2013

POST-TRAUMATIC SLEEP FOLLOWING DIFFUSE TRAUMATIC BRAIN INJURY

Rachel K. Rowe
University of Kentucky, rachel.rowe@uky.edu

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained and attached hereto needed written permission statements(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine).

I hereby grant to The University of Kentucky and its agents the non-exclusive license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless a preapproved embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's dissertation including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Rachel K. Rowe, Student

Dr. Jonathan Lifshitz, Major Professor

Dr. Wayne Cass, Director of Graduate Studies

POST-TRAUMATIC SLEEP FOLLOWING DIFFUSE TRAUMATIC BRAIN INJURY

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Rachel Kathleen Rowe

Lexington, Kentucky

Director: Dr. Jonathan Lifshitz, Faculty of Anatomy and Neurobiology

Lexington, KY

2013

ABSTRACT OF DISSERTATION

POST-TRAUMATIC SLEEP FOLLOWING DIFFUSE TRAUMATIC BRAIN INJURY

By
Rachel Kathleen Rowe

Traumatic brain injury (TBI) is a major cause of death and disability throughout the world with few pharmacological treatments available for individuals who suffer from neurological morbidities associated with TBI**.** Cellular and molecular pathological processes initiated at the time of injury develop into neurological impairments, with chronic sleep disorders (insomnia, hypersomnolence) being among the somatic, cognitive and emotional neurological impairments. Immediately post-injury, TBI patients report excessive daytime sleepiness, however, discordant opinions suggest that individuals should not be allowed to sleep or should be frequently awoken following brain injury. To provide adequate medical care, it is imperative to understand the role of acute post-traumatic sleep on the recovery of neurological function after TBI.

The aim of this thesis was to examine post-traumatic sleep after experimental TBI, defined as an increase in sleep during the first hours post-injury. In these studies, we non-invasively measured sleep activity following diffuse brain injury induced by midline fluid percussion injury to examine the architecture of post-traumatic sleep in mice. We detected significant injury-induced increases in acute sleep for six hours regardless of injury severity or time of day injury occurred. We found concurrent increases in cortical levels of the sleep promoting inflammatory cytokine interleukin 1-beta. We extended the timeline of post-injury sleep recording and found increases in post-traumatic sleep are distinctly acute with no changes in chronic sleep following diffuse TBI. Further, we investigated if post-traumatic sleep was beneficial to neurological outcome after brain-injury by disrupting post-traumatic sleep. Disruption of post-traumatic sleep did not worsen functional outcome (neuromotor, sensorimotor, cognition) at one week after diffuse TBI. With sufferers of TBI not always seeking medical attention, our final studies investigated over-the-counter analgesics and their effect on post-traumatic sleep and functional outcome. Acute administration of analgesics with varying anti-inflammatory properties had little effect on post-traumatic sleep and functional outcome.

Overall, these studies demonstrated translational potential and suggest sleep after a concussion is part of the natural recovery from injury. While disrupting sleep does not worsen outcome, it is in no way beneficial to recovery. Additionally, a single

analgesic dose for pain management following concussion plays little role in short term outcome.

KEYWORDS: TBI, sleep, sleep-disruption, inflammation, concussion

POST-TRAUMATIC SLEEP FOLLOWING DIFFUSE TRAUMATIC BRAIN INJURY

By

Rachel Kathleen Rowe

Dr. Jonathan Lifshitz
Director of Dissertation

Dr. Wayne A. Cass
Director of Graduate Studies

12/20/2013

Dedicated to my parents and sister

It's supposed to be hard; if it wasn't hard everyone would do it. The hard is what makes it great.

~A League of Their Own

Acknowledgments

Completion of the following dissertation would not have been possible without the ongoing encouragement, guidance, and assistance from my parents and my sister. Their love and unwavering support is the backbone of my accomplishments as a scientist. My mother shared her love for science by teaching me to ask questions, be curious, and explore nature. My father engrained in me the work ethic needed to finish the studies of this dissertation, and taught me how to be confident in myself and my capabilities. Lastly, my sister taught me to be a strong woman, and that quitting is never an option, regardless of how difficult the task at hand may seem.

The successes of the outlined studies would not have been possible without the guidance of my mentor Dr. Jonathan Lifshitz, who has played an instrumental role in shaping my scientific thinking. He helped me set reasonable goals for my experiments, while allowing me the necessary independence to learn, understand, and implement my own scientific thought. I am truly grateful for his mentorship throughout the years and look forward to collaborations on scientific endeavors throughout the upcoming years. Also, I would like to thank Dr. Bruce O'Hara, who has contributed extensive knowledge to these studies. Next, I wish to thank my co-principal investigator, Dr. Ed Hall, as well as my committee members, Dr. Linda Van Eldik, and Dr. Scott Livingston who provided insight, guided my research project, and also challenged my thinking along the way. I would like to thank Dr. Bradley Taylor for taking the time to be my outside examiner.

I need to thank Dr. Theresa Thomas for her nurturing and loving mentorship which kept me motivated to keep pressing forward. I am truly grateful for her wisdom and advice throughout the years. Most importantly, I would like to thank Jordan Harrison for his devotion to this project. He has shared with me many of the trials and tribulations encountered while completing these studies. His scientific contribution as well as his friendship and personal support truly made this work possible. He inspires me to be a great scientist and I look forward to future collaborations with him. Thanks to the past and present members of the Lifshitz lab, with a special thanks to Kelley Hall, for providing an enjoyable work environment. Lastly, thank you to Sean Murphy for showing me how to be a hard working scientist, how to stand up for myself, and for providing me with the emotional support needed to survive graduate school.

I am grateful to have been funded under the following grants: R01NS065052-S, R21NS072611, and KSCHIRT 10-5A.

Table of Contents

List of Tables

List of Figures

Chapter One: Introduction

Traumatic Brain Injury

Traumatic brain injury (TBI) is a major cause of death and disability throughout the world (Langlois et al. 2006; Reilly 2007; Roozenbeek et al. 2013). In the United States between 2002 and 2006, the Centers for Disease Control and Prevention estimated 52,000 deaths, 275,000 hospitalizations, and 1,365,000 emergency department visits resulting from TBI each year (Faul et al. 2010b). It is also estimated that as high as 42% of TBIs are not included in these statistics because 1.2-4.3 million survivors of mild TBI annually do not seek medical attention (Setnik and Bazarian 2007). TBI is a heterogeneous disorder leading to varying degrees of symptoms based on mode and severity of injury. Brain injury can lead to both short and long-term impairment, including cognitive (Albensi and Janigro 2003), and behavioral (Yeates et al. 2002) deficits as well as increasing the risk for developing neurodegenerative disease (Masel and DeWitt 2010), post-traumatic headaches (Theeler et al. 2013), and/or psychiatric disorders (Arciniegas et al. 2000). There is no approved pharmacological treatment for TBI and current medical care focuses primarily on controlling physiological parameters including intracranial pressure and blood pressure (Wang et al. 2006). Severity of TBI is categorized based on the Glasgow Coma Scale (GCS) which reliably classifies the severity of TBI based on clinical symptoms with a total GCS score classifying their injury as mild (score: 13-15), moderate (score: 9-12) or severe (score: <9) (Prins et al. 2013). The studies of this thesis will focus on mild (non-severe) brain injury.

There are two principal classifications for TBI delineated by injury mechanisms. Focal brain damage is caused by contact injury resulting in contusion and laceration and diffuse brain injury is caused by acceleration/deceleration injury resulting in diffuse axonal injury and swelling (Werner and Engelhard 2007). TBI is characterized by two pathological phases: cellular injury resulting from primary impact and the ensuing secondary injury mediated by pathological processes (Werner and Engelhard 2007). Secondary injury occurs over time post-injury with a gradual onset beginning minutes to hours after impact and contributes to the clinical morbidities associated with TBI. Little can be done to mitigate the mechanical disruption associated with the primary insult and the biochemical cascades initiated shortly after the time of injury can impair physiological function and ultimately worsen long-term outcome (Gentleman 1999).

The complex neurovascular responses after TBI require investigations that involve the immune, circulatory, and central nervous systems of live animals. The long term consequences of TBI include a host of emotional, cognitive, and sensory deficits that can degrade quality of life. Specific aspects of brain injury, such as cell death, have been successfully modeled with *in vitro* neural injury (Morrison et al. 1998; Geddes et al. 2003). However, *in vitro* models cannot be sustained over chronic time points to evaluate injury progression and lack the complex interactions among systems that characterize TBI neuropathy. Additionally, current computer models cannot reproduce the complicated pathophysiology of TBI. A wide range of well-accepted animal models are

available for neurotrauma investigation and the use of whole animal models is justified for TBI research and deemed appropriate for conduct of pre-clinical studies (Chen et al. 2008). Therefore, neurotrauma research necessitates live animal models of human TBI, which must be employed within the existing animal welfare regulatory environment. To study TBI pre-clinically, a range of experimental models of TBI are used in research differing in primary injury mechanisms. To generate injuries with characteristics of mild to severe TBI, the most commonly used animal models include fluid percussion (FP), controlled cortical impact (CCI), and weight-drop injury (Prins et al. 2013). While the initial impact in these models differ, all produce secondary injury mechanisms with characteristic physiological responses common to human TBI (Prins et al. 2013). The studies of this thesis utilize a fluid percussion animal model of diffuse brain injury to answer specific scientific questions.

Among secondary injury mechanisms, TBI depletes adenosine-5'-triphosphate (ATP) causing failure of energy dependent membrane ion pumps, increases reactive oxygen species (ROS), increases intracellular concentrations of free radicals caused by the activation of lipid peroxidases, and increases inflammatory mediating cytokines (Fan et al. 1995; Werner and Engelhard 2007). Fluid percussion injury decreases ATP levels in both the cortex and hippocampus of rats starting as early as two hours post-injury with declines in ATP levels remaining up to 24 hours post-injury (Lifshitz et al. 2003; Aoyama et al. 2008). An impact acceleration model of TBI has also shown decreases in ATP levels in rats immediately following brain injury (Signoretti et al. 2001). Similarly,

secondary injury mechanisms after brain injury increase oxidative stress through enhanced production of ROS, free radicals, and lipid peroxidation (Reimund 1994; Gopalakrishnan et al. 2004).

Neuroinflammatory cascades are also initiated as part of the secondary injury following TBI (Pleines et al. 2001). The resident macrophages of the central nervous system, microglia, respond immediately following brain injury and release inflammatory mediators which include inflammatory cytokines and chemokines (Davalos et al. 2005; Nimmerjahn et al. 2005; Bachstetter et al. 2013). Elevated cytokine signaling has been observed across experimental models and human TBI, highlighting their involvement in pathological and reparative processes triggered by injury (Morganti-Kossmann et al. 2001; Frugier et al. 2010; Semple et al. 2010; Ziebell and Morganti-Kossmann 2010).

Sleep Disturbances Following TBI

Secondary injuries of TBI, consequences of ongoing cellular events, often cause further damage and lead to physiological consequences (Werner and Engelhard 2007; Prins et al. 2013). Pathological processes initiated at the time of injury develop into neurological impairments, with sleep disturbances among the somatic, cognitive and emotional neurological impairments (Castriotta et al. 2007; Kempf et al. 2010). Among other neurological consequences of TBI, sleep disturbances are commonly reported in the acute phase of TBI, some of which persist through more chronic periods (Castriotta et al. 2007; Verma et al. 2007; Kempf et al. 2010). Published reports indicate an incidence as high as 70% of TBI survivors suffer from sleep-wake disturbances (Cohen et al. 1992; Orff et al.

2009). Similar sleep disorders develop across the spectrum of TBI, including children and adolescents (Tham et al. 2012), ultimately impacting the quality of life. Proper identification and treatment of sleep-wake disturbances following TBI can facilitate outcomes in vigilance, working memory, and capacity of language processing (Wiseman-Hakes et al. 2013).

Excessive daytime sleepiness is the most common sleep-wake disturbance reported among TBI patients (Castriotta et al. 2007; Kempf et al. 2010; Baumann 2012) and is characterized primarily by an increase in sleep propensity. Post-traumatic hypersomnia, an increased need in sleep over a 24 hour period, is also reported among the most common sleep-wake disturbances reported following TBI (Baumann 2012; Billiard and Podesta 2013). Other commonly reported disorders include narcolepsy, delayed sleep phase, insomnia, and fatigue (Ouellet and Morin 2006; Verma et al. 2007; Kempf et al. 2010; Baumann 2012). These sleep disturbances in TBI survivors make an impact on rehabilitation of patients after injury and can exacerbate symptoms such as pain and cognitive deficits (Mathias and Alvaro 2012; Bhalerao et al. 2013).

Functions of Sleep

Sleep is a state of immobility with reduced responsiveness, differing from coma or anesthesia by the ability to rapidly be reversed. While sleep is an evolutionarily conserved phenomenon that is essential for survival (Banks and Dinges 2007), the function of sleep is not completely understood. In the absence of sleep, however, there are significant detriments in cognitive function (Krueger

et al. 1999). Early hypotheses suggest the function of sleep is restoration, eliminating waste and restoring depleted energy sources (Oswald 1980), and energy conservation (Walker and Berger 1980; Xie et al. 2013). More recent hypotheses conclude sleep homeostasis reflects synaptic changes, highlighting a cellular need for sleep and indicate plasticity and learning as the major functions of sleep (Tononi and Cirelli 2003; Huber et al. 2004).

There are substantial variations in the amount of daily sleep required from mammal to mammal, with some animals demanding 18 to 20 hours of sleep and others needing a minimal 3 to 4 hours (Siegel 2005). Ecological studies suggest a correlation between a species' diet and sleep time and a link between body mass, brain size, and duration of cumulative sleep cycle (Zepelin et al. 2005). A possible explanation for this relationship is the inverse correlation of metabolic rate with both body and brain mass (Siegel 2005). High metabolic rates, linked to biochemical changes which modulate sleep, result in the generation of reactive oxygen species (ROS) by mitochondria, and sleep time may be a defense against this oxidative stress (Siegel 2005). The relationship between sleep and defense against oxidative damage has been outlined in studies showing sleep deprivation in the rat increases oxidative stress in the hippocampus, subcortical regions, and peripheral tissue (Eiland et al. 2002; Ramanathan et al. 2002; Everson et al. 2005; Siegel 2005).

Adenosine, a byproduct of energy metabolism is also a regulator of brain energy (Brown et al. 2012). During sleep, adenosine may serve as a homeostatic regulator of brain energy (Benington and Heller 1995; Scharf et al. 2008).

Wakefulness requires a higher metabolic rate than sleep, leading to increased levels of extracellular adenosine as a result of ATP breakdown (Pull and McIlwain 1972; Madsen and Vorstrup 1991; Maquet 1995). Sleep reduces neuronal activity which correlates to a surge in ATP (Dworak et al. 2010). Together, these studies suggest sleep-induced increases in ATP allow for increased anabolic processes during sleep, highlighting the restorative biosynthetic function of sleep (Dworak et al. 2010; Brown et al. 2012).

There is an accumulation of evidence supporting sleep plays a role in synaptic homeostasis, regulating the synaptic weight of the brain (for review, see (Tononi and Cirelli 2003; Tononi and Cirelli 2006)). During wakefulness there is synaptic potentiation in cortical circuits which accumulate as a net increase in synaptic weight, and it is hypothesized this accumulation of synaptic potentiation during wakefulness leads to increases in sleep (Tononi and Cirelli 2006). This is supported by animal studies which conclude increasing wakefulness in rats by gentle handling increases the expression of synaptic potentiation markers (Cirelli and Tononi 2000).

Lastly, sleep has a role in the modulation of the immune system. Sleep is coupled to immunological responses such as inflammation with observations suggesting sleep patterns may predict clinical outcome of disease processes (Toth 1995). Long periods of enhanced sleep in rabbits inoculated with *E. coli*, *S. aureus*, or *C. albicans* lead to a more favorable prognosis and less severe clinical signs when compared to a microbial challenge followed by short periods of enhanced sleep then prolonged sleep suppression (Toth and Krueger 1988; Toth

et al. 1993). Sleep promoting stimuli, such as cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF), expressed transiently after microbial challenge, likely contribute to the sleep enhancement observed during microbial infections (Toth 1995). Furthermore, inflammatory mediating cytokines produced *in vivo* by infection parallel the cytokines produced by secondary injury cascades following TBI (Chensue et al. 1991; Clark et al. 1991; Wakabayashi et al. 1991; Bjork et al. 1992; Ziebell and Morganti-Kossmann 2010). These data suggest sleep may naturally be promoted following brain injury.

Thesis Outline

While acute sleep disturbances are among one of the most commonly reported clinical neurological impairments following TBI, there is a lack of investigations into the duration, manipulation, and implications of this post-traumatic sleep. The overall goal of this thesis was to identify the role of post-traumatic sleep in recovery from diffuse brain injury. The overarching hypothesis was that diffuse brain injury leads to increases in acute sleep, and that manipulation of that sleep leads to worsened outcome.

To test the hypothesis that injury-induced sleep enhances recovery of neurological function following diffuse brain injury, the studies described in this dissertation used a novel non-invasive sleep monitoring cage system to gain insight into acute sleep immediately following TBI in the mouse. The most fascinating finding was that sleep is significantly increased following brain injury independent of injury severity or time of day injury occurs. Also, injury-induced increases in sleep extend into the first week post-injury but do not develop into

chronic sleep disturbances using our midline fluid percussion injury model. These data, in chapter two and three, are the first to provide insight into post-traumatic sleep following diffuse brain injury.

Chapter Four further explores acute post-traumatic sleep by investigating the relationship between immediate disruption of post-traumatic sleep and functional outcome following diffuse brain injury in the mouse. In summary, these studies were designed to disrupt the acute injury-induced increase in sleep observed in Chapter Two, and to elucidate the potential relationship between acute sleep and functional outcome following TBI. Taken together our results from these studies indicate there is a recovery of neurological function despite immediate sleep disruption following diffuse brain injury in the mouse.

Despite the large portion of mild TBI survivors not seeking medical attention (Setnik and Bazarian 2007) who likely self-medicate for post-traumatic headache, the role of over-the-counter (OTC) pain relief medicines in the course of brain injury is not completely understood. Finally, Chapter Five focused on acute OTC pharmacological intervention following TBI. We demonstrated that a one-time dose administered at the time of injury did not adversely affect behavioral outcome following diffuse TBI in the mouse. While there were drug-induced alterations of sleep profiles within the first 24 hours post-injury, changes in sleep did not result in changes in functional outcome. In addition, further implications of drug intervention and TBI outcome are covered in the Appendix.

Collectively, the data of these studies have a translational impact which will help inform clinical recommendations for the at-home treated population of mildly concussed TBI survivors. With TBI individuals not always seeking medical attention, it is important that standardized messages about acute at-home care are properly delivered following investigations into the nature of post-traumatic sleep. If sleep is restorative and promotes plasticity, then post-traumatic sleep may aid in recovery of function following injury, which could change the standards of care for brain-injured patients. The studies of this thesis are comprised of five manuscripts submitted (Chapter 3, Chapter 5) or accepted (Chapter 2, Chapter 4, Appendix) for publication in peer-reviewed journals.

Preface to Chapter Two

The aim of the experiments in Chapter Two was to investigate acute sleep following diffuse brain injury. Using non-invasive sleep monitoring cages significant increases in sleep were detected over the first six hours post-injury which were independent of both injury severity and time of injury. Injury-induced increases in cortical IL-1β were measured over the first nine hours post-injury. In conclusion, it was demonstrated that secondary injury cascades may contribute to the overall increase in acute sleep measured following experimental diffuse brain injury in the mouse.

Chapter Two: Diffuse Brain Injury Induces Acute Post-Traumatic Sleep

Summary

Clinical observations report excessive sleepiness immediately following TBI, however, there is a lack of experimental evidence to support or refute the benefit of sleep following a brain injury. The aim of this study is to investigate acute post-traumatic sleep. Sham, mild or moderate diffuse TBI was induced by midline fluid percussion injury (mFPI) in male C57BL/6J mice at 9:00 or 21:00 to evaluate injury-induced sleep behavior at sleep and wake onset, respectively. Sleep profiles were measured post-injury using a non-invasive, piezoelectric cage system. In separate cohorts of mice, inflammatory cytokines in the neocortex were quantified by immunoassay, and microglial activation was visualized by immunohistochemistry. Immediately after diffuse TBI, quantitative

measures of sleep were characterized by a significant increase in sleep (>50%) for the first 6 hours post-injury, resulting from increases in sleep bout length, compared to sham. Acute post-traumatic sleep increased significantly independent of injury severity and time of injury (9:00 vs 21:00). The pro-inflammatory cytokine IL-1β increased in brain-injured mice compared to sham over the first 9 hours post-injury. Iba-1 positive microglia were evident in brain-injured cortex at 6 hours post-injury. Post-traumatic sleep occurs for up to 6 hours after diffuse brain injury in the mouse regardless of injury severity or time-of-day. The temporal profile of secondary injury cascades may be driving the significant increase in post-traumatic sleep and contribute to the natural course of recovery through cellular repair.

