sleep disturbances associated with TBI are characterized by fragmentation of sleep – wake states and an inability to sustain long, consolidated periods of wakefulness. TBI patients exhibit excessive daytime sleepiness (EDS), whether assessed objectively (Multiple Sleep Latency Test)[11–16] or subjectively (Epworth Sleepiness Scale),[6,15,17] which results in high prevalence of daytime napping.[11,18] Some studies reveal that TBI patients have greater total sleep time,[11,12] with increased light non-rapid eye movement (NREM) sleep[16] or in slow wave sleep.[11,19–21] Although they sleep more, TBI patients generally report poor sleep quality,[17,22–29] and objective measurements demonstrate that sleep of individuals with TBI is more fragmented than controls.[5,19,20,30] The majority of studies do not report differences in the time TBI patients spend in REM sleep.[11–13,20,21,29] In addition to the amount of sleep and/or characteristic changes in sleep architecture, spectral properties of the EEG, especially in the delta power frequency band, may also be altered after TBI: delta power may increase during NREM sleep[12] or during wakefulness.[29] Because delta power is associated with sleep pressure,[31,32] these increases may be related to EDS and reflect increased sleep need in TBI patients.

Rodent models of TBI produce disturbances of sleep and wakefulness that are similar to those of human patients. TBI decreases wakefulness acutely (within 7 days of injury)[33–35] and chronically.[36–

[38] In general, sleep is fragmented and bouts of sleep and wake are shortened. [34,38,39,40–42] The amount of time spent in REM sleep is not altered, at least as reported in animal models to date.[36–39,41,42] After TBI, delta power may be increased during NREM sleep[36,37] or during wakefulness.[39] Brain injured mice also have significantly more EEG slow waves during wakefulness, potentially indicating greater sleep pressure.[43]

In humans, many sleep-wake regulatory neurotransmitters and hormones are altered by TBI, including hypocretin,[44–46] histamine,[44] acetylcholine,[47,48] serotonin,[49] noradrenaline,[49] and melatonin.[20,50] Of these potential transmitter systems, hypocretinergic dysfunction is likely a key contributor to post-TBI sleep-wake disturbance. Most studies demonstrate that hypocretin, a neuropeptide that is essential for promoting wakefulness and stabilizing sleep-wake states, is dysregulated after TBI. In cases of fatal TBI in humans, the number of hypocretin neurons is significantly reduced.[44,45] During the acute phase of TBI, 95% of patients with moderate to severe TBI have abnormally low levels of hypocretin in the cerebrospinal fluid (CSF).[46] Furthermore, at 6 months post-TBI, CSF hypocretin concentrations are significantly lower in patients with EDS than in the CSF of those without EDS.[51]

Hypocretinergic dysfunction is also evident in animal models of TBI. Within the first three days after TBI, hypocretin dynamics are significantly altered in mice, with reduced hypocretin in the hypothalamus and hippocampus.[34] The diurnal rhythm of hypocretin is blunted in brain injured animals, with much less of a peak-to-trough amplitude difference between the light and dark phases.[34] We previously demonstrated that at chronic time points (15 days post-injury and later), there is a significant decrease in the number of hypocretin-pr1oducing cells in the hypothalamus of mice subjected to TBI.[37,41]

To our knowledge, no direct genetic manipulation of the hypocretinergic system has been used in pre-clinical TBI models. In the present study, we used genetically-modified mice that lack hypocretin (HCRT KO) to further investigate the role of hypocretin in post-TBI sleep and wake

disturbances. We now report that, unlike C57BL/6J control mice, the sleep of HCRT KO mice is not significantly affected by TBI. These new data support the hypothesis that disruption to the hypocretinergic system is necessary for altered sleep-wake behavior after brain injury in this model.

Materials and Methods

Animals

Hypocretin knockout mice on a C57BL/6 background (HCRT KO) were kindly provided by Dr. John Peever (University of Toronto; Toronto, Ontario, CN), and adult male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were group housed until surgery (~3-4 months old at time of use), after which they were single housed. Mice were maintained on a 12:12 light:dark cycle at 29 ± 1º C with food and water provided *ad libitum*. All procedures involving the use of animals were approved by the University of Washington IACUC in accordance with the U. S. Department of Agriculture Animal Welfare Act and the National Institutes of Health policy on Humane Care and Use of Laboratory Animals.

EEG Recording and Determination of Sleep-Wake Behavior

Sleep-wake behavior of mice was determined based on the electroencephalogram (EEG) and cage activity patterns. EEG signals were amplified, filtered, and recorded for offline processing using custom software written in LabView for Windows (ICELUS, M. Opp, University of Washington; National Instruments, Austin, TX) as previously described.[52,53] EEG and cage activity records were visually scored in 10-second epochs. Artifact-free EEG signals were subjected to fast Fourier transformation, yielding power spectra between 0.5 and 40 Hz in 0.5-Hz frequency bins. Arousal states were determined as previously described and classified as non-rapid eye movement (NREM) sleep, rapid eye movement (REM) sleep, or wakefulness (WAKE) based upon published criteria.[52–54]

Experimental Design

A schematic representation of the protocols used in this study is presented in **Figure 1.**

Experiment 1: Effects of TBI on Sleep-Wake Behavior of Hypocretin KO Mice

C57BL/6J mice (n=18) and HCRT KO mice (n=17) were surgically implanted with EEG recording electrodes under isoflurane anesthesia. The leads from the screw electrodes were soldered to pins of a plastic connector (Digi-Key, ED85100-ND) to allow coupling to the recording system. Dental acrylic (Integrity Caulk, Dentsply) covered the electrodes and formed a headpiece to which the flexible recording tether could be connected. The section of the skull over the left parietal cortex was not covered with dental acrylic at this time. The incision was closed with sutures, and a subcutaneous injection of an analgesic (0.5mg/kg buprenorphine) was given at the end of the surgery. Mice were allowed 7 days to recover before they were attached to a flexible tether for habituation to the recording system. After 3 days of habituation to the tether and recording environment, 48-hour undisturbed baseline recordings were obtained.

After the 48-h baseline recordings, two mice were dropped from the study due to poor quality of the EEG signals. Remaining mice were then randomized into four groups: C57BL/6J sham (n=8), C57BL/6J TBI (n=9), HCRT KO sham (n=8), and HCRT KO TBI (n=8). TBI was induced using controlled cortical impact (CCI), as previously described.[37,55] We previously demonstrated that 1mm depth CCI produces moderate TBI and significant changes in sleep-wake behavior,[37] and we used the same parameters in this study. All mice (sham, TBI) received a craniotomy over the left parietal cortex using a 5 mm trephine, approximately -2 mm relative to bregma and 2.5 mm lateral to the midline. The skull fragment was removed without disrupting the underlying dura, and TBI was induced in the experimental group using the Leica Impact One system (Richmond, IL) equipped with

an electrically-driven 3-mm diameter metal piston controlled by a linear velocity displacement transducer. CCI parameters were: 5.0 m/s impact velocity; 100 msec dwell time; and impact depth of 1.0 mm (moderate TBI). Sham (control) animals received identical anesthesia and craniotomy without the CCI injury. After the CCI, a sterilized polystyrene disc created from a weighing boat was placed over the craniotomy and covered with dental acrylic. We[37,55] and others[56] have used this or a similar technique to protect the brain after craniotomy. After the incision was closed with sutures, mice received a subcutaneous injection of analgesic (0.5mg/kg buprenorphine) and were returned to their home cages. Animals were closely monitored after surgery and none displayed overt signs of infection.

Additional 48-h recordings were obtained from all mice on post-craniotomy/TBI days 3-4, 7-8, 14-15, and 30-31. Sleep-wake behavior was determined and the EEG subjected to fast Fourier transformation to produce power spectra between 0.5 and 40 Hz in 0.5 Hz bins as described previously.[52] Power in the delta frequency band (0.5 – 4.5 Hz) during NREM sleep was analyzed in a subset of mice (n=5-6 mice per group). NREM delta power was normalized to the total state-specific power (NREM sleep), summed across all frequency bins from 0.5 to 40 Hz for the light and dark periods and expressed as a percent of total power (see [53]).

Experiment 2: Effects of TBI on Numbers of Hypocretin Neurons

After the day 30-31 post-craniotomy/TBI recording, mice were perfused and brains removed for immunohistochemical assessment of TBI effects on hypocretin neurons. Brains were sectioned on a Leica cryostat at 14 μm and mounted on Superfrost Plus slides. Briefly, slides were washed in phosphate buffered saline (PBS) three times for five minutes each, then blocked for 30 minutes with a 1:20 dilution of normal donkey serum in PBS with 1% bovine serum albumin (BSA). Slides were then incubated overnight at 4°C in rabbit anti-mouse orexin-A (H-003-30; Phoenix Pharmaceuticals, Inc.;

1:1,000 dilution). Slides were then rinsed with PBS six times for five minutes each. Slides were then incubated for 30 minutes in the secondary antibody solution (Alexa Fluor 488 donkey anti-rabbit; Jackson ImmunoResearch; 711-545-152; 1:400 dilution). Slides were rinsed with PBS six more times for five minutes each and then coverslipped.