Introduction

TBI is a major cause of death and disability throughout the world with little pharmacological treatment for the individuals who suffer from lifelong neurological morbidities associated with TBI. Brain injury can lead to both short and long-term impairment, including cognitive (Albensi and Janigro 2003), and behavioral (Yeates et al. 2002) deficits as well as increasing the risk for developing neurodegenerative disease (Masel and DeWitt 2010) and/or psychiatric disorders (Arciniegas et al. 2000). Little can be done to mitigate the mechanical disruption associated with the primary insult and the biochemical cascades initiated shortly after the time of injury can impair physiological function and ultimately worsen long-term outcome (Gentleman 1999).

Clinical studies have provided evidence to support the hypothesis that brain injury contributes to chronic sleep disturbances as well as leads to excessive daytime sleepiness (Baumann et al. 2007; Castriotta et al. 2007; Kempf et al. 2010; Baumann 2012). Far less is known about the acute relationship between TBI and sleep. Immediately after TBI, secondary injury mechanisms may impair physiological functions associated with the homeostatic regulation of sleep. For example, secondary injury processes result in glia activation and initiation of marked inflammatory responses. Injury-induced inflammation is mediated by the production of cytokines, such as the pro-inflammatory, cytokine interleukin-1 beta (IL-1β), which can have dual roles as sleep regulatory substances (SRSs) (Krueger and Majde 1995; Krueger et al. 2007). Elevated cytokine signaling has been observed across experimental models and human TBI, highlighting their involvement in pathological and reparative processes triggered by injury (Morganti-Kossmann et al. 2001; Frugier et al. 2010; Semple et al. 2010; Ziebell and Morganti-Kossmann 2010). However, cytokines which are SRSs can also modulate sleep-wake behavior, primarily enhancing sleep by acting on sleep circuits of the brain (Krueger et al. 2001a; Krueger et al. 2007).

Secondary injury mechanisms of TBI deplete ATP causing failure of energy-dependent membrane ion pumps, increase reactive oxygen species (ROS), increase intracellular concentrations of free radicals caused by activation of lipid peroxidases, as well as increase inflammatory mediating cytokines (Fan et al. 1995; Werner and Engelhard 2007). A low energy state, high ROS, and the

increase of certain cytokines have all been implicated in increased sleep propensity and sleep duration (Dworak et al. 2010; Chikahisa and Sei 2011). Thus, secondary injury processes following TBI have the potential to induce post-traumatic sleep. The magnitude and duration of the induction of post-traumatic sleep are unknown. Further, as these cellular processes continue, chronic sleep issues may develop.

The biological function of sleep remains controversial, however, prevailing hypotheses suggest the function of sleep is restorative, conservative, and adaptive (Tononi and Cirelli 2006; Chokroverty 2010). In the absence of sleep, humans exhibit deficits in attention, memory, learning, and higher cognitive processes (McCoy and Strecker 2011). Sleep is regulated by homeostatic processes as well as circadian processes (Borbely and Achermann 1999) such that injury may disrupt the signaling required to maintain a healthy sleep profile. If sleep is regenerative in function, then acute post-traumatic sleep may improve outcome from brain injury. This study is the first of its kind to investigate acute sleep following diffuse brain injury.

To characterize acute sleep patterns in brain-injured mice, a non-invasive sleep monitoring cage system was used to continuously record post-traumatic sleep. The sleep monitoring cage system allows reliable, immediate and continuous sleep monitoring by using piezoelectric materials configured as highly-sensitive pressure detectors incorporated into the bottom of the animal cage to determine rest-activity based on motion and breathing patterns (Donohue et al. 2008). Sleep is discriminated from wake every 2 seconds from tapered 8

second overlapping windows based on classification algorithms that exploit the limited mouse sleeping postures and distinct respiratory patterns consistent with sleep, compared to rest and activity (Flores et al. 2007; Donohue et al. 2008). This technology is also adapted to measure the polycyclic sleep pattern of mice, accounting for short interruptions and brief arousals in sleep. The cage system recognizes short bouts (a single sleep episode) lasting only seconds as well as extended bouts lasting longer than a minute.

Sleep can complicate the understanding of injury processes following TBI (Baumann 2012). The investigation of post-traumatic sleep may lead to rational interventions to mitigate damage. The present study was designed to examine injury-induced alterations in acute sleep following TBI. Clinical observations indicate that patients report excessive sleepiness immediately following TBI (Castriotta et al. 2007). In view of these observations, we hypothesized that diffuse brain injury would induce acute post-traumatic sleep in the mouse. The lack of biomedical research surrounding the controversial question whether one should sleep or be frequently awoken immediately following brain injury adds to the importance of investigating post-traumatic sleep, specifically in the acute period. In neurosurgical wards TBI patients are frequently awoken during the first day after injury to check for possible worsening of their consciousness, a condition that requires immediate action. In more mild conditions, the practice of keeping a brain-injured individual awake is controversial. Here we demonstrate significant increases in acute sleep post-injury regardless of injury severity or

time of day. Post-traumatic sleep occurs during the same time as increased cortical expression of SRS cytokines and inflammation.

Methods

Animals

Male C57BL/6J mice (Harlan Laboratories, Inc., Indianapolis, IN) were used for all experiments (n=75). The animals were housed in a 14 h light/10h dark cycle at a constant temperature (23°C ± 2° C) with food and water available ad libitum according to the Association for Assessment and Accreditation of Laboratory Animal Care International. Animals were acclimated to their environment following shipment for at least three days prior to any experiments. After surgery, animals were evaluated daily for post-operative care by a physical examination and documentation of each animal's condition. All studies were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC Protocol Number: 2007-0142). All surgery was performed under isoflurane anesthesia, and efforts were made to minimize suffering.

Midline Fluid Percussion Injury (mFPI)

Mice (20-24g) were subjected to midline fluid percussion injury (mFPI) consistent with methods previously described (Lifshitz 2008). Mice were anesthetized using 5% isoflurane in 100% oxygen for five minutes and the head of the animal was placed in a stereotaxic frame with continuously delivered isoflurane at 2.5% via nosecone. While anesthetized, the animal's body temperature was maintained using a Deltaphase® isothermal heating pad (Braintree Scientific Inc., Braintree, MA). A midline incision was made exposing

bregma and lambda, and fascia was removed from the surface of the skull. A trephine (3 mm outer diameter) was used for the craniotomy, centered on the sagittal suture between bregma and lambda without disruption of the dura. An injury cap prepared from the female portion of a Luer-Loc needle hub was fixed over the craniotomy using cyanoacrylate gel and methyl-methacrylate (Hygenic Corp., Akron, OH). The injury cap was closed using a Luer-Loc cap and mice were placed in a heated recovery cage and monitored until ambulatory before being returned to their sleep cage.

For injury induction 24 hours post-surgery, animals were re-anesthetized with 5% isoflurane delivered for five minutes. The cap was removed from the injury-hub assembly and the dura was visually inspected through the hub to confirm it was intact with no debris. The hub was then filled with normal saline and attached to a tube connected to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). An injury of moderate severity (1.4 atm) was administered by releasing the pendulum onto the fluid-filled cylinder. Sham-injured animals underwent the same procedure except the pendulum was not released. Animals were monitored for the presence of a forearm fencing response and righting reflex times were recorded for the injured animals as indicators of injury severity (Hosseini and Lifshitz 2009). The righting reflex time is the total time from the initial impact until the animal spontaneously rights itself from a supine position. The fencing response is a tonic posturing characterized by extension and flexion of opposite limbs that has been validated as an overt indicator of injury force magnitude

(Hosseini and Lifshitz 2009). The injury hub was removed and the brain was inspected for uniform herniation and integrity of the dura. The dura was intact in all animals, none were excluded as technical failures. The incision was cleaned using saline and closed with sutures. Moderate brain-injured animals had righting reflex recovery times greater than six minutes and a positive fencing response. Sham injured animals recovered from anesthesia within 20 seconds. After spontaneously righting, animals were placed in a heated recovery cage and monitored until ambulatory (approximately 5 to 15 minutes) before being returned to their sleep cage. Adequate measures were taken to minimize pain or discomfort. Following all surgical procedures the investigators were blinded to experimental groups to prevent bias in latter animal studies.

Sleep Recordings

The non-invasive sleep cage system (Signal Solutions, Lexington, KY) used in this study consisted of 16 separate units that could simultaneously monitor the sleep and wake states over several days. The cage system classified sleep and wake behavior based on methods previously described (Flores et al. 2007; Donohue et al. 2008). Each cage unit housed the mice individually with separate 18x18 centimeter walled compartments and attached food and water structures (Donohue et al. 2008). The cages had open bottoms that allowed them to be placed on a base with a Polyvinylidine Difluoride (PVDF) sensor on the cage floor (Donohue et al. 2008). The PVDF sensors were coupled to an input differential amplifier and pressure signals were generated and classified by the non-invasive high-throughput classifier as motions consistent with either wake

activity or the inactivity associated with sleep (Donohue et al. 2008). Sleep was characterized primarily by periodic pressure measurements with regular amplitudes, typical of respiration from a still mouse. In contrast, movements characteristic of wake were both the absence of the characteristic sleep signal and higher amplitude, irregular spiking associated with volitional movements (Figure 2.3). The piezoelectric signals were classified by the automated sleep scoring system in two second epochs as “sleep” or “wake”. The tapered segmentation window was advanced every two seconds and features associated with the characteristics just described were computed. A linear discriminant classifier based on these features was applied to assign a binary label given to each point associated with the center of the window (see dashed line in Figure 2.3) (Donohue et al. 2008). Data collected from the cage system were binned over specified time periods (e.g. 5 minutes, 1 hour) using a rolling average of the percent sleep, as well as binned by length of individual bouts of sleep and the median bout lengths were calculated.

Mice were acclimated to the cages and sensors were tested for 8 days prior to injury (Figure 2.1A). The animals were removed from their home cages for the midline craniotomy surgery and were placed back into their specific cage following the surgery. The following day the mice were removed from their home cage and were subjected to mFPI or sham injury. As soon as the mice were ambulatory (approximately 5 to 15 minutes), they were returned to their original sleep cage and the sleep recordings started to measure sleep continuously for 7 days (Donohue et al. 2008). Previous validation of the sleep cages in agreement

with human observation resulted in classification rates of 90% or higher (Donohue et al. 2008). This sleep cage system was a valid method for monitoring acute sleep in injured animals without confounding the injury. Placement of invasive EEG equipment for recordings can compromise the dura and adds external weight to the head of the rodent possibly exacerbating the brain-injury.

A two-way analysis of variance (ANOVA) including repeated measures was used to compare the mean percent sleep of brain-injured mice to uninjured shams over time followed by a Bonferroni post-hoc test. Statistical significance was assigned when $p < 0.05$.

Tissue preparation and cytokine measurement

At selected time points (1, 3, 9, 12, 24, 48, 168 hours) post-injury or sham operation, mice were given an overdose of sodium pentobarbital and transcardially perfused with phosphate buffered saline (PBS) (Figure 2.1A). Mice were decapitated and the brains were dissected on ice and snap frozen in liquid nitrogen then stored at -80° C until used. The protein levels of a panel of inflammatory cytokines were measured in the neocortex by Meso Scale Discovery (MSD) multiplex immunoassay (sector imager 2400, Meso Scale Discovery; Gaithersburg Maryland) (Figure 2.1B) as previously described (Bachstetter et al. 2011). Brain cortex was homogenized using high shear homogenizer (Omni TH115), in a 1:10 (w/v) of ice-cold lysis buffer consisting of PBS containing 1µg/ml Leupeptin, 1mM PMSF, and 1mM EDTA. The cortical homogenate was centrifuged at 14,000xg for 20 minutes at 4°C in a microcentrifuge. Fifty microliters of the resulting supernatant was loaded per well

of the custom MSD plate, and IL-1β levels were determined by MSD assay (Mouse Proinflammatory 7-Plex Ultra-Sensitive (K15012C)). IL-1β levels in the cortex were normalized to the total amount of protein in the sample loaded as determined by BCA Protein Assay (Pierce).

Cytokine levels of IL-1β were compared between uninjured sham and brain-injured mice. Increases in cytokine levels in the brain-injured mice were analyzed over a time course using a one-way ANOVA and selected comparisons were made using the Bonferroni post hoc test.

Tissue preparation for immunohistochemistry

At 6 hours post-injury or sham operation, mice were given an overdose of sodium pentobarbital and transcardially perfused with 4% paraformaldehyde after a (PBS) flush. Brains were removed and placed in 4% paraformaldehyde overnight. Brains were immersed in serial dilutions (10%, 20%, and 30%) of sucrose for 24 hours each. The brains were removed from the 30% sucrose and frozen at -20° C. After freezing, brains were sectioned in the coronal plane at 20 µm, mounted onto glass slides, and stored at -80° C.

IBA-1 immunohistochemistry

Slides were removed from -80° C, placed in an oven at 60° C for approximately 4 hours and then rinsed three times for 5 minutes each in PBS. Next, the slides were incubated in 4% goat serum blocking solution for 1 hour. The slides were incubated with the primary antibody (rabbit anti-ionized calcium binding adaptor molecule 1 (IBA-1), 1:1000, Wako Chemicals 0199-19741) and

stored at room temperature overnight. Slides were rinsed three times in PBS and the secondary antibody (biotinylated horse anti-rabbit, 1:250, Vector Laboratories) was added then slides were incubated on a rocker at room temperature for 1 hour. The slides were washed in PBS three times for 5 minutes each and tertiary stain was applied (streptavidin Alexa© Fluor 594, 1:1000, Jackson Laboratories) and slides were incubated for 1 hour at room temperature. Lastly, slides were rinsed three times in PBS and coverslipped with Fluoromount-G anti-fade medium (Southern Biotech). The cortex was examined for microglia activation in response to brain-injury using a Zeiss Axio Scope with attached digital camera.

Results

Diffuse TBI induces acute post-traumatic sleep in the mouse

Immediately after diffuse TBI, mean percent sleep was significantly increased in brain-injured animals compared to sham for the first 6 hours post-injury ($F(1, 45)=6.545$, $p=0.00007$) (Figure 2.2A). After 6 hours post-injury, the mean percent sleep of the injured mice (n=31) normalized and was indistinguishable from sleep in the sham (n=16) through 7 days post-injury (data not shown). A more detailed analysis was performed by calculating the mean percent sleep over five minute intervals for the first hour post-injury to examine the increase in sleep observed acutely post-injury. The mean percent sleep showed a significant time-dependent increase in sleep over the first hour post-injury ($F(11,495)=8.22$, $p<0.0001$) (Figure 2.2B) until maximum sleep was reached. In addition, analysis over the first hour-post injury showed a significant

group effect ($F(1,45)=37.00$, $p<0.0001$) (Figure 2.2B) indicating TBI induced a significant increase in mean percent sleep compared to the uninjured sham. Increase in acute post-traumatic sleep in the diffuse brain-injured mouse was associated with increased median bout lengths of sleep (Figure 2.2C). We observed a significant increase in the median bout length of brain-injured mice compared to sham for the first 4 hours post-injury ($F(1,45)=2.9138$, $p=0.032$). This increase in bout length indicated that the increase in mean percent sleep observed acutely post-injury (Figure 2.2A) could result from mice sleeping for longer durations during each bout, as opposed to sleeping more bouts after diffuse TBI.

Post-traumatic sleep bouts are interrupted by volitional movement and arousal similar to uninjured shams

Immediately after diffuse TBI, mean percent sleep was significantly increased in brain-injured mice compared to uninjured sham. To analyze the signals used to classify sleep, the raw piezoelectric sensor data was extracted and compared between brain-injured and sham mice within the first hour post-injury. Uninjured sham mice showed a periodic rhythm associated with the motion of breathing, approximately 3 Hz, with regular amplitude typical of sleep in the mouse (Figure 2.3A). These sleep bouts were interrupted by higher frequency and amplitude signals that correspond to movements consistent with wake activity (Figure 2.3A). The sleep-wake classifier plotted with the decision threshold (Figure 3, below raw signal) were classified as sleep activity above the threshold and as wake activity below the threshold. In brain-injured mice, sleep

activity showed similar rhythmic breathing classified as sleep (Figure 2.3B). As in the uninjured sham, sleep was interrupted by high amplitude and frequency signals corresponding to volitional movement. Interruptions of sleep bouts by volitional movement indicate the brain-injured animals terminate sleep bouts in a similar manner to uninjured mice, suggesting that brain-injured mice are responsive, capable of movement, and not in a comatose state of unresponsiveness. As shown, sleep bouts in brain-injured mice are longer in duration than in uninjured mice (Figure 2.3B).

Increase in acute post-traumatic sleep in the diffuse brain-injured mouse was independent of injury time of day

Sham or brain injury was administered at transitional time points (9:00 or 21:00) in the light/dark cycle (Figure 2.1A). We observed an increase in the mean percent sleep of brain-injured mice compared to sham when mice were injured at 9:00, following the onset of the light cycle (Figure 2.4A). Brain-injury resulted in a significant increase in mean percent sleep for the first 3 hours following injury as compared to the mean percent sleep of sham ($F(1,25)=15.95$, $p=0.0005$). Similarly, we observed an increase in post-traumatic sleep when mice were subjected to injury at 21:00, following the onset of the dark cycle (Figure 2.4B). We recorded a significant increase in mean percent sleep for the first 3 hours following brain-injury compared to uninjured shams ($F(1,17)=4.42$, $p=0.0506$). Regardless of injury time of day, post-traumatic sleep was increased to comparable levels (45-65%) and became indistinguishable from sleep in the sham after 3 hours post-injury. In contrast, diurnal pressures associated with the

change in the light/dark cycle were evident on the mean percent sleep of uninjured sham animals, as expected. The mean percent sleep of uninjured sham mice in the 9:00 group was significantly higher than the mean percent sleep of sham mice in the 21:00 group ($F(1,15)=6.303$, $p=0.0240$). This finding is representative of the nocturnal activity of mice.

Increase in acute post-traumatic sleep in the diffuse brain-injured mouse was independent of injury severity

Two levels of experimental injury severity were used to test the effects of injury severity on post-traumatic sleep. We define injury severity as mild (0.8 atm) and moderate (1.2-1.3 atm) according to the righting-reflex and the fencing response (see methods). Post-traumatic sleep was not significantly different between mild and moderate brain-injured mice (Figure 2.5). A significant increase in post-traumatic sleep was observed acutely following both mild and moderate injury compared to the uninjured sham ($F(2,44)=3.4773$, $p=0.00037$). No significant difference was found between mild brain-injured mice and moderate brain-injured mice, indicating the significant increase in post-traumatic sleep is independent of injury severity.

Secondary injury responses temporally associate with the increase in acute post-traumatic sleep in the diffuse brain-injured mouse

Following brain injury there is an up-regulation of pro-inflammatory cytokines (Helmy et al. 2011) that include IL-1β (Fan et al. 1995; Frugier et al. 2010). IL-1β is a cytokine with sleep regulatory substance activity (Krueger and Majde 1995; Fang et al. 1998; Krueger et al. 2001a) which could partially explain

post-traumatic sleep. A temporal profile of IL-1β indicated that cortical levels increased rapidly following moderate injury as compared to uninjured sham (Figure 2.6A). Levels of IL-1β peak at or near 9 hours post-injury and return to baseline levels by 12 hours post-injury. There was a significant increase in IL-1β levels in brain-injured animals compared to sham ($F(7,21)=6.474$, $p=0.0004$) and selected comparisons using the Bonferroni post-hoc analysis indicated a significant increase between sham and brain-injured mice at 1, 3 and 9 hours post-injury.

Microglia morphology, an indicator of microglia activation, was examined after diffuse brain injury in the mouse using Iba-1 immunohistochemistry. Iba-1 labels all microglia, however, distinct morphological differences in Iba-1 stained microglia were observed in brain-injured (mild and moderate injury, 09:00, Figure 2.6 C,D) compared to uninjured (Figure 2.6B) cortex at 6 hours post-injury. Microglia in brain-injured cortex showed morphologies consistent with activated microglia, including amoeboid cell bodies with thick, densely labeled processes (denoted by arrowheads). In contrast, thin, highly ramified processes of ramified microglia (denoted by arrows) were present in the uninjured sham cortex.

Discussion

Brain injury survivors report varying degrees of sleep disturbances (Orff et al. 2009), however, the contribution of acute post-traumatic sleep to the injury itself remains unclear. To achieve this long-term goal, we undertook the present study to measure the acute sleep response to diffuse TBI, which we term post-traumatic sleep. We chose to focus on acute sleep post-injury, because sleep

itself may be restorative and aid in the recovery of function following injury. By non-invasively recording sleep immediately following diffuse brain injury, we were able to document the induction of post-traumatic sleep. Altogether, our data, for the first time, support the hypothesis that diffuse brain injury promotes acute post-traumatic sleep in the mouse, and secondary injury related cellular processes coincide with this increase.

Current sleep research associated with TBI has focused on chronic sleep disorders in the sequelae of human injury (Verma et al. 2007; Orff et al. 2009; Boone et al. 2012). The lack of studies investigating acute sleep following TBI heightens the importance of studies in this field, because evidence promoting or disrupting sleep after TBI may change the standard of care for brain-injury patients. By investigating the role of post-traumatic sleep, interventions can be developed to mitigate damage. This study used a non-invasive sleep monitoring cage system which reliably measures injury-induced alterations in sleep (Donohue et al. 2008) immediately following injury. Without the surgical procedures required for EEG recordings, we avoided the contraindications of an electrode in the brain at the time of injury which would create contusion or cavitation and most importantly allowed for post-injury sleep to be measured within minutes of the initial injury. The piezoelectric sensor cages, in conjunction with computer algorithms, recorded the sleep of each individual mouse (injury and sham) and created a detailed sleep profile that included mean percent sleep and median bout length. The cage system allows for sleep profiles of brain-injured and sham mice to be measured in exactly the same way. We found that

post-traumatic sleep was significantly increased after brain injury, regardless of time of day or injury severity, with longer median sleep bouts underlying the overall increased post-traumatic sleep.

Despite the limitation of not being able to discriminate stages of sleep (rapid-eye-movement-sleep, slow wave sleep), the piezoelectric cage system is capable of accurately distinguishing wake from sleep (Flores et al. 2007). While the sleep cage system has been well validated in distinguishing sleep from wake with comparisons between EEG/EMG and human observation in normal mice (Donohue et al. 2008), it is possible that sleep could be over-estimated in TBI mice. If this were true, we would expect percent sleep times to be greatest in the first hour after injury and for moderate TBI to show a greater sleep increase than mild TBI. Since TBI mice were able to make postural adjustments and voluntary movements that signal wake, post-traumatic sleep is likely to be sleep, rather than a more severe condition. While the post-traumatic sleep bears many hallmarks of normal sleep, we cannot rule out that the increased sleep time is in part due to non-convulsive seizures with behavioral and temporal dynamics similar to sleep. We discount non-convulsive status epilepticus (NCSE) as a component of post-traumatic sleep because the development of epilepsy following experimental brain injury does not occur within the first week post-injury (D'Ambrosio et al. 2004). Following severe lateral fluid percussion in rats, EEG recordings indicated no injury-induced seizure within the first six hours post-injury (Kharatishvili et al. 2006). Furthermore, EEG/EMG signal features used to determine sleep stages in normal mice may be compromised after brain injury

and affect sleep-wake scoring. It should also be noted EEG/EMG recordings can result in false positive or negative sleep determination leading to possible error when used as the sole arbiter of sleep. For the purposes of this study, sleep determination did not rely fundamentally on EEG/EMG measures, but more simply is associated with a reversible perceptual disengagement from the environment, marked by a suspension of voluntary bodily functions. Ongoing work with the sleep cage system suggests that signal processing can distinguish REM from NREM sleep based on more irregular breathing during REM stages. Future work will determine whether the increases we observed in post-traumatic sleep arise from increases in REM or NREM sleep, or most likely from increases in both.

In order to explore the impact of diffuse TBI on natural sleep, mice were subjected to injury at two time points in their circadian rhythms. By conducting the injuries at the light/dark transition, we investigated whether post-traumatic sleep was a result of the brain injury or an interaction with natural biological tendencies to sleep. The 9:00 time point followed the onset of the light cycle, a time when nocturnal mice were expected to sleep. The 21:00 time point followed the onset of the dark cycle, a time when nocturnal mice are most active. Sleep patterns of uninjured mice showed circadian related pressures. Acute post-traumatic sleep significantly increased in comparison to the sleep of uninjured shams independent of the time of day mice were subjected to injury. This degree of increase in sleep following TBI is similar to the mean percent sleep of mice following 6 hours of sleep deprivation (Huber et al. 2000). It is possible that the

circadian clock itself or its outputs are dysregulated by TBI (Boone et al. 2012), which would contribute to injury-induced sleep being independent of the time at which the injury occurs. However, immediate permanent pathology is unlikely, as sleep parameters return to sham levels beyond 6 hours post-injury.

We also examined the relationship between injury severity and post-traumatic sleep. Severity of the initial injury is considered a major determining factor for magnitude of secondary injury processes and outcome following TBI (Curry et al. 2011), which led to the hypothesis that injury severity would directly impact post-traumatic sleep. Contrary to our hypothesis, both mildly and moderately brain-injured mice showed similar significant increases in post-traumatic sleep compared to sham values. Even mild injury significantly increased sleep, which urges continued investigation into the contribution of post-traumatic sleep to the natural course of the injury. The possibility exists to induce an even milder injury, which may have less impact on post-traumatic sleep; however this may reduce/eliminate all other cellular hallmarks of TBI as well. Unfortunately, because mildly and moderately injured mice have comparable increases in acute post-traumatic sleep, the utility of sleep to serve as a diagnostic biomarker for injury severity is limited.

Our data also exclude the possibility of an injury-induced coma, as an extreme manifestation of sleep. Severe TBI can lead to coma, however, our brain-injured mice exhibit a brief period of non-responsiveness measured by the suppression of the righting reflex. Also, the maximum median bout length, measured in seconds, was 30 seconds, followed by periods of wake activity,

which excludes the possibility of an injury-induced coma since mice voluntarily woke between sleep bouts. These periods of wake activity during the interbout interval between sleep activity were clearly shown by the piezoelectric sensor data.

TBI is characterized by two pathological phases: cellular injury resulting from primary impact and the ensuing secondary injury mediated by pathological processes (Werner and Engelhard 2007). Secondary injury occurs over time post-injury with a more gradual onset beginning minutes to hours after impact and contributes to the clinical morbidities associated with TBI. Post-traumatic sleep in 5 minute intervals showed that the increase in mean percent sleep over the first hour post-injury is time dependent (Figure 2.2B). If the primary impact solely contributed to post-traumatic sleep, then an immediate increase in post-traumatic sleep to a maximum level would have been observed. The secondary injury cascades that play a role in inducing sleep following diffuse TBI likely include post-traumatic signaling that activate glia, as evidenced by increased production of pro-inflammatory cytokines, such as IL-1β, in both animal models and human head injury patients (Fan et al. 1995; Helmy et al. 2011).

Activated microglia can contribute to the production of IL-1β after TBI. Once produced, IL-1β acts locally to affect neuronal assemblies, altering their functional status, as well as acting on sleep regulatory circuits (Krueger et al. 2007). Our group has accumulated evidence for circuit disruption, dismantling and reorganization in the diffuse-injured cortex, particularly the whisker sensory circuit of the rat (Lifshitz et al. 2007; Hall and Lifshitz 2010; McNamara et al.

2010; Cao et al. 2012; Lifshitz and Lisembee 2012). Microglia likely act as the effectors of circuit disruption (Cao et al. 2012) by producing cytokines and as a consequence influence the functional state of those circuits. The impact of microglia can then extend beyond local circuits to sleep regulatory circuits and ultimately induce sleep (Krueger and Majde 1995; Krueger et al. 2001a; Krueger 2008). In the injured mouse brain, these microglial signaling processes that influence sleep last 6 hours post-injury and remain to be determined in the human condition.

Our data show that IL-1β was upregulated in the cortex following diffuse TBI, and previous studies have reported that humans undergoing IL-1β therapy report excessive sleepiness (Krueger et al. 2007). Injections of IL-1β enhance NREM sleep (Krueger et al. 2001a) and application of IL-1β to the somatosensory cortex leads to enhanced EEG delta wave activity (Yasuda et al. 2005). These data indicate a mechanistic link between IL-1β and sleep. In the injured cortex, IL-1β continues to increase, peaking at or near 9 hours post-injury and returning to sham levels by 12 hours post-injury (Figure 2.6A), similar to the increase in mean percent sleep in brain-injured mice. Collectively, these data suggest that post-traumatic sleep may involve inflammatory mediated processes and the upregulation of pro-inflammatory cytokines that can act as sleep regulatory substances. Our immunohistological staining indicated activation of microglia in the cortex of mild and moderate brain-injured mice compared to uninjured sham at 6 hours post-injury, coinciding with elevated cytokine levels in moderate injury and the end of post-traumatic sleep. Activated microglia produce

pro-inflammatory cytokines, including those with dual roles as SRSs (IL-1β, IL-6, TNFα) (Wisor et al. 2011), as indicated by pharmacological inhibition of microglia reducing levels of pro-inflammatory cytokines. The infiltration and activation of microglia may be a potential source of sleep regulatory factors in the injured brain (Alder et al. 2011; Cao et al. 2012; Jin et al. 2012), regardless of injury severity. We argue that increases in sleep following TBI may result from the inflammatory response associated with the secondary injury in which elevated cytokine levels are associated with activation of microglia after brain injury. Future studies are needed to examine the mechanistic relationship between changes in cytokine levels and sleep.

Conclusion

The current study demonstrated that acute sleep was increased following diffuse TBI, and injury-induced cellular cascades may contribute to this increase. The increase in sleep was independent of time of day that the injury occurred and the injury severity. Increases in median bout length contributed to the overall increase in sleep observed post-injury. Further studies need to determine the cellular benefit or detriment of acute post-traumatic sleep on recovery following TBI (and other neurological conditions) by disrupting acute sleep. Understanding the role of acute post-traumatic sleep on outcome can begin to answer the controversial question, “Should one sleep, be frequently awoken or left uninterrupted after a concussion?”

Chapter Two has been accepted for publication in the following manuscript:
Rowe RK, Striz M, Bachstetter AD, Van Eldik LJ, Donohue KD, O'Hara BF, and Lifshitz J. (2013). Diffuse brain injury induces acute post-traumatic sleep. PLOS ONE

Chapter Two: Figures

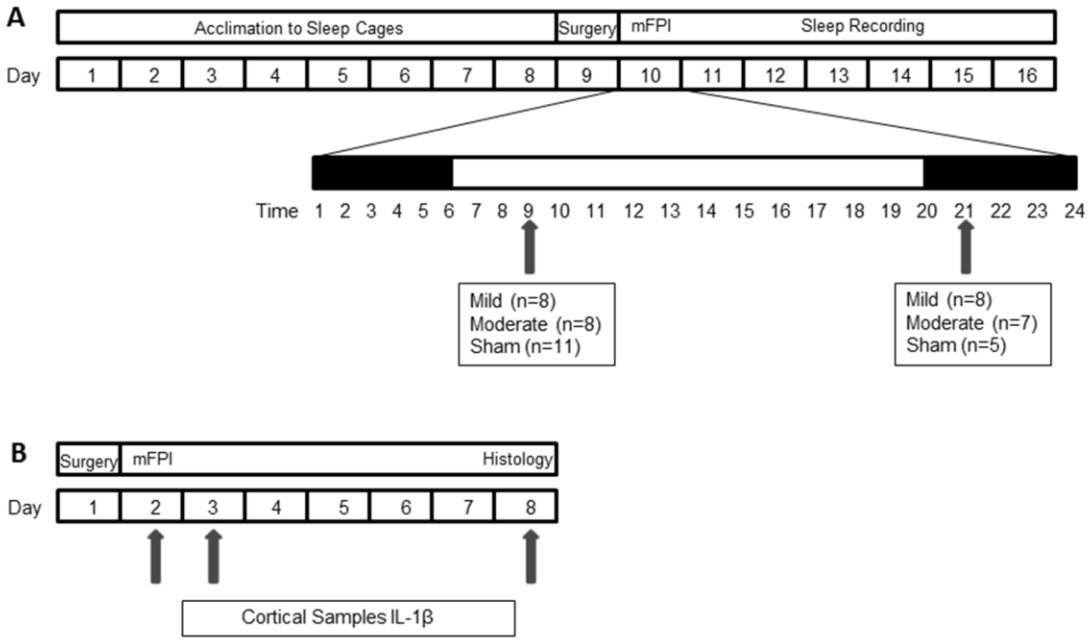
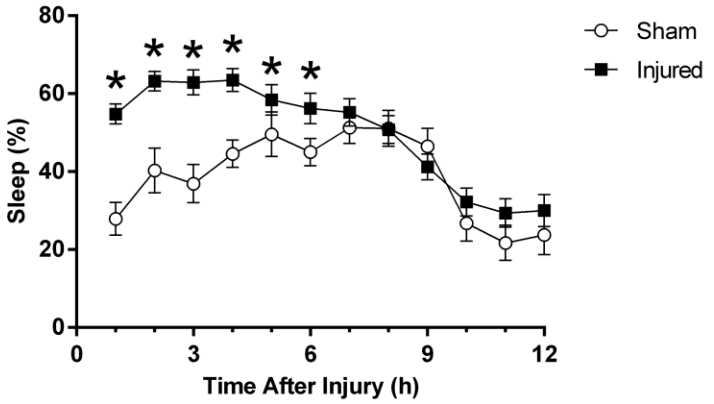


Figure 2.1 Schematic of the study design.

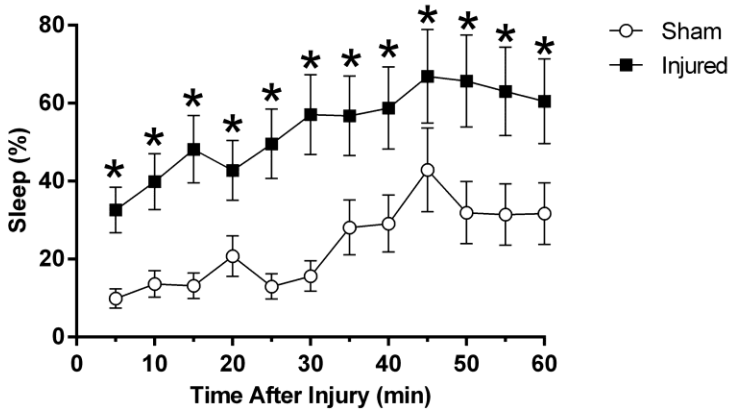
Two cohorts of mice were used based on experimental outcome measures: **(A)** sleep recordings and **(B)** cortical samples and histology. **(A)** Mice were acclimated to piezoelectric sleep cages for 8 days while sample sleep recordings were monitored to test signal integrity. All mice received a midline craniotomy one day prior to brain or sham injury. Mice were divided into 2 groups based on the time of day they were subjected to injury (9:00, 21:00). Within each group, mice were selected at random and subjected to sham, mild (0.8 atm) or moderate (1.2-1.3 atm) diffuse brain injury by midline fluid percussion (mFPI) (n=47). Following injury, mice were placed back into piezoelectric sleep cages and post-traumatic sleep was recorded for 7 days. **(B)** For biochemistry and histology, mice received a midline craniotomy one day prior to injury or sham injury. Mice were subjected to sham, or moderate (1.2-1.3 atm) diffuse brain

injury (9:00) and cortical samples were retrieved at 1, 3, 9, 12, 24, 48, 168 hrs (n=25). Tissue was also collected and prepared for histology 6 hrs post-injury (n=3).

A


Sham
Injured
Sleep (%)
Time After Injury (h)
0
20
40
60
80
0
3
6
9
12

B


Sham
Injured
Sleep (%)
Time After Injury (min)
0
20
40
60
80
0
10
20
30
40
50
60

C

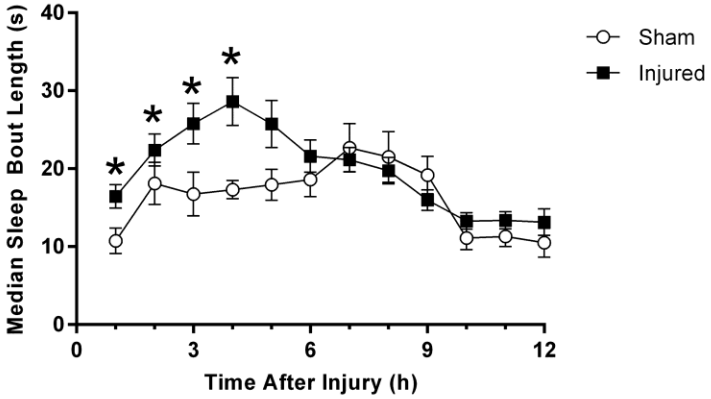

Sham
Injured
Median Sleep Bout Length (s)
Time After Injury (h)
0
10
20
30
40
0
3
6
9
12

Figure 2.2 Diffuse TBI in the mouse disrupts acute post- traumatic sleep parameters compared to uninjured sham.

(A) A multivariate ANOVA showed a significant increase in mean percent sleep over the first 6 hours post-injury compared to the uninjured sham (mean ±SEM; sham n=16; injured n=31; $F(1, 45)=6.545$, $p=0.00007$). After 6 hours post-injury, the mean percent sleep of injured mice normalized to sham mean percent sleep levels and remained comparable for 7 days post-injury (data not shown). **(B)** A detailed analysis of the acute post-traumatic sleep (in the first hour) following diffuse TBI indicated a significant time dependent effect on the increase in sleep. A multivariate ANOVA of the rolling average of the mean percent sleep over 5 min intervals showed post-traumatic sleep significantly increased over the first hour post-injury with a significant effect of time (mean ±SEM; sham n=16; injured n=31; $F(11,495)=8.22$, $p<0.0001$) and group (mean ±SEM; sham n=16; injured n=31; $F(1,45)=37.00$, $p<0.0001$). Bonferroni post hoc analysis was used (*, $p < 0.05$). **(C)** Acutely post-injury, the brain-injured mice showed an increase in median bout length compared to shams. A multivariate ANOVA revealed an increase in bout length significant over the first 4 hours post-injury (mean ±SEM; sham n=16; injured n=31; $F(1,45)=2.9138$, $p=0.032$). This increase in bout length suggested that the increase in mean percent sleep observed acutely post-injury could result from mice sleeping for longer durations, as opposed to sleeping more bouts after diffuse TBI.