Estimating Cell Numbers

Cell numbers were estimated using quantitative methods for unbiased stereology.[57] Briefly, positively stained cells were visualized on an Olympus BX-51 fluorescent stereoscope using Stereo Investigator 10 (MBF Biosciences, Williston, VT). Hypocretin cell number estimates were obtained from 7 sections in a 1:9 series spanning approximately -1.20 mm to -2.10 mm from bregma (Paxinos and Franklin, 2001). The contour for the perifornical-lateral hypothalamic region was outlined using a 4x objective. Cells were then counted using the 20x objective and optical fractionator, with a counting frame of 50x50 microns and a grid size of 100x100 microns. All cell counts were obtained from the hemisphere ipsilateral to injury. TBI-induced changes in cellular and tissue outcomes (cell death, inflammatory cytokine expression, presence of immune cells, etc.) are typically most severe on the side ipsilateral to injury[58,59] and previous studies using unilateral CCI have examined hypocretin neuron number and function in the hypothalamus ipsilateral to injury.[34]

Statistical Analyses

To determine the impact of TBI and genotype on sleep wake behavior, percent time spent in wake, NREM sleep, or REM sleep during the light or dark periods was analyzed using a one-way ANOVA within time block. If significant effects of injury or genotype were detected, post-hoc comparisons were made using Tukey's HSD to determine which groups contributed to statistical differences.

We also determined the effect of injury and genotype on the temporal distribution of sleep-wake behavior over the course of 24-h recording periods. The percent time spent in wake, NREM sleep, and REM sleep were determined for each recording hour and then grouped into 4 h time blocks. Values within each 4 h time block were evaluated using a one way ANOVA across conditions (injury, genotype). If significant effects of injury or genotype were detected, post-hoc comparisons were made using Tukey's HSD to determine differences between/among groups.

To assess the effects of injury and genotype on sleep architecture, bouts of wakefulness were sorted by length into one minute bins up to 10-min. Wake bout lengths of 10 – 20 minutes, and wake bout lengths greater than 20 minutes, were grouped into two separate bins. For each bout length bin, a one-way ANOVA was performed separately for light period and dark period values across injury and genotype groups for baseline and each post-surgical time point. Post-hoc comparisons were made using Tukey's HSD to determine differences between groups.

To assess effects of injury and genotype on delta power, normalized NREM delta power was calculated for each hour across the 24-h period. Statistical analyses using one-way ANOVA were then conducted separately for 12 hours of the light and dark periods for baseline and each post-surgical time point. If significant differences were revealed within time point, post-hoc comparisons were made using Tukey's HSD to determine which conditions contributed to these effects.

Because HCRT KO mice lack hypocretin, the effect of TBI on numbers of hypocretin neurons was assessed only in C57BL/6J mice. Estimated numbers of hypocretin neurons in mice subjected to sham and TBI were compared using a non-paired Student's t test.

All analyses were performed using SPSS for Windows (IBM Corporation, Armonk, NY). Data are presented as mean ± SEM, unless otherwise indicated. An alpha value of $p < 0.05$ was accepted as indicating a significant difference between or among groups.

Results

Sleep-wake Behavior is Altered by TBI in a Genotype-dependent Manner

Under baseline (undisturbed) conditions prior to craniotomy/TBI surgeries, the amount of time mice spent in wakefulness, NREM, and REM sleep differed by genotype: HCRT KO mice spent significantly less time awake during the light and dark period, and more time in NREM and REM sleep during the dark period as compared to C57BL/6J mice (**Figure 2**). Sleep-wake behavior of mice of the same genotype that were subsequently randomized into sham or TBI groups did not differ during pre-surgical baseline recordings in terms of total amount of time spent in each state (**Figure 2**) or in temporal distribution (**Figure 3**).

TBI did not alter sleep of HCRT KO mice (**Figure 2**). No differences were detected between HCRT KO sham and TBI groups in the total amount of time spent in wake, NREM or REM sleep at any post-surgical time point. Furthermore, the temporal distribution of sleep-wake behavior was not altered by TBI in HCRT KO mice (**Figure 3**).

In contrast to HCRT KO mice however, TBI altered sleep of C57BL/6J mice (**Figure 2**) with changes most pronounced during the dark period at 15 and 30 days post-injury. During these chronic post-TBI time points, relative to control mice that received sham surgeries, the total amount of time C57BL/6J mice spent awake was reduced during the dark period. Reduced wakefulness during the dark period was concomitant with increased time spent in NREM sleep (**Figure 2**). The temporal distribution of sleep-wake behavior of C57BL/6J mice was altered by TBI, with effects apparent during the dark period at 7 days post injury and becoming more profound at 15- and 30 days post-injury. Specifically, reduced wakefulness and increased NREM sleep were first apparent during the early dark period at 7 days post-injury, and these changes progressively extended to encompass the entire 12-h dark period by 30 days post-injury (**Figure 3**).

There were minimal genotype differences in the total amount of time spent in wakefulness and NREM sleep after TBI, although HCRT KO mice spent less time awake during the light period 3 days

after injury (**Figure 2**) and during early parts of the dark period (**Figure 3**). At chronic time points, the total amount of NREM sleep and wakefulness of C57BL/6J mice subjected to TBI did not differ from that of HCRT KO mice with TBI (**Figure 2**).

The total amount of time spent in REM sleep differed between genotype irrespective of whether or not mice had TBI (**Figure 2**). In general, HCRT KO mice had more REM sleep during any time point across the 24 h recording periods (**Figure 3**).

Wake Bout Length is Altered by TBI in a Genotype-dependent Manner

There were genotype differences in the duration of wake bouts, which were most apparent for very short (one minute or less) and very long (more than twenty minute) bouts (**Figure 4**). Under baseline conditions during the light and dark periods, HCRT KO mice had more very short bouts and fewer very long bouts than did C57BL/6J mice. TBI did not alter the distribution of wake bout lengths in HCRT KO mice (**Figure 4**). In contrast, 3 days after TBI, C57BL/6J mice had more very short bouts during the light and dark periods than did sham C57BL/6J mice. At 7 days post-surgery, and at the 15- and 30-day time points, C57BL/6J mice with TBI had significantly fewer very long wake bouts than did sham C57BL/6J animals during the dark period, and fewer very long wake bouts during the light period at 3 and 30 days post-surgery compared to C57BL/6J sham mice.

Delta Power is Transiently Altered by TBI

Due to artifacts in the EEG, some mice were not included in analyses of spectral characteristics of the EEG. In these cases, the signal was sufficient for manual determination of sleep-wake state, but artifacts or spikes produced large erroneous changes in calculated power. NREM delta power was analyzed separately for the light and dark periods (**Figure 5**).

During baseline recordings, there were no significant differences between groups in NREM delta power. At 3- and 7 days post-surgery, TBI increased NREM delta power during both the light and dark periods in C57BL/6J mice as compared to C57BL/6J mice that had sham surgeries. Increased NREM delta power was not detected at 15 or 30 days post-surgery in C57BL/6J mice. NREM delta power was increased after TBI in HCRT KO mice relative to HCRT KO sham mice only at 15 days post-surgery (light period) and 30 days post-surgery (dark period). Differences between genotypes in NREM delta power after TBI were transient, only observed at 3 days post-surgery, and as such may be residual effects of anesthesia and surgery (**Figure 5**).

Estimated Numbers of Hypocretin Neurons are Reduced by TBI

At 32 days post injury, C57BL/6J animals with TBI had significantly fewer hypocretin neurons in the lateral hypothalamus than did sham-operated C57BL/6J mice (**Figure 6**). As expected, HCRT KO mice had no evidence of hypocretin immunoreactivity.

Discussion

Sleep-wake disturbances after TBI have been documented extensively in humans.[6,10,16,51,60,61] In spite of the large body of literature on post-TBI sleep and wake disturbances, there is a lack of effective treatments. Stimulants like methylphenidate, modafinil, and armodafinil have mixed efficacy in normalizing sleep-wake behavior in TBI patients.[62–66] Antidepressants and melatonin agonists are not always effective in improving nighttime sleep quality.[62,67,68] Sleep hygiene interventions are ineffective,[69] and although small trials of blue light therapy[70] or modified cognitive behavioral therapy[71,72] have shown some promise, few of these interventions have been attempted, and these treatments are expensive and time-consuming.