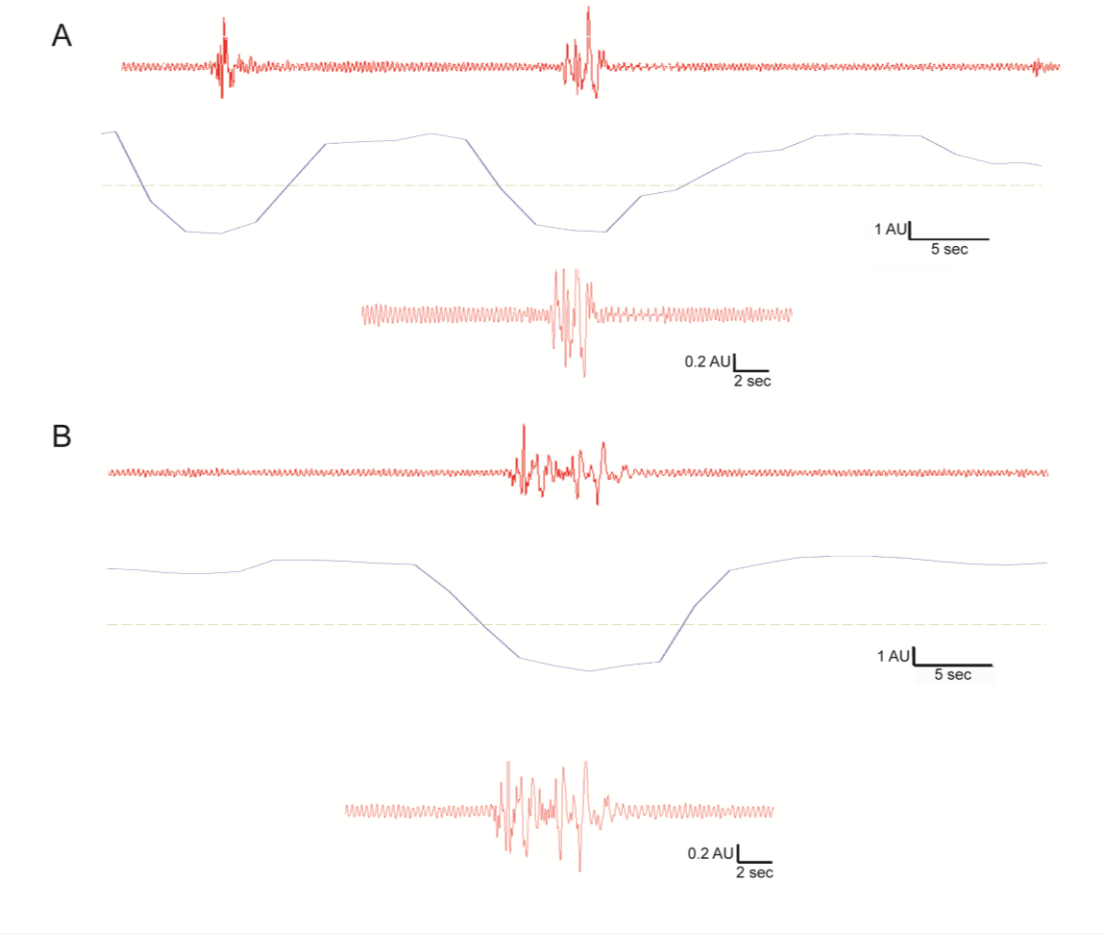


Figure 2.3 Representative sleep-wake recordings in the first hour post-injury showed sleep bouts interrupted by brief arousal and movement.

Uninjured sham mice showed a periodic rhythm of breathing motion (~3 Hz) with regular amplitude typical of sleep, interrupted by high frequency and amplitude signals corresponding to movement consistent with an awake mouse **(A)**. Diffuse brain-injured mice showed similar rhythmic breathing classified as sleep interrupted by frequency and amplitude variations corresponding to movement during interbout intervals of sleep **(B)**. The red lines represent the raw

piezoelectric sensor data over a one minute (top) or 25 second (bottom) interval. The discontinuous blue line indicates the decision classifier over two second intervals to classify sleep activity from wake activity. The broken green line delineates the threshold (in arbitrary units) to determine sleep activity (above the threshold) from wake activity (below the threshold).

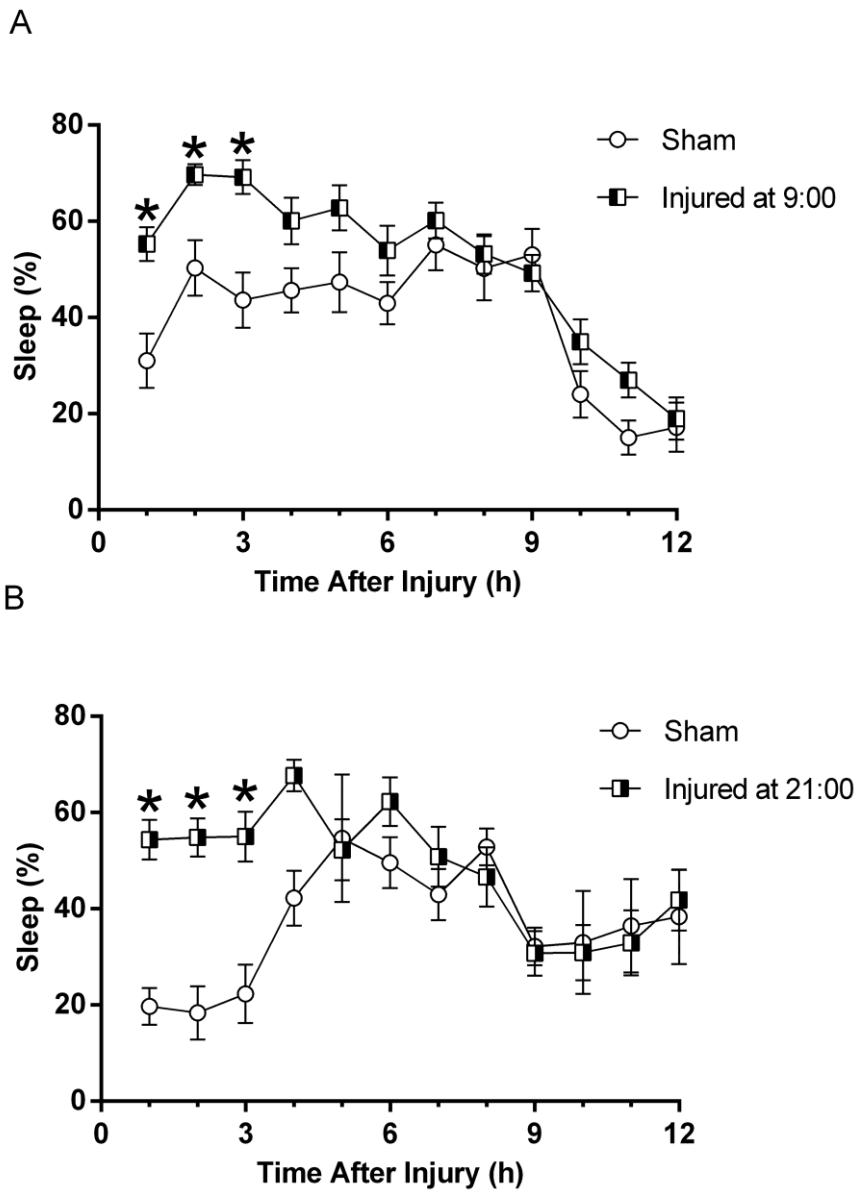


Figure 2.4 Significant increase in post-traumatic sleep is independent of the time of day of the injury.

Mice subjected to mild or moderate injury at 9:00 **(A)**, following the dark/light transition showed significant increases in acute post-traumatic sleep compared to uninjured sham. A multivariate ANOVA and Bonferroni post-hoc analysis was used (mean ±SEM; sham n=12; injured n=17; $F(1,25)=15.95$); *, $p < 0.05$). Mice subjected to mild or moderate injury at 21:00 **(B)**, following the light/dark transition also showed significant increases in acute post-traumatic sleep compared to sham. A multivariate ANOVA and Bonferroni post-hoc analysis was used (mean ±SEM; sham n=5; injured n=14; $F(1,17)=4.42$; *, $p < 0.05$). An increase in sleep is observed acutely following TBI and is observed over the course of the first 3 hours in injured mice compared to sham. After 3 hours, sleep began to normalize in the injured animals and became indistinguishable from sleep in the sham. Mean percent sleep of uninjured sham mice in the 9:00 group was significantly higher than the mean percent sleep of sham mice in the 21:00 group ($F(1,15)=6.303$, $p=0.0240$), as expected.

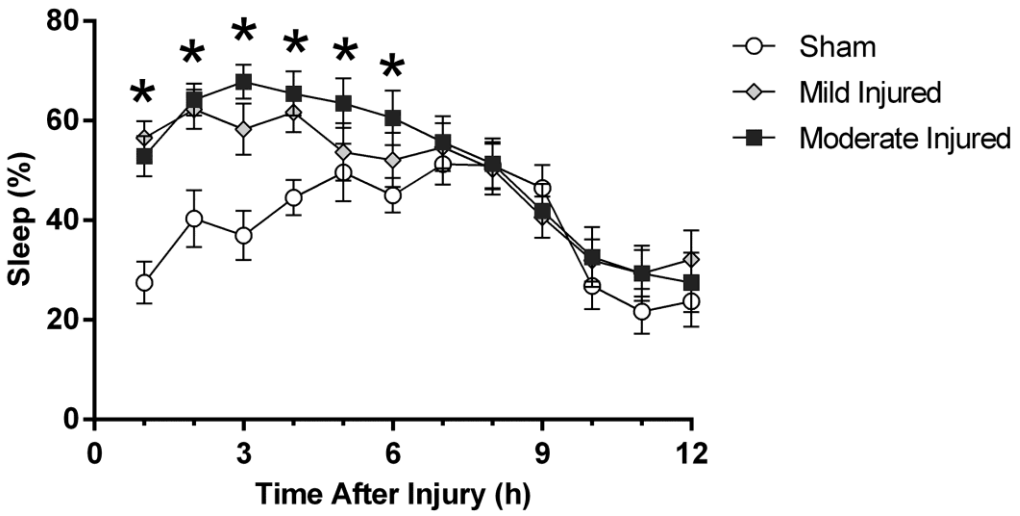


Figure 2.5 The significant increase in post-traumatic sleep is observed acutely following both mild and moderate injury.

A multivariate ANOVA showed a significant increase in mean percent sleep between injured mice and uninjured shams over the first 6 hours post-injury with no significant difference between mildly injured mice compared to moderately injured mice (mean ±SEM; sham n=16; mild n=16; moderate n=15; $F(2,44)=3.4773$, $p=0.00037$).

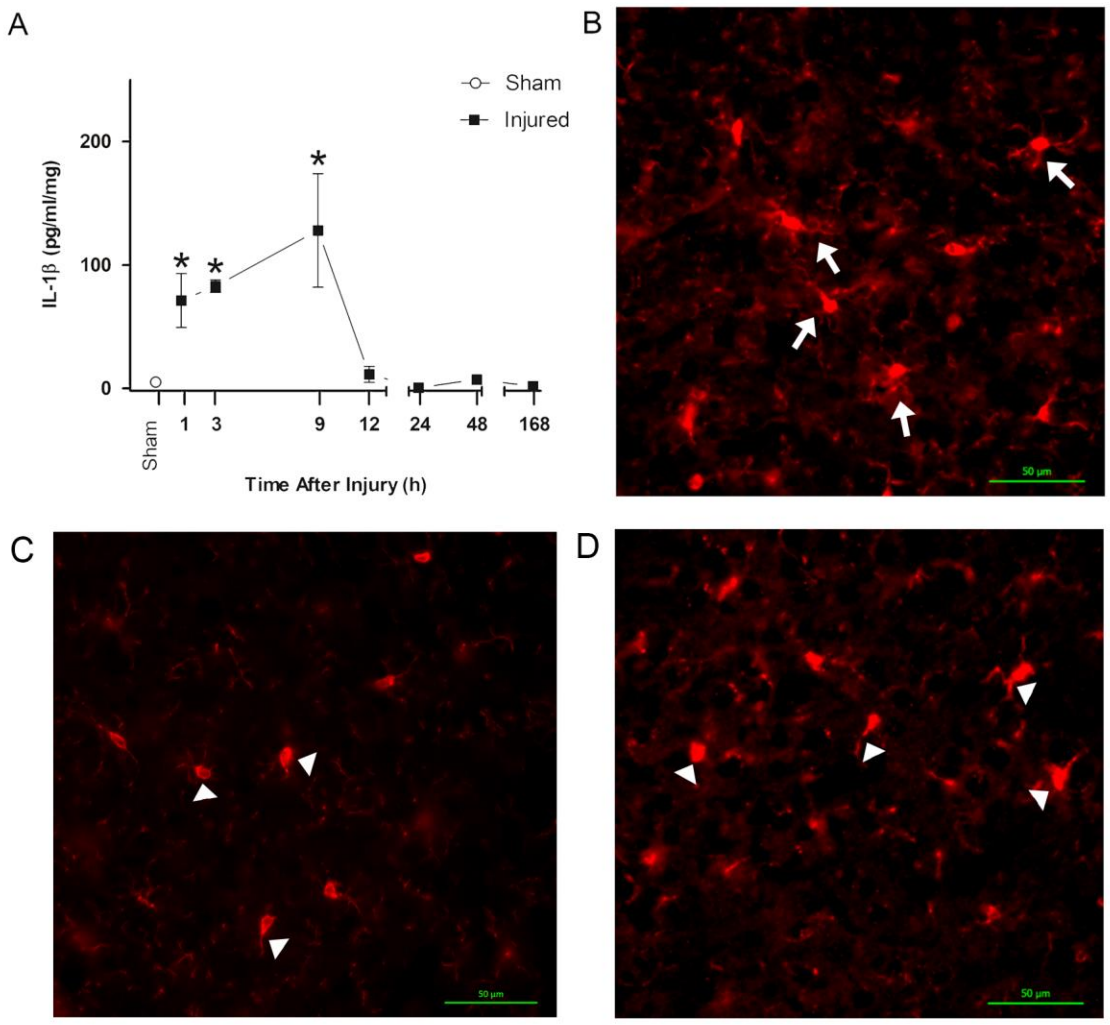


Figure 2.6 Inflammation following diffuse TBI in the mouse.

(A) Temporal profile of IL-1β. The temporal profile indicated that levels in the cortex increase rapidly following moderate injury (9:00) as compared to uninjured sham. Levels of IL-1β peak at or near 9 hours post-injury and return to baseline levels by 12 hours post-injury (One-way ANOVA, mean ±SEM; sham n=7; injured n=22; $F(7,21)=6.474$; $p=0.0004$). Selected comparisons were made (Bonferroni post-hoc), asterisk denotes significance (*, $p < 0.05$) compared to sham. **(B, C,**

D) Microglia morphology, an indicator of microglia activation, was examined after mFPI in the mouse using Iba-1 immunohistochemistry. Iba-1 labels all microglia, however, tissue from a 6 hr sham (40x) **(B)** compared to a 6 hr mild injury (40x) **(C)** and a 6 hr moderate injury (40x) **(D)** show distinct differences in microglia morphology. Microglia in sham **(B)** demonstrated thin ramified processes (denoted by arrows) strongly contrasting the larger cell bodies and thicker processes (denoted by arrowheads) characteristic of activated microglia observed in the diffuse injured mouse **(C, D)**.

Preface to Chapter Three

The aim of the experiments in Chapter Three was to test if our current model of diffuse brain injury produced chronic sleep disturbances similar to those reported by TBI patients. Using non-invasive sleep cages, sleep was measured following diffuse brain injury and injury-induced increases were detected in sleep during the first week post-injury. Furthermore, it was shown that these increases did not extend beyond week one, and were not present in weeks two through five.

Chapter Three: Diffuse brain injury does not affect chronic sleep patterns in the mouse

Summary

The objective of this study was to test if our current model of diffuse brain injury produces chronic sleep disturbances similar to those reported by TBI patients. Adult male C57BL/6 mice were subjected to moderate midline fluid percussion injury (n=7; 1.4 atm; 6-10 min righting reflex time) or sham injury (n=5). Sleep-wake activity was measured post-injury using a non-invasive, piezoelectric cage system. Chronic sleep patterns were analyzed weekly for increases or decreases in percent sleep (hypersomnia or insomnia) and changes in bout length (fragmentation). During the first week after diffuse TBI, brain-injured mice exhibited increased mean percent sleep and mean bout length compared to sham-injured mice. Further analysis indicated the increase in mean percent sleep occurred during the dark cycle. Injury-induced changes in sleep,

however, did not extend beyond the first week post-injury and were not present in weeks 2-5 post-injury. Previously, we showed that the midline fluid percussion model used in this study immediately increased post-traumatic sleep. The current study extended the timeline of investigation to show that sleep disturbances extended into the first week post-injury, but did not develop into chronic sleep disturbances. However, the clinical prevalence of TBI-related sleep-wake disturbances warrants further experimental investigation.

Introduction

Sleep disturbances are commonly reported neurological impairments in the acute phase of TBI, some of which persist through more chronic periods (Castriotta et al. 2007; Verma et al. 2007; Kempf et al. 2010). Pathological processes initiated at the time of injury develop into neurological impairments, with chronic sleep disturbances among the somatic, cognitive and emotional neurological impairments (Castriotta et al. 2007; Kempf et al. 2010). According to the literature, an incidence as high as 70% of TBI survivors suffer from sleep-wake disturbances (Cohen et al. 1992; Orff et al. 2009). Similar sleep disorders develop across the spectrum of TBI, including children and adolescents (Tham et al. 2012). The high prevalence of sleep disorders and impact on quality of life reported in both the adult and pediatric population of TBI survivors warrants investigation of this injury-induced neurological impairment.

Excessive daytime sleepiness is a common sleep-wake disturbance reported among TBI patients (Castriotta et al. 2007; Verma et al. 2007; Baumann 2012) and is characterized primarily by an increase in sleep propensity. Post-

traumatic hypersomnia, an increased need to sleep over a 24 hour period, is reported equally frequent following TBI (Baumann 2012; Billiard and Podesta 2013). These disturbances of increased sleepiness and fatigue can remain several years after injury, becoming chronic impairments (Beaulieu-Bonneau and Morin 2012; Tham et al. 2012; Billiard and Podesta 2013) and overall lowering the quality of life of TBI patients. Injury-induced sleep disturbances also potentially affect the course of recovery (Rao and Rollings 2002; Tham et al. 2012) and hinder rehabilitation (Mathias and Alvaro 2012). Chronic sleep disturbances not only compromise recovery, but can intensify comorbidities including anxiety, depression, cognitive deficits, and pain (Dean et al. 2012; Mathias and Alvaro 2012; Bhalerao et al. 2013; Khoury et al. 2013). For these reasons, exploring animal models that recapitulate aspects of injury-induced sleep problems is timely.

With compromised sleep affecting patient outcome and quality of life, understanding the role of sleep in recovery from brain injury is an important health concern. Focusing on pre-clinical experimentation can potentially accelerate the understanding of the relationship between brain injury and sleep. Experimental models of TBI can be used to evaluate secondary injury mechanisms underlying the pathophysiology of the injury and may be a useful model to investigate chronic post-traumatic sleep. Our lab has recently shown diffuse brain injury in mice increases sleep during the first six hours post-injury (Rowe et al. 2013c). Here we investigate whether the same experimental model shows chronic sleep disturbances associated with TBI. In this study we examine

the relationship between TBI and chronic sleep using non-invasive piezoelectric sleep cages to measure sleep for five weeks after midline fluid percussion injury (mFPI) (Dixon et al. 1987). We hypothesize our model of diffuse brain injury will produce chronic sleep deficits relevant to those reported by TBI patients, thereby permitting further exploration of the role of sleep in recovery from brain injury.

Methods

Animals

Male C57BL/6 mice (Harlan Laboratories, Inc., Indianapolis, IN) were used for all experiments (n=12). The animals were housed in a 12h light/12h dark cycle at a constant temperature (23°C ± 2°C) with food and water available *ad libitum* according to the Association for Assessment and Accreditation of Laboratory Animal Care International. Animals were acclimated to their environment following shipment for at least three days prior to any experiments. After surgery, daily post-operative care was provided, including physical examination and documentation of each animal's condition. Animal use was approved by the Institutional Animal Care and Use Committee at St. Joseph's Hospital and Medical Centre (Phoenix, AZ). All animals used in this study were singly housed in the non-invasive sleep-monitoring cage system (Signal Solutions, Lexington, KY).

Midline Fluid Percussion Injury (mFPI)

Mice (20-24g) were subjected to mFPI consistent with methods previously described (Lifshitz 2008). Mice were anesthetized using 5% isoflurane in 100% oxygen for five minutes and the head of the animal was placed in a stereotaxic

frame with continuously delivered isoflurane at 2.5% via nosecone. While anesthetized, the animal's body temperature was maintained using a Deltaphase® isothermal heating pad (Braintree Scientific Inc., Braintree, MA). A midline incision was made exposing bregma and lambda, and fascia was removed from the surface of the skull. A trephine (3 mm outer diameter) was used for the craniotomy, centred on the sagittal suture between bregma and lambda without disruption of the dura. An injury cap prepared from the female portion of a Luer-Loc needle hub was fixed over the craniotomy using cyanoacrylate gel and methyl-methacrylate (Hygenic Corp., Akron, OH). The injury cap was closed using a Luer-Loc cap and mice were placed in a heated recovery cage and monitored until ambulatory before being returned to their sleep cage. For injury induction 24 hours post-surgery, animals were re-anesthetized with 5% isoflurane delivered for five minutes. The cap was removed from the injury-hub assembly and the dura was visually inspected through the hub to confirm it was intact with no debris. The hub was then filled with normal saline and attached to a tube connected to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). An injury of moderate severity (1.4 atm) was administered by releasing the pendulum onto the fluid-filled cylinder. Sham-injured animals underwent the same procedure except the pendulum was not released. Animals were monitored for the presence of a forearm fencing response and righting reflex times were recorded for the injured animals as indicators of injury severity (Hosseini and Lifshitz 2009). The righting reflex time is the total time from the

initial impact until the animal spontaneously rights itself from a supine position. The fencing response is a tonic posturing characterized by extension and flexion of opposite limbs that has been validated as an overt indicator of injury force magnitude (Hosseini and Lifshitz 2009). The injury hub was removed and the brain was inspected for uniform herniation and integrity of the dura. The dura was intact in all animals, none were excluded as technical failures. The incision was cleaned using saline and closed with sutures. Moderate brain-injured animals had righting reflex recovery times greater than six minutes and a positive fencing response. Sham injured animals recovered from anesthesia within 20 seconds. After spontaneously righting, animals were placed in a heated recovery cage and monitored until ambulatory (approximately 5 to 15 minutes) before being returned to their sleep cage. Adequate measures were taken to minimize pain or discomfort.

Sleep Recordings

The non-invasive sleep cage system (Signal Solutions, Lexington, KY) used in this study consisted of 16 separate units which simultaneously monitor sleep and wake states, as previously published (Rowe et al. 2013c). Each cage unit housed a single mouse inside 18 x 18 centimetre walled compartments with attached food and water structures (Donohue et al. 2008). The cages had open bottoms resting on Polyvinylidine Difluoride (PVDF) sensors serving as the cage floor (Donohue et al. 2008). The non-invasive high-throughput PVDF sensors were coupled to an input differential amplifier and pressure signals were generated and classified by the classifier as motions consistent with either wake

activity or the inactivity and regular breathing movements associated with sleep (Donohue et al. 2008). Briefly, sleep was characterized primarily by periodic (3 Hz) and regular amplitude signals recorded from the PVDF sensors, typical of respiration from a sleeping mouse. In contrast, signals characteristic of wake were both the absence of characteristic sleep signals and higher amplitude, irregular spiking associated with volitional movements. The piezoelectric signals in two second epochs were classified by a linear discriminant classifier algorithm based on frequency and amplitude to assign a binary label of “sleep” or “wake” (Donohue et al. 2008). Mice sleep in a polycyclic manner (more than 40 sleep episodes per hour) (McShane et al. 2010) and so mouse sleep was quantified as the minutes spent sleeping per hour, presented as a percentage for each hour. Data collected from the cage system were binned over specified time periods (e.g. 1 hour) using the average of percent sleep, as well as binned by length of individual bouts of sleep and the median bout lengths were calculated.

Sleep data were collected continuously for five weeks and organized into week long intervals for analysis. Daily percent sleep was calculated by averaging the percent sleep at each of 24 hours for all days of a given week post-injury.

Statistical Analysis

Data are shown as mean ± SEM and analyzed using statistical software (GraphPad-Prism 6). Differences in mean percent sleep and mean bout length were determined with a repeated measures two-way analysis of variance (ANOVA) followed by Sidak’s multiple comparison test. Statistical significance was assigned when $p<0.05$.

Results

Diffuse TBI impacted percent sleep and mean bout length during the first week post-injury.

We have previously reported significant increases in mean percent sleep in brain-injured mice compared to uninjured shams over the first six hours post-injury (Rowe et al. 2013c). In the present study, we repeated this observation and brain-injured mice slept significantly more over the first six hours compared to uninjured shams ($F(1,10)=7.209$, $p=0.0229$; sham n=5, injury n=7; data not shown). Overall, brain-injured mice slept significantly more than sham during the first week post-injury ($F(1,10)=17.61$, $p=0.0018$; sham n=5, injury n=7; Figure 3.1A). To investigate "excessive daytime sleepiness", we evaluated sleep propensity during the dark phase. Mice are nocturnal, sleeping more during the day with prolonged wakefulness at night, and therefore the mouse equivalent of "excessive daytime sleepiness" in humans would most likely occur at night. Mean percent sleep was evaluated over the dark cycle (the time the lights went off at night until they turned on again the following morning). Our data indicate a significant increase in mean percent sleep in brain-injured mice compared to uninjured shams ($F(1, 10)=11.29$, $p=0.0072$; sham n=5, injury n=7; Figure 3.1B) during the dark cycle. Brain-injured mice had the typical initial bout of high wakefulness at dark onset (low percentage sleep), but did not sustain this wakefulness to same degree as uninjured mice (Fig. 3.1A, 3.1B). By 01:00 in the dark cycle, brain-injured mice were sleeping significantly more than uninjured sham. Further, mean bout length was significantly increased in brain-injured mice

compared to uninjured shams during the first week post-injury ($F(1, 10)=5.186$, $p=0.0460$; sham n=5, injury n=7;Figure 3.1C), suggesting that the increase in mean percent sleep observed was due to mice sleeping for longer durations each bout, as opposed to sleeping more bouts, during the first week post-injury (Figure 3.1A, 3.1C).

Diffuse TBI did not induce sleep disturbances between weeks two and five post-injury.

Sleep recordings were extended beyond week one for the same mice to investigate chronic sleep patterns post-injury. Identical percent sleep, dark cycle sleep and mean bout length analyses were also conducted for weeks 2-5 post-injury. No significant injury-dependent effect on daily percent sleep was detected in brain-injured mice (n=7) compared to sham mice (n=5) during post-injury week two ($F(1,10)=2.206$, $p=0.1683$; Figure 3.2A), week three ($F(1,10)=0.4557$, $p=0.5150$; Figure 3.2B), week four $F(1,10)=0.7659$, $p=0.4020$; Figure 3.2C), or week five ($F(1, 10) = 0.1282$, $p=0.7277$; Figure 3.2D).

Further analysis of mean percent sleep focused on sleep only during the dark cycle, placing emphasis on the nocturnality of mice. No injury-dependent effect during the dark cycle on percent sleep was detected in brain-injured mice compared to sham mice during post-injury week two ($F(1,10)=0.07426$, $p=0.7908$; Figure 3.3A), week three ($F(1,10)=0.2760$, $p=0.6108$; Figure 3.3B), week four $F(1,10)=0.01892$, $p=0.8933$; Figure 3.3C), or week five ($F (1, 10) = 0.4322$, $p=0.8395$; Figure 3.3D).

No significant injury-dependent effect on daily mean bout length was detected in brain-injured mice compared to sham mice during post-injury week two ($F(1,10)=0.3694$, $p=0.5569$; Figure 3.4A), week four ($F(1,10)=0.8686$, $p=0.3733$; Figure 3.4C), or week five ($F(1,10)=0.2344$, $p=0.6387$; Figure 3.4D). During week three, brain-injured mice slept significantly longer average bouts than shams $F(1,10)=8.437$, $p=0.0157$; Figure 3.4B), without specific post-hoc differences at particular hours. Overall, brain-injury did not result in consistent chronic changes in mean percent sleep or mean bout lengths of sleep.

Discussion

Sleep research associated with TBI has focused on sleep disturbances in human injury (Verma et al. 2007; Orff et al. 2009; Baumann 2012). These chronic disturbances are present in both pediatric and adult cases of TBI extending up to three years after injury (Kaufman et al. 2001; Kempf et al. 2010). Commonly reported sleep-wake disturbances after TBI include excessive daytime sleepiness, fatigue, hypersomnia, and insomnia (Ouellet and Morin 2006; Verma et al. 2007; Kempf et al. 2010; Baumann 2012). The high prevalence and subjective nature of clinically reported chronic sleep disturbances warrant the investigation of chronic sleep following experimental TBI. Our previous studies showed a significant increase in percent sleep of brain-injured mice compared to uninjured shams over the first six hours following diffuse TBI (Rowe et al. 2013c), termed post-traumatic sleep. Injury-induced changes in sleep patterns were limited to the first week after diffuse brain injury in the mouse, not extending into chronic time points. Only in the first week post-injury did the total percentage

sleep, sleep during the dark cycle and sleep bout length increase in brain-injured compared to uninjured sham mice.

Excessive daytime sleepiness and post-traumatic hypersomnia, characterized primarily by an increase in sleep propensity, have been reported to be among the most common sleep-wake disturbances following TBI (Baumann 2012). Hypersomnia, an increased need for sleep over a 24 hour period, differs from excessive daytime sleepiness in which the increased need for sleep is exclusively in the daytime (Baumann 2012). In the current study, mean percent sleep was evaluated to determine if experimental brain injury resulted in hypersomnia. After diffuse brain injury, an increase in overall mean percent of sleep, hypersomnia, was observed only in the first week post-injury in brain-injured mice compared to uninjured shams. This increase did not extend in to the chronic period 2-5 weeks post-injury. To investigate excessive daytime sleepiness, sleep propensity was analyzed during the dark phase, as mice typically have prolonged wakefulness at night. Brain-injured mice slept significantly more during the dark phase compared to uninjured shams only within the first week post-injury. Taken together, injury-induced increases in sleep are restricted to the first week post-injury. The first week becomes a critical window for future investigations of brain-injury induced sleep. Sleep may contribute to the natural recovery process following injury, which may be most salient in the first week post-injury. Further investigation of the increase in sleep observed over the first week may continue to inform clinical decisions and improve treatment of TBI survivors.

In the current study, mean bout length was analyzed as an indicator of sleep fragmentation. Sleep fragmentation, an increase in awakenings during sleep, leads to excessive daytime sleepiness and can cause changes in daytime function similar to those found following sleep deprivation (Stepanski et al. 1984; Stepanski 2002). Interruptions in sleep may prevent the benefit of the period of sleep prior to the arousal (Bonnet 1985; Bonnet 1986; Stepanski 2002). Short sleep bout lengths are indicative of arousals during the sleep cycle and could potentially confound the reparative processes following brain injury. In this study, sleep bout lengths in diffuse brain-injured mice were significantly longer than sham mice during the first week post-injury, but not beyond. Hence, brain-injured mice had longer periods of uninterrupted sleep compared to uninjured shams; whether this is beneficial to outcome remains unknown. After controlled cortical impact in mice, electroencephalography (EEG) recordings showed in the first three days post-injury, brain-injured mice exhibit reduced ability to maintain prolonged wakefulness (Willie et al. 2012). Together, these data indicate experimental TBI increases sleep bout length and decreases prolonged wakefulness, which could contribute to recovery from brain injury. It is possible sleep bout length is only increased during the first week post-injury, because the bulk of cellular recovery occurs during this period; an increase in sleep beyond this time point may not be necessary.

The exact pathophysiology of post-traumatic sleep-wake disturbances remains elusive. Excessive daytime sleepiness has been correlated to the injury itself, concomitant with other pathophysiology associated with TBI, including

damage to the hippocampus (Baumann et al. 2007; Baumann 2012). A prospective patient study showed as many as 43% of patients have sleep wake disturbances directly related to the injury itself (Baumann et al. 2007). The secondary injury processes which affect sleep following diffuse TBI likely include processes such as ATP depletion, an increased reactive oxygen species (ROS), higher intracellular concentrations of free radicals, and elevated inflammation mediating cytokines (Fan et al. 1995; Werner and Engelhard 2007). Impaired signalling of sleep-wake modulating systems such as the hypocretin (orexin) neuropeptide system, may also contribute to both acute and chronic sleepiness following TBI (Baumann et al. 2005; Baumann et al. 2007; Willie et al. 2012). Future studies can focus on the relationship between these signalling process and sleep in the first week post-injury.

The reparative function of sleep is associated with increased brain ATP levels (Dworak et al. 2010; Chikahisa and Sei 2011). Blocking ATP depletes energy and increases sleep (Kalinchuk et al. 2003), suggesting that increases in sleep following TBI may result from depletions in ATP. Decreases in ATP alter cellular function contributing to cell death, as demonstrated in experimental TBI (Headrick et al. 1994; Signoretti et al. 2001; Lifshitz et al. 2003; Aoyama et al. 2008). Fluid percussion injury decreases ATP levels in both the cortex and hippocampus of rats starting as early as two hour post-injury with declines remaining up to 24 hours post-injury (Lifshitz et al. 2003; Aoyama et al. 2008). Similarly, an impact acceleration model of TBI decreases ATP levels in rats two hours following a moderate injury and as early as ten minutes following a severe

injury (Signoretti et al. 2001). If decrease in ATP is an immediate response to brain injury, post-traumatic sleep may increase brain ATP levels. Similar secondary injury mechanisms after brain injury increase oxidative stress through enhanced production of ROS, reactive radicals, and lipid peroxidation (Ansari et al. 2008), which could increase sleep. Sleep subsequently could remove accumulated free radicals (Reimund 1994; Gopalakrishnan et al. 2004).

In diffuse brain injury, cortical levels of interleukin 1-beta (IL-1β) immediately increased and then normalized by 12 hours post-injury through 1 week (Rowe et al. 2013c). In controlled cortical impact in the rat, interleukins (IL-4, IL-5, IL-13) and tumour necrosis factor alpha (TNF-α) levels increased acutely and recovered to baseline levels by three days post-injury (Dalgard et al. 2012). A temporal profile of inflammatory cytokines following human TBI confirms the translational relevance of these findings with peak levels of IL-1β, IL-6 and TNF occurring within the first three days post-injury(Helmy et al. 2011). The pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF, have dual roles as sleep regulatory substances (Krueger and Majde 1995; Krueger et al. 2001b; Krueger et al. 2007), which may contribute to the acute increase in sleep post-injury.

A limitation of the present study is the inability to distinguish between REM (rapid eye movement) and nonREM sleep. While overall percent sleep and bout length were not impacted at chronic time points, injury-induced alterations in sleep architecture could occur, but not be measured by the non-invasive monitoring system used in this study. Clinical studies show brain injury contributes to changes in both types of sleep (Prigatano et al. 1982; Frieboes et

al. 1999). Increases in REM sleep in the second sleep cycle have been reported close to a year after TBI (Frieboes et al. 1999), while data suggest TBI patients have less stage one nonREM sleep (Prigatano et al. 1982). Further analysis of REM and nonREM sleep may reveal more subtle injury-induced chronic sleep disturbances.

Our literature search showed numerous clinical reports of chronic sleep-wake disturbances associated with TBI (Ouellet and Morin 2006; Verma et al. 2007; Kempf et al. 2010; Baumann 2012), however, experimental studies to date have been terminated too early to evaluate chronic sleep disturbances (Helmy et al. 2011; Willie et al. 2012; Rowe et al. 2013c) . To more completely understand chronic sleep disorders in TBI survivors, future studies could investigate secondary injury processes at chronic time points, focusing on processes that may confound sleep physiology (e.g. ATP, free radicals, cytokines). A secondary insult, such as a second brain injury, may be necessary to induce chronic sleep disturbance in the mouse. Moreover, the present study disregarded the contraindications of psychiatric sequelae, such as depression and anxiety, which contribute to the development, if not maintenance, of sleep-wake disturbances in TBI patients (Baumann 2012).

Conclusion

In conclusion, sleep was increased following diffuse TBI during the first week post-injury. These injury-dependent changes in sleep were not maintained thereafter. Further studies are needed to understand the contribution of sleep on recovery following TBI, as well as other neurological conditions.

Chapter Three is in review in the following manuscript:
Rowe RK, Harrison JL, O'Hara BF, and Lifshitz J. (2013). Diffuse brain injury does not affect chronic sleep patterns in the mouse.

Chapter Three: Figures

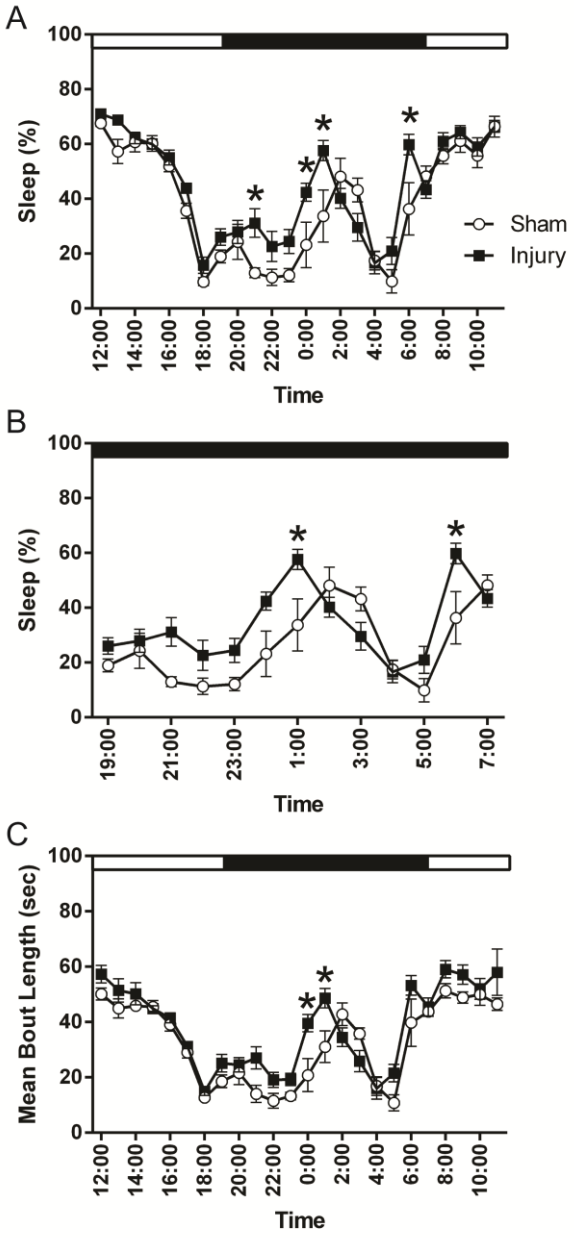


Figure 3.1 Diffuse TBI impacted percent sleep and mean bout length in the first week post-injury

(A) Overall, brain-injured mice slept significantly more than shams during the first week post-injury (mean ±SEM; $F(1,10)=17.61$, $p=0.0018$; sham n=5, injury n=7). Percent sleep was calculated by averaging the percent sleep at each of 24 hours for all days of a given week post-injury, and this average is represented in the graph. Differences between brain-injured mice and uninjured shams as indicated (Sidak's multiple comparison test, *, $p<0.05$). **(B)** Percent sleep in brain-injured mice increased significantly compared to uninjured shams (two-way ANOVA, mean ±SEM; $F(1, 10)=11.29$, $p=0.0072$; sham n=5, injury n=7) during the dark cycle, with significant differences at 01:00 and 06:00 (Sidak's multiple comparison test; *, $p<0.05$). **(C)** Overall, the average bout length of sleep was significantly longer in brain-injured mice compared to uninjured shams during the first week post-injury (mean ±SEM; $F(1, 10)=5.186$, $p=0.0460$; sham n=5, injury n=7), with significantly longer sleep bouts in the middle of the dark cycle for brain-injured compared to uninjured mice (Sidak's multiple comparison test; *, $p<0.05$).