The lack of effective therapeutics is likely due, at least in part, to a lack of understanding of the precise causes of post-TBI sleep and wake disturbances. Many have hypothesized that changes in neurotransmitters, neuropeptides and hormones that regulate sleep and wakefulness are responsible for post-TBI disturbances. Hypocretin,[37,38,45,46] histamine,[36,44] serotonin,[49] noradrenaline,[49] acetylcholine,[37,47,73] and melatonin[50] are the transmitters for which data suggest potential roles as mediators of post-TBI sleep and wake disturbances. Melatonin is relatively easy to assay in saliva or plasma, but determining levels of neurotransmitters in living human patients is much more difficult. Although neurotransmitters can be measured in the cerebrospinal fluid (CSF) with a lumbar puncture as a proxy for levels in brain, this process is invasive, and not without risk.

Given the limitations in obtaining appropriate samples from living patients, some studies have determined neurotransmitter concentrations in cases of fatal TBI.[44,45,49] Although post-mortem collection provides access to samples for the assay of neurotransmitters, the extent to which a brain that has undergone a fatal injury resembles a brain that has been subjected to mild or moderate injury and then survived is not known. Utilizing appropriate animal models to identify potential neurotransmitters or other systems that are potential contributors to, or mediators of post-TBI sleep and wake disturbances ameliorates some of the limitations that are inherent in the study of patients subjected to brain injury. Animal models are especially useful in the context of TBI as injury location and severity can be precisely controlled, and the brain can be assessed at selected time points after injury.[74–78] Furthermore, genomic manipulation in animal models is a powerful tool that can be used to determine the functional role of transmitter substances in exacerbating or ameliorating the secondary injury or reparative processes that occur after TBI.

To our knowledge, this study is the first to use genetic ablation of hypocretin to determine its role in responses to TBI. In this study, we subjected HCRT KO mice to moderate TBI, and now report that hypocretin plays a critical role in sleep responses to brain injury in this model. Sleep-wake behavior of HCRT KO mice is not altered by TBI relative to values obtained from HCRT KO mice

subjected to sham surgery. Furthermore, although TBI reduces wakefulness and increases NREM sleep of C57BL/6J (control) mice at later post-injury time points (15 and 30 days), the amount of time spent in these arousal states does not differ from those of HCRT KO mice.

Similarly, although they have significantly more very short wake bouts and fewer very long wake bouts during baseline conditions than do C57BL/6J mice, TBI does not change the distribution of wake bouts in HCRT KO mice. In contrast, after TBI, C57BL/6J mice have significantly more very short and fewer very long wake bouts than do mice of the same genotype that had sham surgeries. Furthermore, at chronic time points (15 and 30 days), the distribution of wake bout lengths of C57BL/6J mice and HCRT KO mice do not differ after TBI.

Although the majority of studies report sleep disturbance after TBI, the exact nature of altered sleep is highly variable, even in animal models. Our present results agree with other pre-clinical studies of TBI that report increased NREM sleep[36–38] and increased sleep fragmentation[34,38,39,41] during the dark period without changes in the amount of REM sleep.[36–39,41,42] Although the timing is variable, our results are also in agreement with other studies that found increased NREM delta power at some times after TBI.[36,37]

Our results are also consistent with some studies in humans that found increases in slow wave sleep[11,19–21] and no differences in time spent in REM sleep.[11–13,20,21,29] In our model, TBI has little impact on REM sleep (this present study and [37]). Although HCRT KO mice normally spend more time in REM sleep than do genetically-intact control mice, there are no consistent changes in this sleep stage after TBI in either genotype. Loss of hypocretin neurons in narcoleptic humans[79–82] and KO mice[83–85] consistently produces REM sleep abnormalities (increased time in REM sleep and sleep-onset REM periods). However, acute modulation of hypocretin neurons has less impact on REM sleep. For example, optogenetic silencing of hypocretin neurons in mice decreases wakefulness and increases NREM sleep and increases sleep-wake state fragmentation [86] without altering REM sleep.[86,87] Similarly, pharmacogenetic suppression of hypocretin neuron activity decreases

wakefulness and increases NREM sleep with no change in REM sleep time.[88] Studies of hypocretin antagonists in humans and animals consistently reveal reduced wakefulness and increased NREM sleep, and although some studies also report increased REM sleep,[89–91] others do not.[92–94]

One possible explanation for inconsistent findings of hypocretin function on REM sleep is that there may be dose-related effects. For example, at low doses, hypocretin antagonists increase NREM sleep, but not REM sleep, whereas high doses of the same antagonist increase NREM and REM sleep.[95] Even at high doses, hypocretin antagonists generally do not induce sleep-onset REM periods, which are a common feature of narcolepsy, i.e., a state of hypocretin loss.[96,97] Furthermore, homozygous HCRT KO mice display an animal form a cataplexy, whereas heterozygous HCRT KO mice do not.[83] Thus, it is possible that the partial loss of hypocretin neurons observed in genetically-intact mice in this and our previous study[37] is sufficient to decrease wakefulness and increase NREM sleep, but not enough to increase REM sleep. Increased REM sleep is only evident when hypocretin is completely absent, as in the HCRT KO mice.

The reduction in the number of very long wake bouts observed in control mice subjected to TBI in this present study suggests an inability to maintain long periods of wakefulness during their active period. This observations is consistent with the high rates of daytime napping observed in humans with TBI.[11,18]

Our results also generally agree with other research on sleep-wake behavior in animals and humans that lack hypocretin. Similar to other reports, HCRT KO mice in our current study exhibit less wakefulness,[83,84] more NREM sleep,[83] more REM sleep,[83,84,98] and fragmented sleep and wake bouts[99,100,85] under pre-injury baseline conditions as compared to genetically intact mice. Like HCRT KO mice, humans with narcolepsy exhibit more NREM sleep,[82] more REM sleep,[82] and more fragmented sleep-wake behavior.[82,101,102] The reduced wakefulness observed in HCRT KO mice during their active period may be analogous to the excessive daytime sleepiness observed in narcolepsy patients.[103–105]

Lastly, we found that numbers of hypocretin-positive cells in genetically intact mice subjected to TBI were reduced at the end of the study relative to sham-injured animals. These data extend findings from our previous work in which hypocretin neurons were significantly reduced by TBI at 7 and 15 days post-injury.[37] These new results suggest that hypocretin neurons are not recovered with time, at least within the one month post-injury period evaluated in this study. These results are consistent with previous human[44,45] and animal studies[41] that report reduced numbers of hypocretin neurons after TBI. Other aspects of hypocretin function also are altered by TBI. For example, hypocretin release[34] and hypocretin neuronal activation[38] are reduced after TBI. Why some studies show that hypocretin neurons disappear while others find that they are merely dysfunctional remains to be determined.

Hypocretin is an attractive therapeutic candidate because, in addition to sleep and wake disturbances, hypocretinergic dysfunction may also be involved in several other sequelae, including impaired cognition,[106] depression/depressive-like behavior,[41,107] and microglial dysregulation.[108,109] Hypocretin is implicated in memory processes[110,111] and hippocampal cell proliferation.[112] In humans[113] and animals,[114] low hypocretin is associated with depression or depressive-like behaviors. Hypocretin is also involved in microglial regulation, steering them towards a less inflammatory phenotype.[115] Thus, therapeutics that attempt to normalize hypocretin might address several behavioral and neuroinflammatory symptoms of TBI.

In conclusion, the present study is in agreement with our previous work, and that of others, which report TBI-induced alterations in sleep. TBI in this model decreases wakefulness, increases NREM sleep, decreases the length of wake bouts, and reduces the number of hypocretin neurons. We extend these observations by providing convincing evidence that hypocretin cell loss, or hypocretin dysfunction, is responsible for post-TBI sleep and wake disturbances. First, sleep-wake behavior of HCRT KO mice is not altered by TBI in this model. Second, although sleep-wake behavior of genetically-intact C57BL/6J mice differs from that of HCRT KO mice prior to injury, after TBI the

time spent in wake and NREM sleep does not differ, and the distribution of very long or very short wake bouts is the same between genotypes. Thus, as genetically-intact mice lose hypocretin cells after TBI, their sleep patterns become similar to HCRT KO mice. As such, the decrease in cell numbers, or change in cell function of hypocretin neurons that occurs after TBI appears to be necessary for the altered sleep-wake behavior observed in this model. Findings from this study indicate the hypocretinergic system is a major contributor to post-TBI sleep and wake disturbances and suggest that hypocretin agonists may be a useful therapeutic intervention for ameliorating these disturbances.