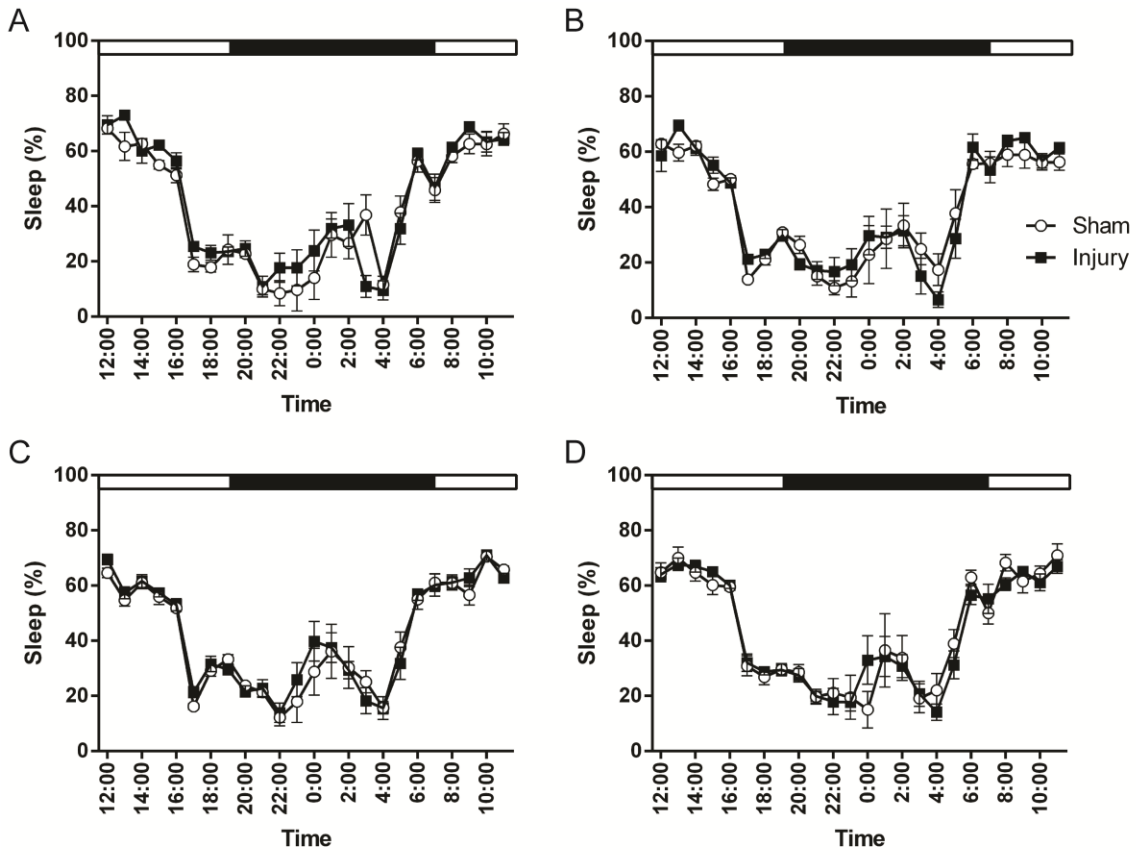


Figure 3.2 Diffuse TBI did not chronically impact percent sleep.

Daily percent sleep was calculated by averaging the percent sleep at each hour for all days of week two **(A)**, three **(B)**, four **(C)**, and five **(D)** post-injury. No significant differences in daily percent sleep were found between brain-injured and sham mice during week two ($F(1,10)=2.206$, $p=0.1683$), week three ($F(1,10)=0.4557$, $p=0.5150$), week four ($F(1,10)=0.7659$, $p=0.4020$), or week five ($F(1, 10) = 0.1282$, $p=0.7277$).

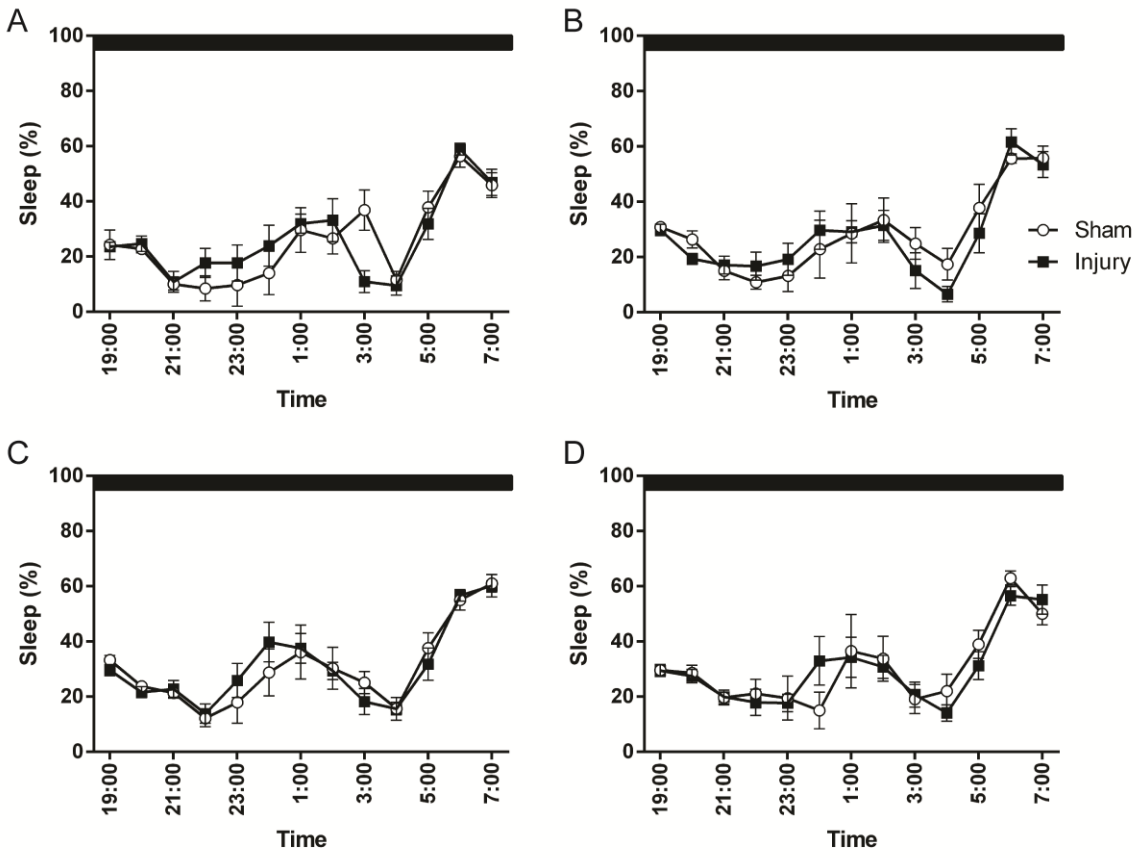


Figure 3.3 Diffuse TBI did not chronically impact percent sleep during the dark cycle.

The mean percent sleep was evaluated during the dark cycle by calculating the average percent sleep at each hour of the dark cycle for all days of week two **(A)**, three **(B)**, four **(C)**, and five **(D)**. No injury-dependent effect during the dark cycle on percent sleep was detected in brain-injured mice compared to sham mice during post-injury week two ($F(1,10)=0.07426$, $p=0.7908$), week three ($F(1,10)=0.2760$, $p=0.6108$), week four $F(1,10)=0.01892$, $p=0.8933$), or week five ($F(1, 10) = 0.4322$, $p=0.8395$).

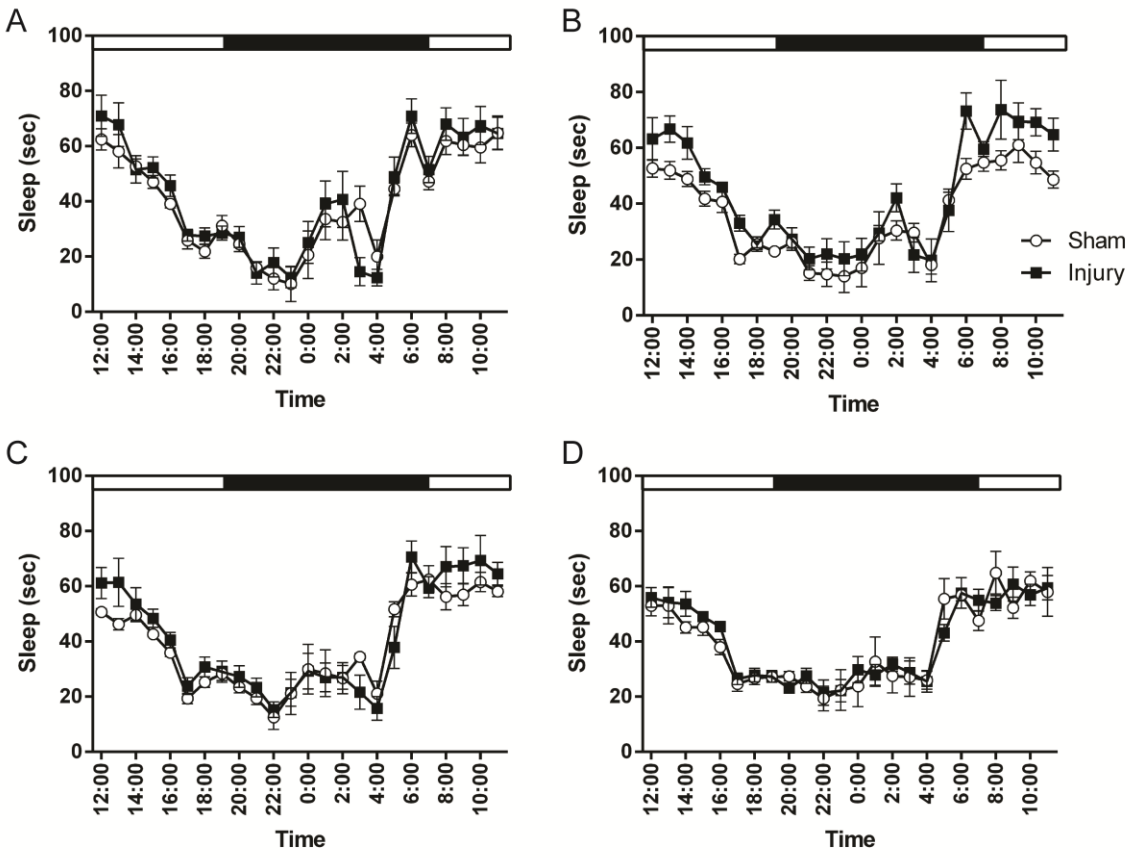


Figure 3.4 Diffuse TBI did not chronically impact mean bout length.

Daily mean bout length was calculated by averaging the mean bout length at each hour for all days of week two **(A)**, week three **(B)**, week four **(C)**, and week five **(D)**. No significant differences in average bout length slept were found between brain-injured and sham mice during week two ($F(1,10)=0.3694$, $p=0.5569$), week four ($F(1,10)=0.8686$, $p=0.3733$), or week five ($F(1,10)=0.2344$, $p=0.6387$). Overall during week three, brain-injured mice slept significantly longer average bouts than shams ($F(1,10)=8.437$, $p=0.0157$).

Preface to Chapter Four

In the previous chapters, it has been demonstrated that diffuse brain injury resulted in increased post-traumatic sleep over the first six hours following injury. The aim of the experiments in Chapter Four was to examine the relationship between immediate disruption of post-traumatic sleep and functional outcome in the diffuse brain injured mouse. Using a gentle handling method of sleep disruption, we showed short duration disruption of post-traumatic sleep did not affect functional outcome, measured by motor and cognitive performance.

Chapter Four: Recovery of neurological function despite immediate sleep disruption following diffuse brain injury in the mouse: clinical relevance to medically untreated concussion

Summary

In this study we investigate the relationship between immediate disruption of post-traumatic sleep and functional outcome in the diffuse brain-injured mouse. Adult male C57BL/6 mice were subjected to moderate midline fluid percussion injury (n=65; 1.4 atm; 6-10 min righting reflex time) or sham injury (n=44). Cohorts received either intentional sleep disruption (minimally stressful gentle handling) or no sleep disruption for six hours following injury. Following disruption, serum corticosterone levels (ELISA) and post-traumatic sleep (non-invasive piezoelectric sleep cages) were measured. For 1-7 days post-injury, sensorimotor outcome was assessed by Rotarod and a modified Neurological Severity Score (NSS). Cognitive function was measured using Novel Object Recognition (NOR) and Morris water maze (MWM) in the first week post-injury.

Disrupting post-traumatic sleep for six hours did not affect serum corticosterone levels or functional outcome. In the hour following the first dark-onset, sleep disrupted mice exhibited a significant increase in sleep, however, this increase was not sustained; there was no rebound of lost sleep. Regardless of sleep disruption, mice showed a time-dependent improvement in Rotarod performance, with brain-injured mice having significantly shorter latencies on day 7 compared to sham. Further, brain-injured mice, regardless of sleep disruption, had significantly higher NSS scores post-injury compared to sham. Cognitive behavioral testing showed no group differences among any treatment group measured by MWM and NOR. Short duration disruption of post-traumatic sleep did not affect functional outcome, measured by motor and cognitive performance. These data may refute post-traumatic sleep as a mechanism of recovery from diffuse brain injury and impact TBI survivors not seeking medical attention.

Introduction

TBI is a major cause of death and disability throughout the world with few pharmacological treatments available for individuals suffering from lifelong neurological morbidities associated with TBI. Vascular, cellular and molecular pathological processes initiated at the time of injury can compound the injury and manifest into functional impairments. In the United States alone, the Centers for Disease Control and Prevention estimated that between 2002 and 2006 there were on average 52,000 deaths, 275,000 hospitalizations, and 1,365,000 emergency department visits related to TBI each year (Faul M 2010). Beyond this, it is estimated that as high as 42% of TBIs are not included in these

statistics, because approximately 1.2-4.2 million survivors of mild TBI do not seek medical attention (Setnik and Bazarian 2007). A common neurological consequence of mild TBI is excessive sleepiness immediately following injury (Castriotta et al. 2007). However, discordant opinions suggest that individuals should not be allowed to sleep or should be frequently awoken following mild brain injury. The intentional sleep disruption employed outside of medical care (e.g. at home) contrasts the unintentional sleep disruption associated with diagnostic procedures in a clinical setting. This controversy is not supported by peer-reviewed biomedical literature and the impact of sleep disruption immediately following TBI upon functional recovery is not understood.

For the first time, this study investigates the contribution of acute post-traumatic sleep on the recovery of neurological function after diffuse TBI. Post-traumatic sleep may be beneficial to recovery from injury, because prevailing hypotheses suggest the function of sleep is restorative, conservative, and adaptive (Tononi and Cirelli 2006; Chokroverty 2010). To investigate the relationship between TBI and acute sleep, the current study uses gentle handling to disrupt sleep after mFPI, an animal model of concussion (Dixon et al. 1987). Following mFPI, with and without sleep disruption, mice can be evaluated for performance in cognitive, neurological, and motor function, using standard behavioral tests (Nakamura et al. 1999; Longhi et al. 2004; Schoch et al. 2012).

Our previous findings indicated an increase in sleep during the first six hours following diffuse brain injury in mice—a period we have defined as post-traumatic sleep (Rowe et al. 2013c). During this period brain-injured mice are

responsive, capable of movement, and eat and groom themselves, indicating they are not in a comatose state of unresponsiveness. Following TBI, secondary injury processes are triggered including the production of cytokines, some of which have dual roles as sleep regulatory substances (SRSs) (Krueger and Majde 1995; Krueger et al. 2007; Krueger 2008). Similar increases in cytokine signaling have been observed across experimental models and in human TBI, highlighting their involvement in pathological and reparative processes triggered by injury (Morganti-Kossmann et al. 2001; Frugier et al. 2010; Semple et al. 2010; Ziebell and Morganti-Kossmann 2010). The increases in sleep promoting cytokines suggest post-traumatic sleep is a natural process. Whether this natural process is beneficial to functional outcome remains to be seen.

Clinical recommendations and at home practices with regard to sleep after TBI cover an array of interventions including total deprivation, frequent awakening, and encouraging sleep. Experimentally, multiple techniques can disrupt or deprive sleep. Sleep deprivation is the complete disruption of one or both types of sleep (rapid eye movement (REM), non-rapid eye movement (NREM)), compared to sleep disruption in which minimal sleep may occur. Deprivation of REM sleep can be achieved using methods that operate when muscle tone is lost as REM sleep begins. In these methods, muscle tone is required for an animal to balance on a platform in water; with the onset of atonia, the animal would fall off the platform into water and wake the animal. Frequently used REM deprivation methods include the flower pot and variations of the multiple platform method (Cohen and Dement 1965), which may also

compromise slow wave sleep (SWS) (Machado et al. 2004). Acute injury-induced motor deficits prevented the use of these deprivation methods. Thus, total disruption of post-traumatic sleep was implemented, allowing only minimal sleep during the disruption period. Experimental methods for disrupting sleep include forced wheel running and gentle handling. Investigations have shown forced and voluntary exercise can positively and negatively affect behavioral and histological outcomes following brain injury (Zhang et al. 2010; Griesbach 2011; Crane et al. 2012; Griesbach et al. 2012; Yang et al. 2012b; Silva et al. 2013). To disrupt sleep, we used the gentle handling method along with cage tapping whenever animals began falling asleep (Patti et al. 2010), which disrupts all stages of sleep.

We are extending our investigations into sleep as a natural response to TBI. Increased post-traumatic sleep, paired with inconsistent recommendations for sleep after TBI, make understanding the role of post-traumatic sleep an important public health concern. Understanding the impact of post-traumatic sleep on functional outcome could inform home care recommendations for the large number of TBI survivors not seeking medical attention. We hypothesize that post-traumatic sleep disruption would result in poor functional outcome following TBI. To test this hypothesis, we used mFPI to model diffuse TBI in mice, disrupted post-traumatic sleep for six hours post-injury, and assessed cognitive, motor and sensorimotor functional outcome over one week post-injury. The goal of this study is to add knowledge for evidence-based clinical recommendations in the treatment of mild TBI.

Methods

Animals

Male C57BL/6 mice (Harlan Laboratories, Inc., Indianapolis, IN) were used for all experiments (n=109). The animals were housed in a 12 h light/12h dark cycle at a constant temperature (23°C ± 2° C) with food and water available ad libitum according to the Association for Assessment and Accreditation of Laboratory Animal Care International. Animals were acclimated to their environment following shipment for at least three days prior to any experiments. After surgery, animals were evaluated daily for post-operative care by a physical examination and documentation of each animal's condition. Animal care was approved by the Institutional Animal Care and Use Committees at the University of Kentucky and the St. Joseph's Hospital (Phoenix, AZ).

Housing

All mice used in this study were singly housed. Mice used for the MWM and NOR studies were housed in standard individually ventilated cages. Mice for all other studies were housed in the non-invasive sleep-monitoring cage system (Signal Solutions, Lexington, KY).

Midline Fluid Percussion Injury (mFPI)

Mice (20-24g) were subjected to midline fluid percussion injury (mFPI) consistent with methods previously described (Lifshitz 2008). Animal numbers are indicated in the results section and figure legends for individual studies. Mice were anesthetized using 5% isoflurane in 100% oxygen for five minutes and the head of the animal was placed in a stereotaxic frame with continuously delivered

isoflurane at 2.5% via nosecone. While anesthetized, the animal's body temperature was maintained using a Deltaphase® isothermal heating pad (Braintree Scientific Inc., Braintree, MA). A midline incision was made exposing bregma and lambda, and fascia was removed from the surface of the skull. A trephine (3 mm outer diameter) was used for the craniotomy, centered on the sagittal suture between bregma and lambda without disruption of the dura. An injury cap prepared from the female portion of a Luer-Loc needle hub was fixed over the craniotomy using cyanoacrylate gel and methyl-methacrylate (Hygenic Corp., Akron, OH). The incision was sutured at the anterior and posterior edges and topical Lidocaine ointment was applied. The injury cap was closed using a Luer-Loc cap and animals were placed in a heated recovery cage and monitored until ambulatory before being returned to their sleep cage.

For injury induction 24 hours post-surgery, animals were re-anesthetized with 5% isoflurane delivered for five minutes. The cap was removed from the injury-hub assembly and the craniotomy was visually inspected through the hub. The hub was then filled with normal saline and attached to a tube connected to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). An injury of moderate severity (1.4 atm) was administered by releasing the pendulum onto the fluid-filled cylinder. Sham-injured animals underwent the same procedure except the pendulum was not released. Animals were monitored for the presence of a forearm fencing response and righting reflex times were recorded for the injured animals as indicators of injury severity (Hosseini and Lifshitz 2009). The righting

reflex time is the total time from the initial impact until the animal spontaneously rights itself from a supine position. The fencing response is a tonic posturing characterized by extension and flexion of opposite arms that has been validated as an overt indicator of injury force magnitude (Hosseini and Lifshitz 2009). The injury hub was removed and the brain was inspected for uniform herniation and integrity of the dura. Animals in which the dura was compromised were excluded from all studies as technical failures. The incision was cleaned using saline and closed using sutures. Moderate brain-injured animals had righting reflex recovery times greater than six minutes and a positive fencing response. Sham injured animals recovered within 20 seconds. After spontaneously righting, animals were placed in a heated recovery cage and monitored until ambulatory (approximately 5 to 15 minutes) before being returned to their sleep cage or subjected to sleep disruption. Adequate measures were taken to minimize pain or discomfort.