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Figures

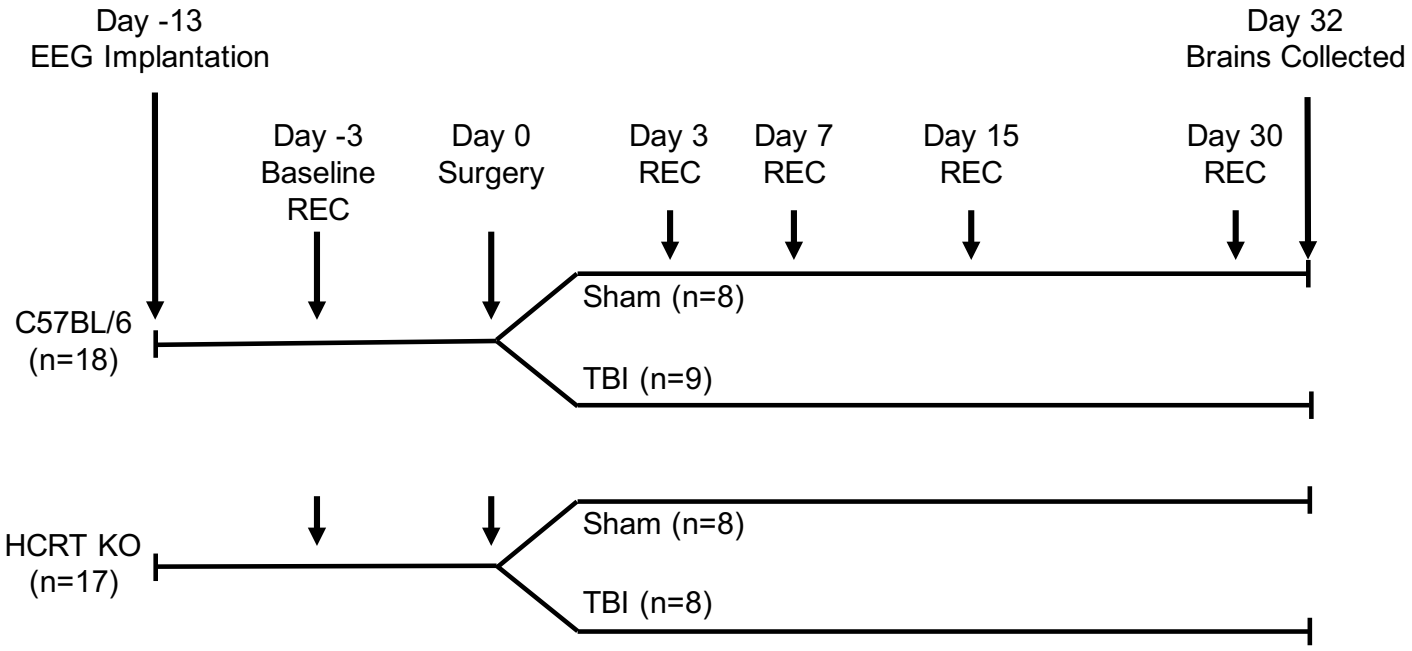


Figure 1: Schematic representation of the protocols used in the present study. C57BL/6J and hypocretin knockout mice were implanted with electroencephalogram (EEG) recording electrodes and allowed to recover. 48 h baseline EEG recordings were obtained from undisturbed mice and two mice were dropped from the study due to poor EEG signals. Mice of each genotype were randomized into either control (sham surgeries) or experimental (moderate TBI surgeries) groups. Moderate TBI was administered using the controlled cortical impact technique at a depth of 1.0 mm. EEG recordings were obtained from all four groups at 3, 7, 15, and 30 days post-surgery. After the last EEG recording (32 days post-surgery), animals were perfused and brains were removed from immunohistochemisitry (IHC).

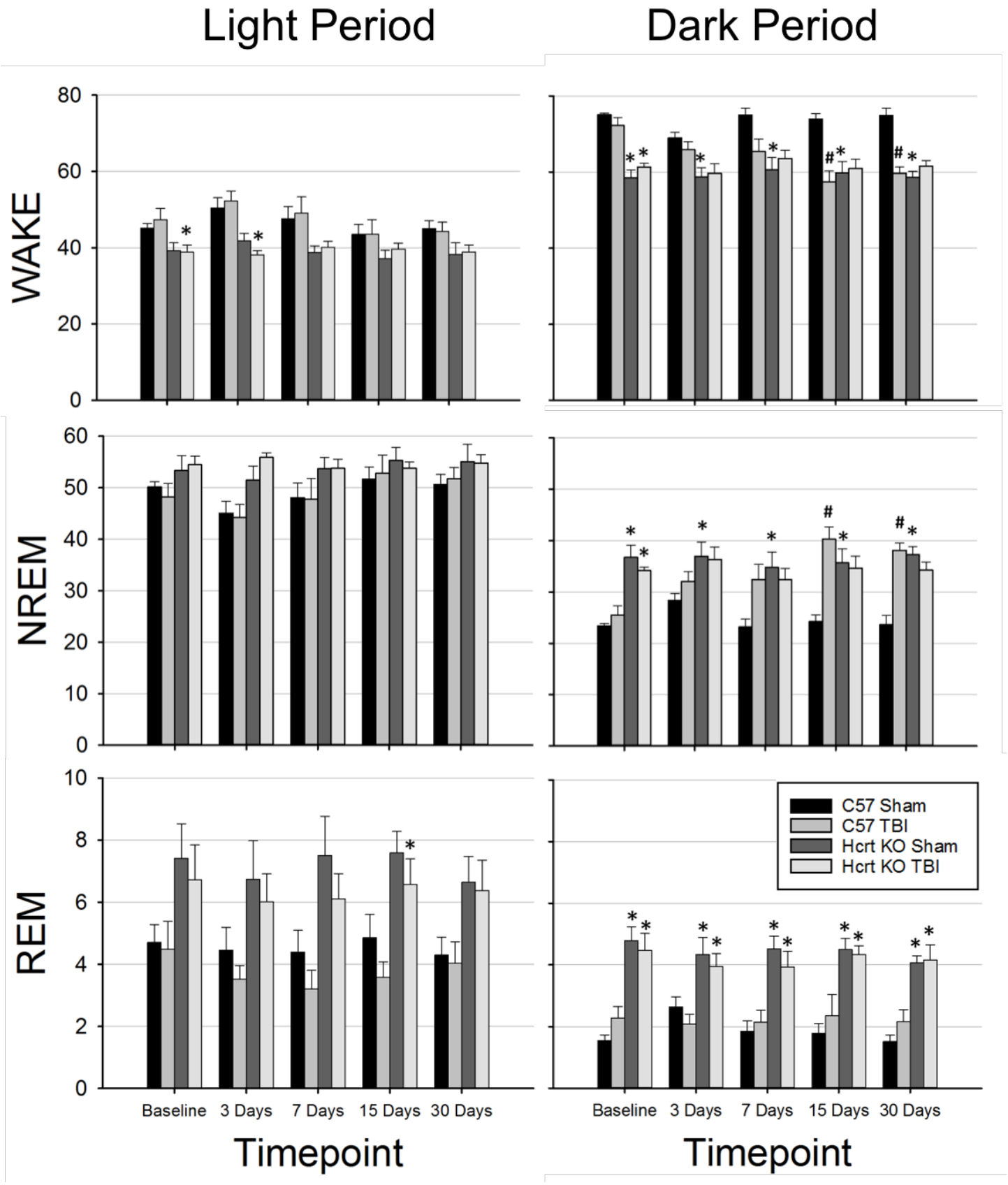


Figure 2: Traumatic brain injury decreases wakefulness and increases non-rapid eye movement sleep in C57BL/6J mice but not in hypocretin KO mice. EEG and activity recordings

were obtained from four groups of mice: sham surgery C57BL/6J mice (n=8), TBI surgery C57BL/6J mice (n=9), sham surgery hypocretin KO mice (n=8), and TBI surgery hypocretin KO mice (n=8). Percent of time spent in wakefulness (WAKE), non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep were determined during the 12 h light and 12 h dark period at baseline, 3 days post-surgery, 7 days post-surgery, 15 days post-surgery, and 30 days post surgery. Values are the mean (± SEM) percentages of time spent in each sleep or wake state. * indicates a statistically significant difference ($p < 0.05$) between genotypes (C57BL/6J vs. hypocretin KO) within the same condition. # indicates a statistically significant difference ($p < 0.05$) between conditions (sham vs. TBI) with the same genotype.

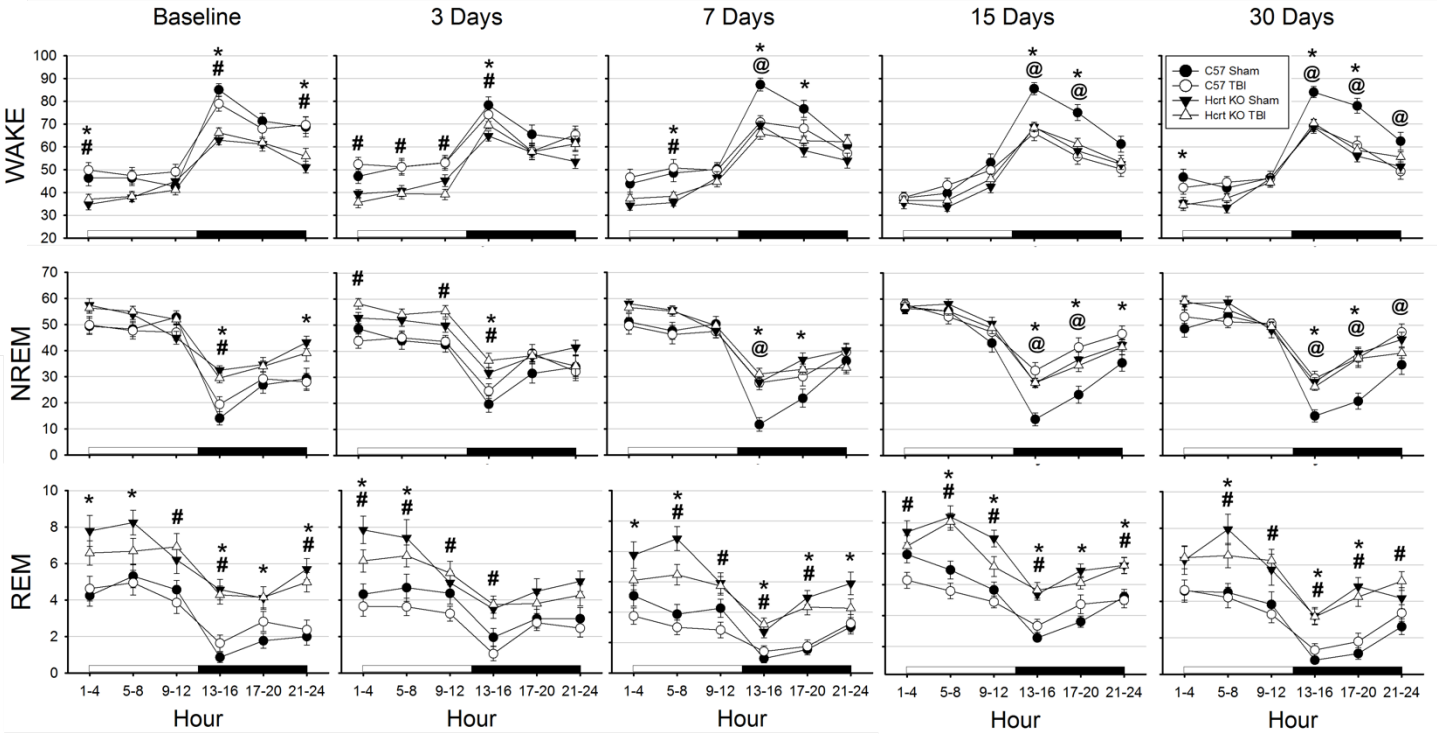


Figure 3: 24 h sleep-wake patterns are affected by genotype and by injury. Values are mean (± SEM) percent recording time spent in wakefulness (WAKE), non-rapid eye movement (NREM) sleep, or rapid eye movement (REM) sleep plotted in 4 hour blocks by sham surgery C57BL/6J mice (n=8), TBI surgery C57BL/6J mice (n=9), sham surgery hypocretin KO mice (n=8), and TBI surgery hypocretin KO mice (n=8). Baseline recordings were obtained from undisturbed animals prior to sham surgeries and surgical procedures to induce injury. Hours 1-12 are the light period and hours 13-24 are the dark period, indicated by open and closed bars on the graph. Statistical analyses were performed on 4 hour time blocks. * and # indicate a statistically significant difference ($p < 0.05$) between genotypes with conditions: * indicates a difference between C57BL/6J and hypocretin KO mice within the sham condition and # indicates a difference between C57BL/6J and hypocretin KO mice within the TBI condition. @ indicates a statistically significant difference ($p < 0.05$) between conditions (sham vs. TBI) within the C57BL/6J animals. There were no statistically significant differences between conditions with the hypocretin KO genotype at any timepoint evaluated.

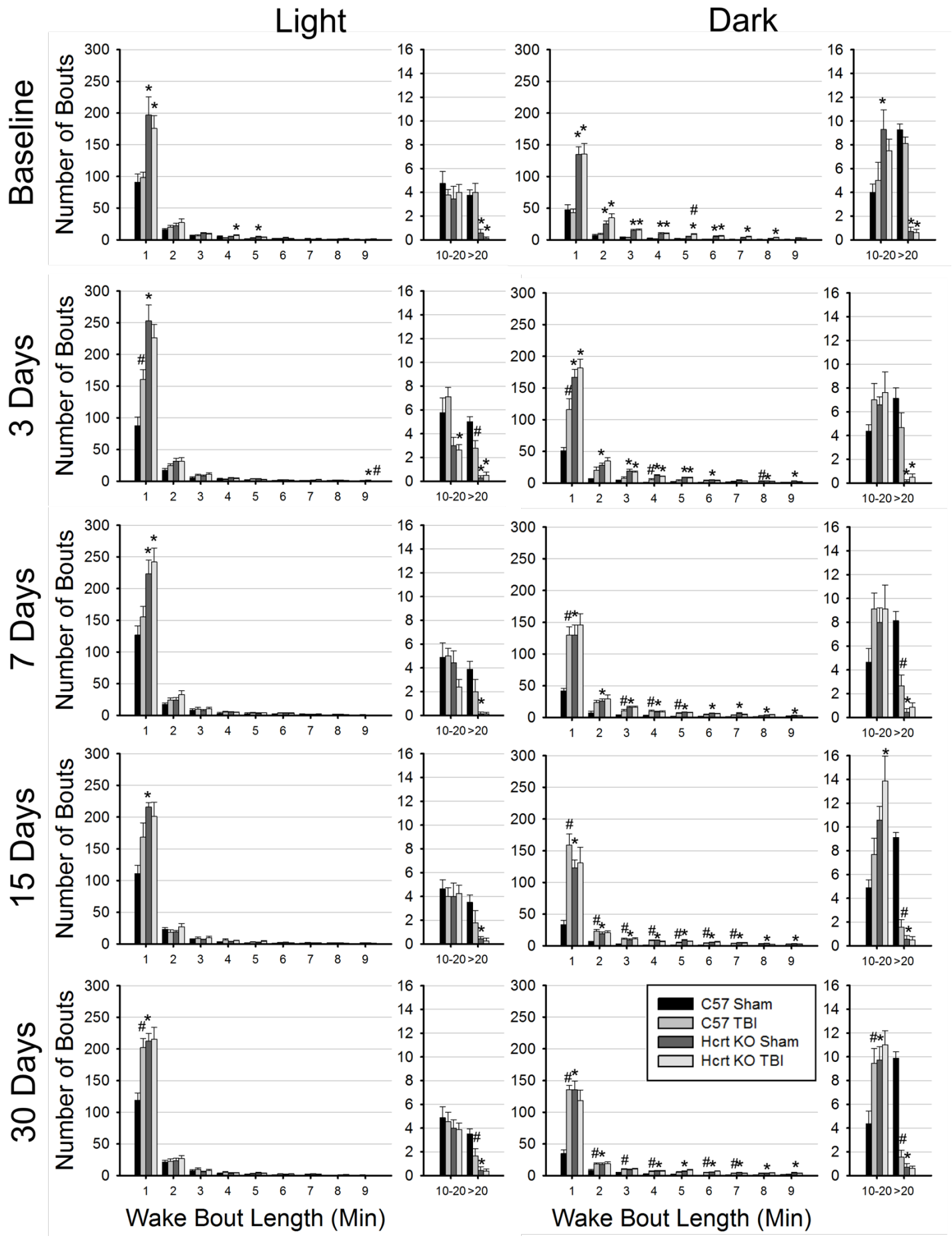
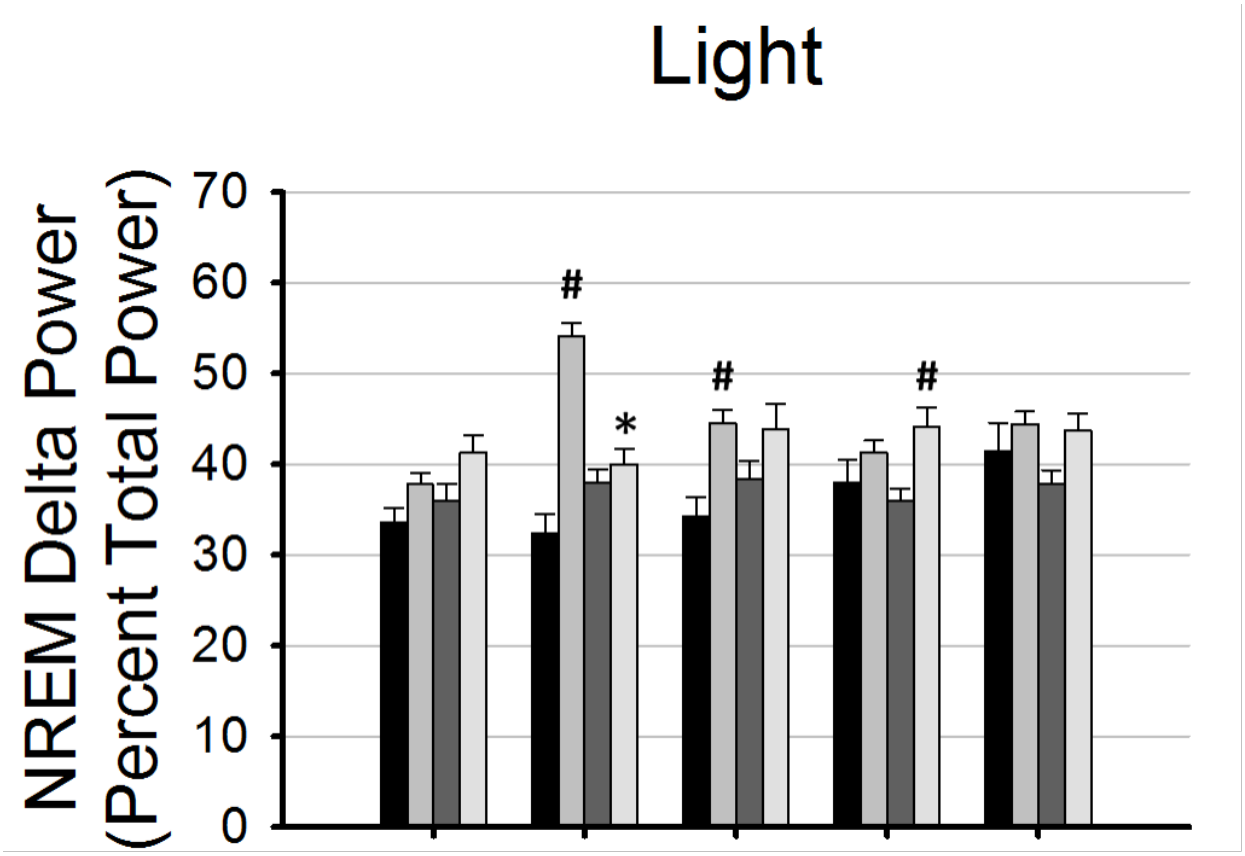