Sleep Disruption

Mice were randomly assigned to a sleep disruption or no sleep disruption group. The mice in the no sleep disruption group were returned to their individual cages as soon as they were ambulatory (approximately 5 to 15 minutes) following the brain injury or sham injury. Mice in the sleep disruption group were placed in individual cages and continuously sleep disrupted for six hours post-injury, the duration over which the mFPI mice sleep in excess of sham controls, as previously observed in our model (Rowe et al. 2013c). Sleep disruption was accomplished using a minimally stressful gentle handling method (O'Hara et al. 1993; Asikainen et al. 1995; Patti et al. 2010), which included tapping on the

cages or gently touching the animal when visible signs (immobile, eyes closed) of sleep were present (Patti et al. 2010). After the disruption period, mice were returned to their individual cages and sleep activity was measured continuously for 24 hours (see below).

Sleep Recordings

The non-invasive sleep cage system (Signal Solutions, Lexington, KY) used in this study consisted of 16 separate units that could simultaneously monitor the sleep and wake states, as previously published (Rowe et al. 2013c). Each cage unit housed a single mouse inside 7x7 inch walled compartments with attached food and water structures (Donohue et al. 2008). The cages had open bottoms resting on Polyvinylidine Difluoride (PVDF) sensors serving as the cage floor (Donohue et al. 2008). The non-invasive high-throughput PVDF sensors were coupled to an input differential amplifier and pressure signals were generated and classified by an algorithm (see below) as motions consistent with either wake activity or the inactivity and regular breathing movements associated with sleep (Donohue et al. 2008). Briefly, sleep was characterized primarily by periodic (3 Hz) and regular amplitude signals recorded from the PVDF sensors, typical of respiration from a still mouse. In contrast, signals characteristic of wake were both the absence of characteristic sleep signals and higher amplitude, irregular spiking associated with volitional movements. The piezoelectric signals in two second epochs were classified by a linear discriminant classifier algorithm based on frequency and amplitude to assign a binary label of “sleep” or “wake” (Donohue et al. 2008). Mice sleep in a polycyclic manner (more than 40 sleep

episodes per hour) (McShane et al. 2010). For experimental studies, mouse sleep was quantified as the minutes spent sleeping per hour, presented as a percentage for each hour. Sleep activity data were binned over specified time periods (e.g. 5 minutes, 1 hour) to calculate the average of percent sleep. Data were binned by length of individual sleep bouts to calculate median bout length.

Corticosterone assay

Immediately following six hours of sleep disruption, mice were euthanized (between 14:00 and 17:00), by an overdose of sodium pentobarbital, and a cardiac blood sample was collected (sham n=2, injury n=4). A separate group of animals was not exposed to sleep disruption prior to blood collection (sham n=2, injury n=4). The blood samples were centrifuged (3000 rpm, 8 min) and the serum was stored at -20ºC. A commercially available competitive immunoassay was followed according to the manufacturer's protocol for the quantitative determination of corticosterone (no. 900-097; Assay Designs, Inc. Ann Arbor, MI). The kit uses an anti-corticosterone polyclonal antibody to bind standards and samples. The enzyme reaction generates a yellow color that is inversely proportional to the corticosterone concentration and is read on a microplate reader (405 nm). All samples were diluted 1:5 (80%) in order to stay within the sensitivity of the assay (32–20,000 pg/ml). Data are presented as levels of serum corticosterone concentration in nanograms/milliliter.

Behavioral Testing

Behavioral testing was performed on two cohorts of animals. One cohort was tested on the rotarod and neurological severity score. A separate cohort was tested by novel object recognition and the Morris water maze.

Neurological Severity Score (NSS). Post-traumatic neurological impairments were assessed using an 8-point NSS paradigm adapted from those previously used in experimental models of TBI (Chen et al. 1996; Semple et al. 2010; Pleasant et al. 2011; Ziebell et al. 2011). Animals were tested at selected time points post-injury (1, 3, 5, 7 days). One point was given for failure on an individual task, and no points were given if an animal completed a task successfully. Mice were observed for hind limb flexion, startle reflex, and seeking behavior (presence of these behaviors was considered successful task completion). Mice traversed in sequence, 3, 2, and 1 centimeter beams. The beams were elevated and mice were given 1 minute to travel 30 centimeters on the beams. The task was scored as a success if the mouse traveled 30 centimeters with normal forelimb and hindlimb position (forelimb/hindlimb did not hang from the beam). Mice were also required to balance on a 0.5 centimeter beam and a 0.5 centimeter round rod for 3 seconds in a stationary position with front paws between hind paws. Non-parametric data are presented as a composite score ranging from 1 to 8 representing performance on all tasks combined. High final NSS scores were indicative of task failure and interpreted as neurological impairment.

Rotarod. Sensorimotor function was assessed using the Economex Rotarod system from Columbus Instruments (Columbus, OH). Animals were pre-trained

(60 sec at 4 rpm for 3 trials) for three consecutive days including the day of the craniotomy. Animals were then tested at selected time points post-injury. Animals were tested either immediately and 1 hour after injury or tested on post-injury days 1, 3, 5, and 7. For the test, animals were placed on the rod with a starting speed of 4 rpm, and the speed was continuously accelerated up to 28 rpm over 5 minutes (for 2 trials), as previously published (Bachstetter et al. 2013). The trial ended when the mouse fell from the rod or 5 minutes elapsed. Data are presented as latency to fall in seconds and total distance traveled in centimeters.

Morris Water Maze (MWM). Learning ability was assessed in the MWM using a paradigm similar to those previously used in experimental models of TBI (Smith et al. 1995; Murai et al. 1998; Smith et al. 1998; Prins and Hovda 2001; Pleasant et al. 2011). The 1-meter diameter MWM was filled with water (19-21°C) and nontoxic white paint (Rich Art Co., Northvale, NJ) was added to hide the platform (6.3 centimeter diameter) that was submerged 0.5 centimeters. At selected time points post-injury (3, 4, 5, 6 days), mice were tested in sets of four trials per day. Mice started from one of four starting points (North, South, East, West) and used visual cues placed on the walls outside the tank to locate the platform. All trials were monitored using overhead video/tracking software (EZVideo version 5.51DV, Accuscan Instruments Inc., Columbus, OH). The latency of the mouse to find the platform was recorded as well as the distance traveled. If a mouse did not find the platform within the 70 second trial, it was placed on the platform for 10 seconds. Data are presented as latency to find the hidden platform in seconds.

Novel Object Recognition (NOR). Cognitive impairment was tested using the NOR test as previously published (Ennaceur and Aggleton 1997; Han et al. 2011). The test consisted of three phases: habituation, training, and testing. On day 3 post-injury, mice were placed in an open field (42 cm, 21 cm, 21 cm) for one hour of habituation. Mice were removed and two identical objects were placed in opposing quadrants of the field for the training phase. Mice were placed in the center of the open field and given 5 minutes to explore the objects. Following training mice were returned to their home cages. Testing began 4 hours after training. One familiar object and one novel object were placed in opposing quadrants of the field. Mice were placed into the center and given 5 minutes to explore. On day 7 post-injury, mice were given 10 minutes of habituation to their previously used open field. After habituation, mice were removed and the familiar object from training and a novel object (distinct from the object on day 3) were placed in opposing quadrants of the field and mice were given 5 minutes to explore. For training and testing the percentage of time spent exploring the novel object was quantified. Exploration of an object included the mice sniffing, touching, or climbing onto an object while facing the object. If an animal climbed onto an object and sniffed into the air, this time was not calculated into the exploration of the novel object. During training and testing trials, mice were required to spend a minimum combined 10 seconds exploring objects. If this time was not met, trial time was extended for that animal until 10 seconds of exploration was achieved (sham: 1 of 12, 30 sec; FPI: 5 of 24, mean

61 ± 17.5 sec). Data are presented as percent of total exploration time spent exploring the novel object.

Statistical Analysis

Data are shown as mean ± SEM and analyzed using statistical software (GraphPad-Prism 6). Differences in righting reflex times were determined by t-test. Differences in rotarod performance immediately following TBI were determined with a repeated measure two-way analysis of variance (ANOVA) followed by Sidak's multiple comparison test. Percent sleep following disruption was analyzed using a repeated measure two-way ANOVA. Differences in rebound sleep were determined using a one-way ANOVA followed by Tukey's post-hoc analysis. Differences in functional performance over time post-injury measured by the rotarod, MWM, and NOR were all determined using a two-way ANOVA, followed by Tukey's post-hoc analysis as needed. Non-parametric NSS data were analyzed by Kruskal-Wallis ANOVA, followed by Dunn's comparison post-hoc test (see results). Statistical significance was assigned when $p<0.05$.

Results

Immediate neurological deficits following diffuse TBI.

We have previously reported a suppression of the righting reflex response in rats following mFPI (Hosseini and Lifshitz 2009), as an injury-induced deficit. Diffuse brain injury resulted in a significant suppression of the righting reflex in brain-injured mice compared to anesthetized, uninjured shams ($t_{31}=3.351$, $p=0.0021$; sham n=28, injury n=33; Figure 4.1A). To assess acute vestibulomotor deficits following diffuse TBI, we used the rotarod task immediately and 1 hour

after the return of the righting reflex (Fox et al. 1998; Hamm 2001; Laurer et al. 2001). There was a significant injury-dependent motor deficit measured by latency on the Rotarod task in brain-injured mice compared to uninjured shams ($F(1, 11)=83.93$, $p<0.0001$; Figure 4.1B) both immediately and 1 hour after return of the righting reflex (sham n=6, injury n=7; Figure 4.1B).

Intentional sleep disruption following diffuse TBI did not result in a rebound of lost sleep but alters activity response to dark-onset.

Corticosterone levels were measured at the conclusion of the six hour disruption period as an indicator of stress related to the sleep disruption. Sleep disruption did not significantly alter corticosterone levels in cardiac blood samples in either sleep disruption shams or sleep disruption injured mice impaired to no disruption shams and no disruption brain-injured mice (Figure 4.2A). Brain-injured mice had significantly lower levels of corticosterone compared to uninjured shams regardless of sleep disruption at the conclusion of the testing period ($F(1,8)=7.57$, $p=0.0250$; Figure 4.2A). As intended, the sleep disruption method developed for these studies did not adversely impact corticosterone levels.

Following six hours of intentional sleep disruption, using the gentle handling method (Patti et al. 2010), there was no significant change in percent sleep over six hours between sleep disruption brain-injured and sleep disruption shams compared to no disruption brain-injured and no disruption shams ($F(3, 32)=2.187$, $p=0.1087$; no disruption sham n=7, no disruption injury n=8, sleep disruption sham n=8, sleep disruption injury n=13; Figure 4.2B). This indicated

that the sleep lost during the six hours of intentional disruption was not recovered by the sleep disrupted groups, at least in terms of total sleep time. However, there was a sleep disruption effect on activity response to dark-onset at 10h post-injury (19:00) ($F(3, 32)=0.9386$, $p=0.0024$; Figure 4.2C). There was a significant difference between no disruption sham compared to both sleep disruption brain-injured and sleep disruption sham mice (Figure 4.2C). Both the sleep disrupted brain-injured and sleep disrupted shams slept significantly more following dark-onset compared to the no disruption brain-injured and no disruption shams. In the absence of sleep disruption, uninjured and brain-injured animals showed increased wake activity following dark onset, as is typical for nocturnal rodents.

Diffuse TBI resulted in neurological impairments independent of acute sleep disruption.

Overall, brain-injured mice showed significant neurological impairments measured by the neurological severity score (NSS) compared to uninjured shams, independent of sleep disruption (no disruption sham n=12, no disruption injury n=13, sleep disruption sham n=10, sleep disruption injury n=13; Figure 4.3). On post-injury days 1, 3 and 5, both sham groups had significantly lower NSS scores compared to both brain-injured groups (Day 1 $KW(4,48)=27.45$, $p<0.0001$; Day 3 $KW(4,48)=18.99$, $p=0.0003$; Day 5 $KW(4,48)=15.63$, $p=0.0013$; Day 7 $KW(4,48)=16.36$, $p=0.001$; Kruskal-Wallis statistic with Dunn's comparison post-hoc; Figure 4.3). There was no significant difference in neurological impairments measured by the NSS between the sleep disruption brain-injured mice and no disruption brain-injured mice at any post-injury time point.

Diffuse TBI reduced motor performance on the rotarod task independent of acute sleep disruption.

To assess motor function we used the rotarod task as previously published (Bachstetter et al. 2013). Following diffuse brain injury, there was a significant injury-dependent effect on latency to stay on the rotarod ($F(3, 44)=3.367$, $p=0.0268$) and a time-dependent improvement in latency ($F(3, 132=41.60$, $p<0.0001$; Figure 4.4A; no disruption sham n=12, no disruption injury n=13, sleep disruption sham n=10, sleep disruption injury n=13). There was a significant injury and disruption effect on rotarod latency between sleep disruption brain-injured mice compared to no disruption sham on post-injury day 7 (Figure 4.4A). There was no significant difference in latency on the rotarod task between the sleep disruption brain-injured mice and no disruption brain-injured mice ($F(1,24)=0.5033$, $p=0.4849$). Further analysis of rotarod performance showed a significant injury-dependent effect on distance traveled ($F(3, 44)=4.009$, $p=0.0132$) and a time-dependent improvement in distance traveled ($F(3, 132)=34.61$, $p<0.0001$; Figure 4.4B). There was a significant injury effect between no disruption sham compared to both injury groups (no disruption and sleep disruption) on post-injury day 5 and 7. There was no significant difference in distance traveled on the rotarod task between the sleep disruption brain-injured mice and no disruption brain-injured mice ($F(1,24)=0.2592$, $p=0.6154$).

Acute sleep disruption following diffuse TBI did not alter cognitive performance.

Performance on the Morris water maze task indicated all groups had a significant time-dependent improvement in latency to find the hidden platform ($F(3, 96)=17.40$, $p<0.0001$; Figure 4.5; no disruption sham n=6, no disruption injury n=12, sleep disruption sham n=6, sleep disruption injury n=12). There was no significant injury-dependent effect or sleep disruption effect on cognitive performance measured by latency to find the platform at the selected acute post-injury time points (days 3, 4, 5, 6). Performance on the novel object recognition task showed no significant injury-dependent effect or sleep disruption effect on recall of the familiar object at the selected acute post-injury time points (days 3, 7). All groups showed significant increase in novel object exploration at 3 days post-injury compared to training ($F(2,64)=15.61$, $p<0.001$; Figure 4.6; no disruption sham n=6, no disruption injury n=12, sleep disruption sham n=6, sleep disruption injury n=12). There was no significant injury effect or sleep disruption effect on novel object recognition.

Discussion

In the diffuse brain-injured mouse, immediate disruption of post-traumatic sleep does not worsen injury-induced motor or cognitive deficits. Our previous studies showed a significant increase in percent sleep of brain-injured mice compared to uninjured shams over the first six hours following diffuse TBI (Rowe et al. 2013c). Disrupting the six hours of post-traumatic sleep was hypothesized to worsen functional outcome after midline fluid percussion injury, because sleep,

in general, is reparative and restorative (Walker et al. 2005; Tononi and Cirelli 2006; Sheth et al. 2008; Cohen et al. 2009; Chokroverty 2010). In the current study, we show that gentle handling for six hours post-injury to disrupt sleep had no effect on stress measured by corticosterone levels at the end of the disruption period and did not result in rebound of lost sleep or worsened functional outcome. It remains possible that sleep disruption lead to an early surge in corticosterone, which was missed by measuring after six hours of disruption, regardless without functional consequences. Anesthetics used at the time of serum collection may have also contributed to alterations in corticosterone (Jacobsen et al. 2012). Our data provide the first evidence that sleep disruption (immediate, short duration) does not affect functional outcome following diffuse brain injury in mice.

Currently, investigations into acute sleep disruption after TBI are lacking, despite the common practice of disturbing sleep acutely following a concussion. Previous attempts to investigate sleep disruption as a neuroprotective intervention following TBI have been presented in short communication, but methodological issues compromise the interpretation of the data (Martinez-Vargas et al. 2012). Following ischemia, however, acute intervention has been shown to be neuroprotective in the rat (Lay et al. 2010). Whisker stimulation in the immediate post-infarct period (within the first hour) can protect the cortex, suggesting a role for cortical activation through sensory stimulation in determining outcome following middle cerebral artery occlusion (Lay et al. 2010). Contradictory data after focal cerebral ischemia in the rat show that sleep

deprivation for 12 hours using a gentle handling protocol increased infarct area (Gao et al. 2010). Also, sleep disturbance (12 hours a day for three consecutive days) following focal cerebral ischemia in the rat worsened behavioral outcome assessed using the single pellet reaching test through 35 days post-ischemia (Zunzunegui et al. 2011). The findings of these studies suggest a role of sleep modulating recovery processes following stroke (Gao et al. 2010; Zunzunegui et al. 2011). Thus, sleep disturbance immediately following ischemia can worsen outcome, but activating discrete circuits through whisker stimulation can be neuroprotective (Gao et al. 2010; Lay et al. 2010; Zunzunegui et al. 2011), suggesting that additional systemic effects of behavioral sleep deprivation (e.g. elevated temperature and blood pressure) may counteract protective effects of local circuit activation. In this study, we pursued sleep disruption in a clinically relevant manner to model the acute period of post-traumatic sleep disruption and found limited impact on functional outcome.

In midline fluid percussion, injury-induced histopathology is uncomplicated by contusion, cavitation, or overt hemorrhage (Povlishock and Katz 2005; McGinn et al. 2009) and microscopically the injury is characterized by traumatic axonal and vascular injury (Singleton et al. 2002; Kelley et al. 2006; Farkas and Povlishock 2007; Greer et al. 2011; Greer et al. 2012; Greer et al. 2013). Diffuse brain injury also leads to significant changes in behavioral and histological outcome (Semple et al. 2010; Bachstetter et al. 2013). We have previously shown that TBI increases sleep in mice over the first six hours post-injury regardless of injury severity (Rowe et al. 2013c). Since our previous data show

that mild and moderate diffuse TBI result in equivalent increases in sleep, the current study did not include mild TBI. Sleep bouts in brain-injured mice are longer in duration than in uninjured mice (Rowe et al. 2013c). Our previous report of the raw data produced by the non-invasive sleep monitoring cages showed sleep was interrupted by high amplitude and frequency signals corresponding to volitional movement in both brain-injured mice and uninjured shams (Rowe et al. 2013c). Interruptions of sleep bouts by volitional movement indicate the brain-injured animals terminate sleep bouts in a similar manner to uninjured mice, suggesting that brain-injured mice are responsive, capable of movement, and not in a comatose state of unresponsiveness.

For our method of sleep disruption, we used gentle handling to continually disrupt sleep for six hours. Immediate motor deficits following our diffuse injury model (Figure 4.1) make it impractical to use the flower pot or multiple platform method for sleep disruption, because the brain-injured animals cannot perform the task (i.e. balance on the flower pot). Previous studies have shown forced and voluntary exercise can positively and negatively affect behavioral and histological outcomes following brain injury (Zhang et al. 2010; Griesbach 2011; Crane et al. 2012; Griesbach et al. 2012; Yang et al. 2012b; Silva et al. 2013) and for this reason, animals were not disrupted with forced wheel running. For example, treadmill exercise following fluid percussion injury in the rat reduced injury-induced seizures but did not protect against neuronal injury (Silva et al. 2013). Similarly, voluntary wheel running following medial frontal cortical contusions in rats exacerbated TBI-induced deficits (Crane et al. 2012). Our data also indicate

that our method of sleep disruption did not significantly alter stress levels, as indicated by stable corticosterone levels at the conclusion of the disruption period. Corticosterone, a glucocorticoid, is released following stress and exposure to high level glucocorticoids may affect brain plasticity and recovery (Sapolsky and Pulsinelli 1985; McEwen 2008). To this end, a sleep disruption protocol minimized stress and thereby impact on outcomes.

Immediately following the six hour disruption period, percent sleep was measured to determine whether sleep-disrupted mice displayed a rebound of lost sleep. A previous study using a similar sleep disruption protocol for six hours prior to ischemia in rats showed a significant rebound of sleep in the dark cycle following insult (Cam et al. 2013). Sleep was disrupted prior to insult to test if sleep rebound is neuroprotective after ischemia. However, in the present studies sleep disrupted mice did not show a rebound in sleep to compensate for the loss of six hours of sleep. Despite the lack of rebounded sleep, short-term disruption was insufficient to adversely affect outcome. It is likely that both rodent and man can recover from transient sleep disruption after brain injury without significant functional consequence. Sleep disruption prior to injury or for longer durations could worsen outcome.