Light
Dark
Baseline
3 Days
7 Days
15 Days
30 Days
Number of Bouts
Wake Bout Length (Min)
10-20>20
C57 Sham
C57 TBI
Hcrt KO Sham
Hcrt KO TBI

Figure 4: Wake bout length is affected by traumatic brain injury in a genotype-dependent manner. Wake bouts were sorted into bins. Values are the mean (± SEM) number of bouts in each bin during the 12 h light and 12 h dark periods at baseline, 3 days post-surgery, 7 days post-surgery, 15 days post-surgery, and 30 days post surgery for sham surgery C57BL/6J mice (n=8), TBI surgery C57BL/6J mice (n=9), sham surgery hypocretin KO mice (n=8), and TBI surgery hypocretin KO mice (n=8). * indicates a statistically significant difference ($p < 0.05$) between genotypes (C57BL/6J vs. hypocretin KO) within the same condition. # indicates a statistically significant difference ($p < 0.05$) between conditions (sham vs. TBI) within the same genotype.


Light
NREM Delta Power
(Percent Total Power)
70
60
50
40
30
20
10
0
#
*
#
#

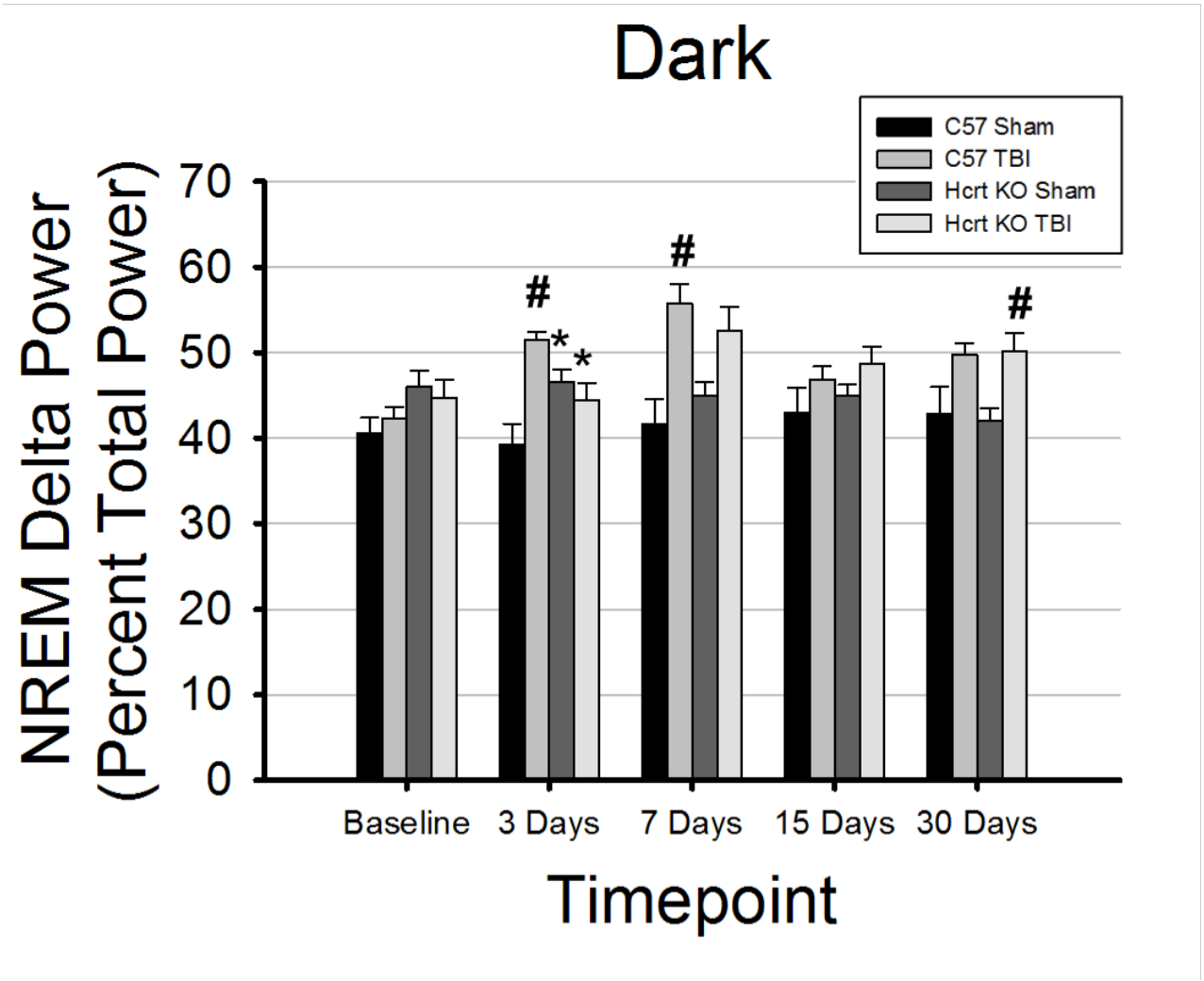

Dark
C57 Sham
C57 TBI
Hcrt KO Sham
Hcrt KO TBI
NREM Delta Power
(Percent Total Power)
70
60
50
40
30
20
10
0
#
*
*
#
#
Baseline
3 Days
7 Days
15 Days
30 Days
Timepoint

Figure 5: Traumatic brain injury transiently increases delta power during non-rapid eye movement sleep in C57BL/6J mice. Power in the electroencephalogram (EEG) delta frequency band (0.5 – 4.5 Hz) was determined during the light period and dark period from artifact-free state-specific epochs. Animals with the least artifact from each group were used: sham surgery C57BL/6J mice (n=5), TBI surgery C57BL/6J mice (n=5), sham surgery hypocretin KO mice (n=6), and TBI surgery hypocretin KO mice (n=6). Delta power was normalized as the percent of the total power and is plotted as mean ± SEM. For the combined graph, light period and dark period delta power values were used so that each animal yielded two data points for each timepoint (baseline, 3 days, etc.). * indicates a statistically significant difference ($p<0.05$) between genotypes within the same condition (sham vs. TBI) and # indicates a statistically significant difference ($p <0.05$) between conditions within the same genotype.

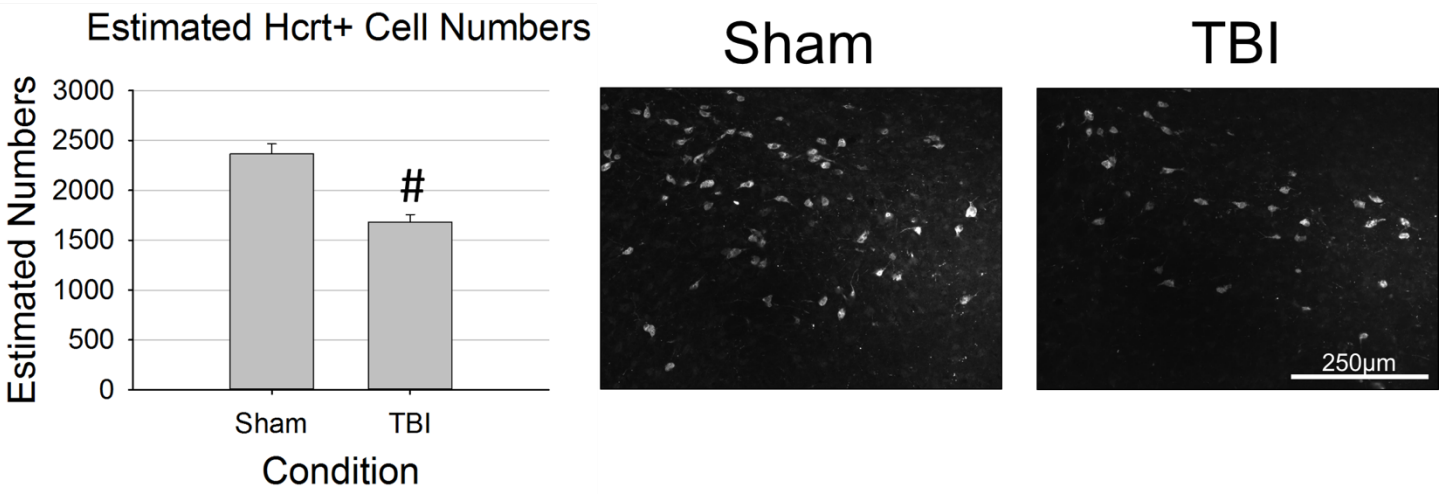


Figure 6: Traumatic brain injury reduces numbers of hypocretin neurons in C57BL/6J (C57) mice. Numbers of hypocretin neurons in the lateral hypothalamus ipsilateral to the injury site were estimated using unbiased stereology and the optical fractionator method. Values are means ± SEM obtained from C57BL/6J sham (n=8) and TBI (n=9) mice at 32 days post-surgery. # indicates a statistically significant difference ($p < 0.05$) between conditions (sham vs. TBI) within the same genotype. Representative photomicrographs were obtained from the lateral hypothalamus ipsilateral to the injury site using immunofluorescence.

Chapter 4: Discussion

Introduction

The findings presented here provide support for the involvement of hypocretin in mediating the effects of traumatic brain injury (TBI) on sleep-wake behavior. These studies used a well-established mouse model of TBI to examine the effects of TBI on sleep-wake behavior and neuronal populations that regulate sleep and wakefulness in wild type (C57BL/6) and hypocretin knockout (KO) mice. This final dissertation chapter will summarize the findings from chapters II and III and discuss the implications of these findings within the broader context of the research fields of sleep and TBI.

TBI, Hypocretin and Sleep-Wake Behavior

The experiments in chapter II and III indicate that TBI significantly alters sleep-wake behavior in wild-type mice. More specifically, moderate TBI increases non-rapid eye movement (NREM) sleep and decreases wakefulness during the active period. This is in agreement with animal studies showing similar changes in sleep macroarchitecture,[1,2] and with studies in humans showing increases in slow-wave sleep[3–6] and excessive daytime sleepiness, post-TBI.[3,7–11] TBI also destabilizes wakefulness: after TBI, animals had a significant increase in short bouts of wakefulness (one minute or less) accompanied by a decrease in long periods of wakefulness (greater than 20 minutes). This is in agreement with human [4,5,12,13] and animal[2,14–16] studies showing a reduction in the length of wakefulness bouts and fragmentation of sleep-wake states.

The experiments in chapter III indicate that these changes in sleep-wake behavior are hypocretin dependent: hypocretin knockout (KO) animals had no changes in sleep-wake behavior in response to TBI. However, at baseline, hypocretin KO animals had significantly more NREM sleep and less wakefulness during the active period than wild-type mice, as well as a skewed distribution of wakefulness bouts such that very short wake bouts were overrepresented. Thus, TBI caused wild-type mice to become more similar to hypocretin KO mice in terms of amounts of wakefulness and NREM sleep as well as in length of wakefulness bouts.

TBI and Arousal-Regulating Neurotransmitters

The experiments in chapter II and III indicate that TBI chronically reduces the population of hypocretin neurons. This is in agreement with other studies showing that TBI results in hypocretin cell loss[17,18,16] or dysfunction.[15,2] The reason why some models indicate that hypocretin cells are lost whereas in others they are merely dysfunctional is unknown and should be investigated in further experiments.

Other neurotransmitter populations were explored in chapter II as well. In agreement with previous studies, we found no change in the number of melanin-concentrating hormone (MCH) neurons[15] and a decrease in cholinergic neurons in the forebrain.[19,20] Interestingly, we found no change in histaminergic neurons in the tuberomammillary nucleus where others did.[1] Future studies should examine this disparity in histaminergic neuron survival and examine the contributions of basal forebrain cholinergic neurons to post-TBI pathology.

Methodological Considerations: Hypocretin KO vs. Hypocretin-Ataxin Mouse Models

The studies presented in this dissertation examined the role of hypocretin in post-TBI sleep-wake behavior using mice that do not produce hypocretin. There are two types of hypocretin lacking mice that have been created and widely studied. Each type has its own set of advantages and disadvantages.

Hypocretin knockout (KO) mice were used in the research presented here. These mice first appeared in a published paper in 1999[21] and have since been widely studied.[22–27] These mice lack the gene for the protein preprohypocretin, the precursor protein that is cleaved to form hypocretin-1 and hypocretin-2. Thus, these mice develop with an absence of hypocretin. The main concern regarding this mouse model is that a lack of hypocretin may cause the brains of these mice to develop abnormally. Although studies have examined the embryonic and early postnatal development

of the lateral hypothalamus,[28,29] to our knowledge no studies have examined the development of this brain region in hypocretin knockout animals.

The second type of hypocretin null animals are hypocretin-ataxin mice, which first appeared in a published paper in 2001.[30] These transgenic mice conditionally express ataxin-3 (a toxin which induces apoptosis) in hypocretin-producing neurons.[30] These hypocretin-ataxin mice are born with hypocretin-producing neurons, but these neurons gradually disappear until they are almost completely absent when mice are 15 weeks old.[30] These mice are a better model for human narcolepsy, in which individuals have normal hypocretin production throughout development, until hypocretin-producing neurons are lost, generally during adolescence or young adulthood. For our purposes, however, this model is less useful. In this model, the majority of hypocretin-producing neurons die – which is problematic as hypocretin neurons also produce other neurotransmitters like glutamate,[31] dynorphin,[32] neurotensin,[33] and leucine-enkephalin.[34] As we sought to specifically examine the role of hypocretin in post-TBI sleep-wake disturbance, the simultaneous decrease in these other neurotransmitters would have been a confound. It should be noted that a conditional hypocretin ablation model does exist.[35] However, this model also results in loss of the entire neuron (with all associated neurotransmitters), making it not ideal for our purposes.

Although few studies have directly compared these two models, most have found that they are quite similar in terms of energy balance abnormalities[36] and protein expression in the hypothalamus.[37] Nevertheless, it would be interesting to repeat the above experiments to determine if hypocretin KO and hypocretin-ataxin mice respond similarly to TBI.

Future Directions

Although the experiments described in this dissertation have provided some insight into the role of hypocretin in the development of post-TBI sleep-wake disturbances, there are still many unanswered questions that were beyond the scope of this dissertation. The role of hypocretin receptor subtypes in responses to TBI should be explored. Furthermore, the role of inflammation in

responses to TBI and its effects on hypocretin and sleep behavior should be more thoroughly examined. Lastly, the role of hypocretin in post-TBI sleep-wake disturbance has important implications for potential therapeutics which should be investigated.

Hypocretin Receptors

The present experiments have provided important evidence supporting the role of hypocretin in post-TBI sleep disorders. However, the hypocretin peptide-receptor system has many complexities that were beyond the scope of the present experiments. There are two different forms of hypocretin: hypocretin-1 and hypocretin-2. Both forms are cleaved from the protein preprohypocretin. Hypocretin knockout animals are generated by deleting the preprohypocretin gene, actually making them preprohypocretin knockouts.[21] To our knowledge, no animal models exist that lack only hypocretin-1 or hypocretin-2. Thus, these peptides have been studied mainly by administering hypocretin-1 or -2 and observing the effects. Many studies have shown that hypocretin-1 promotes wakefulness,[38–45] whereas none have specifically examined the role of hypocretin-2 in sleep-wake behavior to our knowledge. The majority of research has focused instead on the roles of the two types of hypocretin receptors. Hypocretin receptor 1 (HcrtR1) preferentially binds hypocretin-1, whereas hypocretin receptor 2 (HcrtR2) is bound by both hypocretin-1 and -2 with roughly equal affinity.[46] Both receptors are G-protein coupled receptors.[46]

The different hypocretin receptors seem to play slightly different, although complementary, roles in the regulation of sleep-wake behavior. Although antagonism of either HcrtR1[47] or HcrtR2[48,49] can promote sleep, antagonism of both HcrtR1 and R2 is more effective for the promotion of sleep than individual antagonism of R1 or R2 alone.[50] Interestingly, one study reported that antagonism of R2 selectively promotes NREM sleep, whereas antagonism of R1 and R2 promotes both NREM and REM, suggesting R1 may play a larger role in the regulation of REM sleep.[51] However, another study found that high doses of a different selective HcrtR2 antagonist increased both NREM and REM, so these effects may be compound- or dose-dependent.[49]

Additionally, the brain areas where these receptors are expressed are slightly different. Although there is significant overlap in many areas including most parts of the hippocampus and thalamus, the dorsal raphe nucleus, and ventral tegmental area, certain areas are distinct.[52] The prefrontal cortex and locus coeruleus contain mostly HcrtR1,[52] whereas the tuberomammillary nucleus contains mostly HcrtR2.[52] Furthermore, in areas important for the regulation of sleep and wake, the receptors seem to be expressed on different types of neurons: HcrtR1 was found on cholinergic neurons, whereas HcrtR2 was found on GABAergic interneurons.[53] Thus, although both receptor subtypes are involved in the promotion of wakefulness, they may contribute in different and complementary ways. Future studies could examine how HcrtR1 and R2 are affected by TBI and the roles that each receptor plays in post-TBI sleep-wake disturbance.

Complex Interactions Between Neuroinflammation, Sleep, and Hypocretin

Any study of TBI and sleep is inevitably complicated by the intricate and not fully understood relationship between sleep and neuroinflammation. TBI unquestionably results in several neuroinflammatory processes and there is a complex bidirectional relationship between neuroinflammation and sleep. Hypocretin also has a bidirectional relationship with neuroinflammation/cell damage, which has implications for sleep-wake behavior.

Briefly, traumatic brain injury sets off a cascade of predominately pro-inflammatory processes and a few anti-inflammatory processes. In the acute phase, there is a dramatic rise of pro-inflammatory cytokines, including interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNFα) in human patients and animal models.[54–57] Blocking the action of these proinflammatory cytokines, especially TNFα and IL-1β, seems to improve outcomes from TBI in animal models.[58–63] These cytokines are known to be involved in neuronal death processes.[64–66]

Inflammation occurs on a cellular level as well, in the form of activated astrocytes and microglia. Cellular inflammatory processes appear to be much longer lasting than the acute cytokine responses, persisting for weeks or even years.[67,68] The roles of activated astrocytes and microglia are

complex and not fully understood. Although the M1/M2 activation of microglia is no longer thought to be an appropriate distinction,[69] it is known that both microglia and astrocytes are capable of actions that are detrimental or beneficial to recovery from TBI.[70,71] Astrocytes can produce the anti-inflammatory agents transforming growth factor β (TGF-β) and interleukin-1 receptor antagonist (IL-1ra)[72,73] but also the inflammatory agents IL-1β, IL-8, and TNFα.[73,74] Similarly, microglia can produce anti-inflammatory agents like TGF-β and IL-10[72,75] as well as inflammatory agents such as interferon γ (IFNγ), IL-1β, and TNFα.[75,76]

These pro- and anti-inflammatory cytokines have complex effects on the structure and function of neurons as well as whole-organism behavior. Importantly, there is a bidirectional relationship between sleep and inflammation. Inflammatory cytokines promote NREM sleep in animal studies.[77–80] Conversely, sleep deprivation promotes cellular inflammation (astrocytosis and microgliosis)[81,82] and also increases levels of proinflammatory cytokines like TNFα and IL-1β.[83,84]

To underscore the interconnectedness of these processes, a bidirectional (and not fully understood) relationship also exists between inflammation and the hypocretinergic system. Inflammation reduces the activity of hypocretinergeric neurons and the production of hypocretin.[85–88] Conversely, hypocretin reduces neuroinflammation and steers microglia to a less inflammatory phenotype.[89–91]

Thus, it is possible that TBI could produce inflammation which downregulates the production of the anti-inflammatory hypocretin, permitting greater activation of inflammatory processes that are detrimental for recovery. Future experiments could investigate this in two ways. First, although the use of anti-inflammatory therapies improve neuronal survival after TBI,[63,92,93] it should be determined whether these therapies specifically improve the survival of hypocretin positive neurons. Secondly, the effect of hypocretin replacement on neuroinflammation after TBI should be examined.

Implications for the Development of Hypocretin Agonist Therapeutics

The evidence presented by others, and in this dissertation, provides support for the hypothesis that hypocretin dysfunction is a major underlying cause of post-TBI sleep-wake disturbance. This has important implications for the development of pharmacotherapies to treat post-TBI sleep-wake disturbance.

Hypocretin antagonists have been studied extensively for their ability to promote sleep.[94–100] Dual hypocretin receptor antagonists have been tested in clinical trials,[101,102] and Suvorexant (Belsomra) has been approved for the treatment of insomnia in the United States.[103]

Conversely, relatively little time has been devoted to the development of hypocretin agonists. Although selective hypocretin receptor 2 agonists do exist[104] and have shown promise in treating animal models of narcolepsy,[105] they have not been extensively evaluated even in preclinical models. Naturally, hypocretin itself serves as a potent agonist, but administration of hypocretin can be difficult. In animal models, hypocretin is very effective at promoting wakefulness when administered by intracerebroventricular (ICV) injection,[39–41,106] but this is not a viable method of administration for human patients.

Intravenous (IV) administration is more suitable for human treatment, but much less effective. Although hypocretin (a lipophilic peptide) can cross from the blood to the brain by diffusion,[107] animal studies show that IV administration has relatively minor effects on behavior.[108,109] Furthermore, as hypocretin receptors are expressed in many peripheral tissues including the gastrointestinal tract,[110,111] adipose tissue,[112] and adrenal glands,[113] systemic administration could have deleterious off-target effects.

Until an orally bioavailable hypocretin agonist is developed, intranasal administration of the hypocretin peptide may be the most promising method of administration. A study in rats using radiolabeled hypocretin peptide showed that intranasally administered hypocretin rapidly penetrated the brain with minimal systemic exposure.[114] Furthermore, animal studies of intranasal hypocretin administration indicate that this method increased activity levels and ameliorated the effects of sleep deprivation.[109,115] Promisingly, intranasal hypocretin ameliorates symptoms of narcolepsy in humans:

patients had fewer wake to REM sleep transitions, improved performance on tests of divided attention, and improved olfaction (olfactory dysfunction in narcoleptics is also thought to be due to loss of hypocretin). [116–118]

It is possible that hypocretin agonists have not been extensively explored due to the relatively small population of narcoleptics – only 25 to 50 per 100,000 people.[119] However, an estimated 5.3 million Americans suffer from a TBI-related disability –many of these including sleep-wake disorders.[120,121] If this much larger population of TBI patients could also be helped by hypocretinergic therapeutics, this would hopefully prompt more research on hypocretin agonists.

Hypocretin is a particularly interesting candidate for the treatment of TBI in that it may be able to address several post-TBI pathological changes, not just sleep-wake disturbance. Other common post-TBI symptoms include impaired cognition,[122] depression/depressive-like behavior,[123,16] and microglial dysregulation.[68,124] Importantly, a reduction in hypocretin may be involved in all of these sequelae. Hypocretin is important for cognition and memory processes[125,126] and hippocampal cell proliferation.[127] Additionally, in humans[128] and animals,[129] low hypocretin is associated with depression or depressive-like behaviors. Lastly, hypocretin is also involved in the regulation of microglia, reducing their proinflammatory output.[89] Thus, therapeutics that normalize hypocretin levels might address several behavioral and neuroinflammatory symptoms of TBI, not just sleep-wake disturbance.

Like any psychotropic agent, hypocretin agonists would not be completely without risk and appropriate dosage would have to be carefully determined. Ectopic overexpression of hypocretin leads to disordered sleep-wake behavior and incomplete atonia during REM sleep.[130] Furthermore, hypocretin plays a large role in reward-seeking behavior and thus in addiction.[131,132] Nevertheless, the potential benefits of hypocretin agonists for people with post-TBI sleep disorders or narcolepsy could be dramatic and would likely outweigh potential side effects.

Conclusion

The studies presented herein provide evidence for the involvement of hypocretin in post-TBI sleep-wake disturbance. To our knowledge, these experiments are the first to examine the effects of TBI in mice lacking hypocretin. These studies have important implications for the development of new hypocretin agonist therapeutics for treatment of TBI. Although post-TBI therapeutics have been extensively studied, they have limited efficacy, and millions of Americans still suffer from TBI-induced disability. This underscores our lack of a comprehensive picture of post-TBI diseases processes and the need for further research. Continuing study of the hypocretinergic system will be crucial for the understanding of post-TBI pathology, with important implications for post-TBI sleep-wake disturbance, and potentially also disturbances of mood and cognition.

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