Clinical data suggest injury-induced circadian rhythm disturbances may contribute to the pathophysiological sequelae of TBI (Ayalon et al. 2007; Paul and Lemmer 2007; Castriotta and Murthy 2011) and inhibit neurogenesis, potentially compromising recovery (Meerlo et al. 2009). Lateral fluid percussion injury has been shown to alter circadian clock gene expression without producing

injury-dependent changes in activity during the first dark cycle post-injury (Boone et al. 2012). Similarly, in our study, we did not observe injury-dependent changes in activity during the first dark cycle. However, sleep disrupted brain-injured and uninjured shams exhibit a delayed activity response during the hour following the first dark-onset post-injury (10 hours post-injury), sleeping significantly more than non-sleep disrupted mice. Sleep disrupted mice slept 10-20% more during this hour, amounting to 6-12 minutes more sleep than the non-sleep disrupted mice. Sleep patterns of the disrupted mice become indistinguishable from the non-disrupted mice by the first light onset (data not shown).

We found that sleep disruption immediately following brain injury did not affect functional outcome. It has been previously shown that experimental diffuse TBI results in significant motor impairment (Bachstetter et al. 2013; Yang et al. 2013). We observed injury-induced sensorimotor impairments measured with the rotarod and neurological severity score tests. Acute sleep disruption, however, neither exacerbated nor attenuated the injury-induced deficits. In our literature evaluation, we found no published reports on the impact of acute sleep disruption on sensorimotor performance in mice.

Similarly, diffuse TBI in mice has been shown to significantly impair cognition as early as two days post-injury with deficits lasting as long as 90 days post-injury (Zohar et al. 2011; Yang et al. 2013). However, cognitive outcome measures in mice following midline fluid percussion injury have not been previously reported. No cognitive impairment following diffuse TBI was measured using the Morris water maze or novel object recognition task, within the first week

post-injury. Following sleep disruption there was no significant change in cognitive performance. It has previously been reported that learning in the MWM is not impacted by six hours of rapid eye movement sleep deprivation (Walsh et al. 2011). Also, sleep disruption before acquisition had no effect on spatial learning and memory components of rodent cognition (Hagewoud et al. 2010; Yang et al. 2012a). These findings are in line with our data indicating six hours of sleep disruption did not alter cognitive function.

Taken together, our data indicated that acute sleep disruption following diffuse TBI did not worsen functional outcome, which precluded further analysis of cellular repair benefits of sleep. These results were not unexpected, since previous studies have reported that six hours of sleep disruption did not impact functional outcome independent of brain-injury (Hagewoud et al. 2010; Walsh et al. 2011). A possible limitation of this study was the disruption of post-traumatic sleep only. Extending the duration of sleep disruption may negatively affect outcome, however, chronic sleep disruption would reduce clinical relevance for the large at-home, non-medically treated population. To address the controversy of whether non-hospitalized individuals should sleep or be frequently awoken following TBI, six hours of sleep disruption following TBI is translationally relevant. Further investigation is needed to determine the impact of sleep disruption on other aspects of post-traumatic symptomology including somatic and emotional function.

Conclusion

In conclusion, the current study demonstrated that disrupting acute post-traumatic sleep following diffuse TBI did not worsen functional outcome. The sleep lost during the disruption period was not recovered by increased sleep time, and there were no long lasting circadian disturbances detected under 12:12 light:dark conditions. Further studies are needed to fully understand the cellular benefit or detriment, if any, of acute post-traumatic sleep on recovery following TBI, as well as other neurological conditions.

Chapter Four has been accepted to be published in the following manuscript:
Rowe RK, Harrison JL, O'Hara BF, and Lifshitz J. (2013). Recovery of neurological function despite immediate sleep disruption following diffuse brain injury in the mouse: clinical relevance to medically untreated concussion.

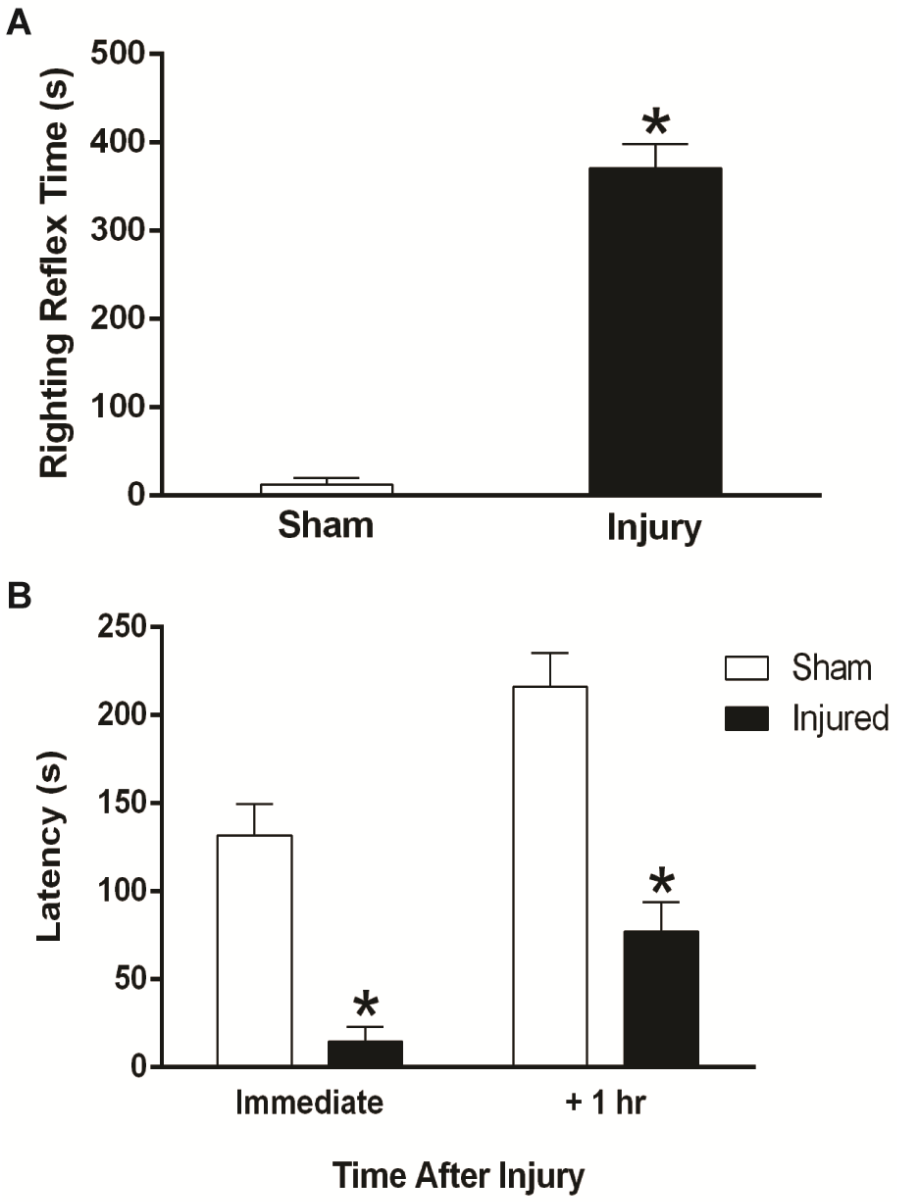


Figure 4.1 Diffuse TBI led to immediate neurological deficits.

(A) Immediately following experimental diffuse TBI in mice there was significant suppression of the righting reflex (mean ±SEM; t(31)=3.351, p=0.0021; sham n=28, injury n=33). **(B)** Immediately following diffuse TBI there was a significant motor deficit measured by latency in the Rotarod task. A repeated-measure two-

way ANOVA showed a significant decrease in latency on the Rotarod in brain-injured mice compared to uninjured shams (mean ±SEM; $F(1, 11)=83.93$, $p<0.0001$). Sidak's multiple comparison test showed a significant difference between brain-injured mice compared to uninjured shams both immediately after injury, and 1 hour after injury (*, $p<0.05$; sham n=6, injury n=7).

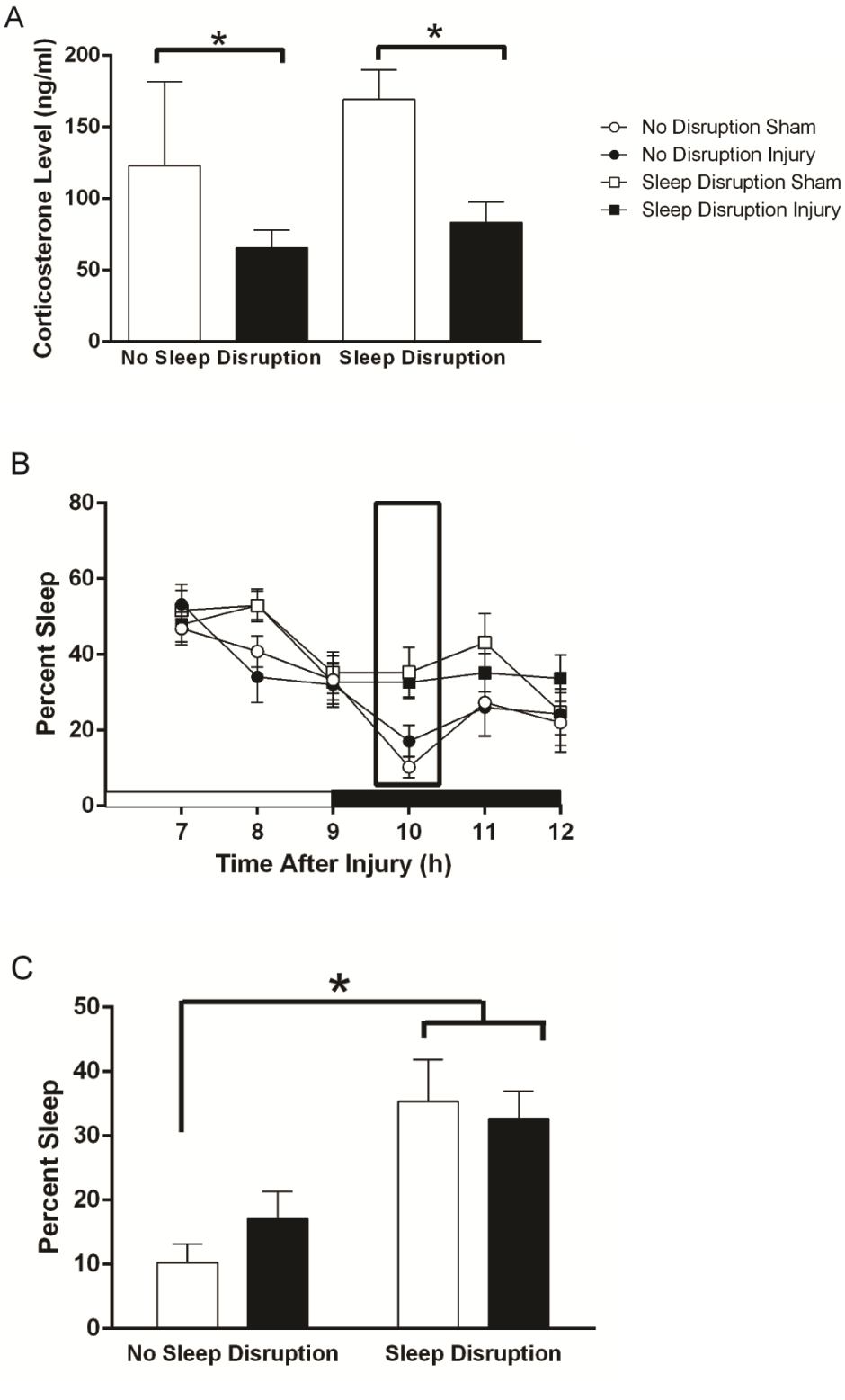

A
Corticosterone Level (ng/ml)
200
150
100
50
0
*
*
No Sleep Disruption
Sleep Disruption
No Disruption Sham
No Disruption Injury
Sleep Disruption Sham
Sleep Disruption Injury
B
Percent Sleep
80
60
40
20
0
7
8
9
10
11
12
Time After Injury (h)
C
Percent Sleep
50
40
30
20
10
0
*
No Sleep Disruption
Sleep Disruption

Figure 4.2 Intentional sleep disruption following diffuse TBI did not result in altered corticosterone levels or a rebound of lost sleep but altered activity response to dark-onset.

(A) Sleep disruption for six hours following diffuse TBI did not alter levels of corticosterone in cardiac blood samples. In both no disruption and sleep disruption groups the brain-injured mice had significantly lower levels of corticosterone compared to uninjured shams (mean ±SEM; $F(1,8)=7.57$, $p=0.0250$). There was no significant difference between sleep disruption and no disruption groups. **(B)** Following six hours of intentional sleep disruption (see methods), a two-way ANOVA showed no significant change in percent sleep between groups (mean ±SEM; $F(3, 32)=2.187$, $p=0.1087$) indicating no rebound of lost sleep. Bar indicates light/dark transition. Box in panel B is enlarged in panel C. **(C)** At dark-onset, a one-way ANOVA showed an effect of sleep disruption (mean ±SEM; $F(3, 32)=0.9386$, $p=0.0024$). Tukey's post-hoc test indicated a difference between no disruption sham compared to both sleep disruption brain-injured and sleep disruption sham mice (*, $p<0.05$; no disruption sham n=7, no disruption injury n=8, sleep disruption sham n=8, sleep disruption injury n=13).

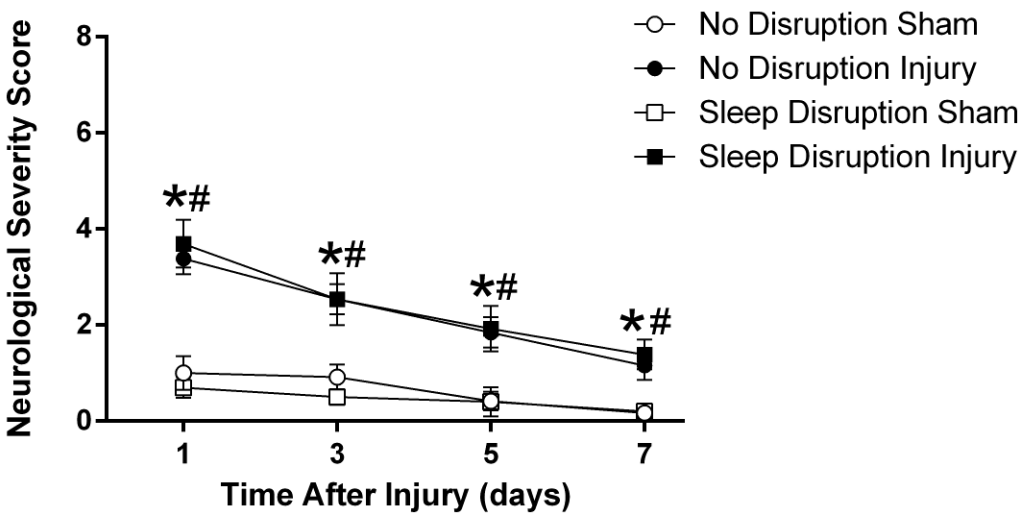


Figure 4.3 Diffuse TBI resulted in neurological impairments independent of acute sleep disruption.

Overall, brain-injured mice showed significant neurological impairments measured by the neurological severity score compared to uninjured shams independent of sleep disruption. Sleep disruption shams showed a significantly lower NSS score compared to both injury groups (sleep disruption injury, no sleep disruption injury) on post-injury days 1,3 and 5 (*; $p<0.05$). On post-injury day 7, sleep disruption shams had a significantly lower NSS score compared to sleep disruption brain-injured mice (*; $p<0.05$). No disruption shams had a significantly lower NSS score compared to both injury groups (sleep disruption injury, no disruption injury) on post-injury days 1 and 7 (#; $p<0.05$). No disruption shams had significantly lower NSS scores compared to no disruption brain-injured mice on post injury days 3 and 5 (#; $p<0.05$). See results for statistics. (no disruption sham n=12, no disruption injury n=13, sleep disruption sham n=10, sleep disruption injury n=13).

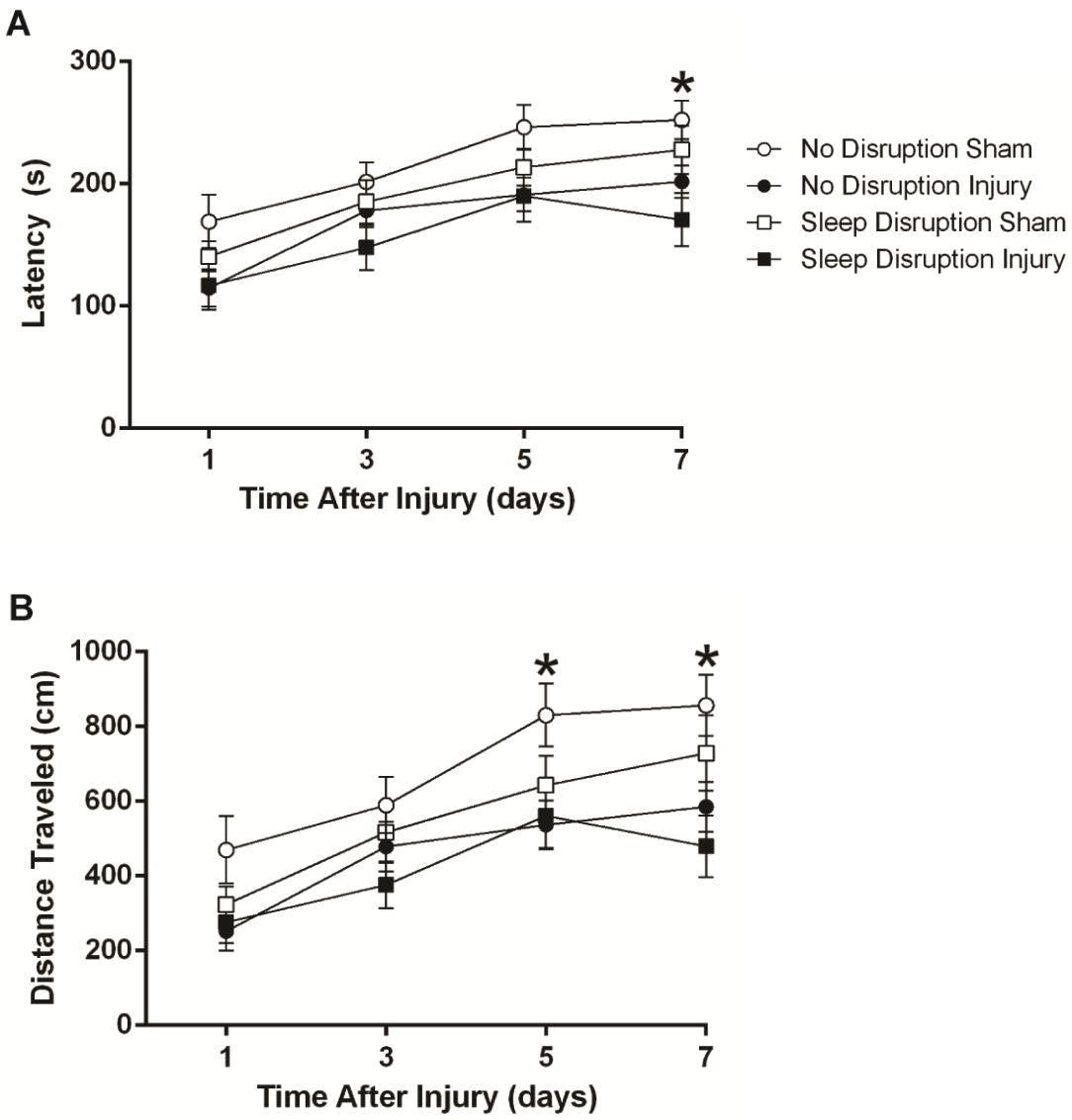


Figure 4.4 Diffuse TBI altered motor performance on the rotarod task independent of acute sleep disruption.

(A) A two-way ANOVA showed a significant injury-dependent effect on latency to stay on the rotarod (mean ±SEM; $F(3, 44)=3.367$, $p=0.0268$) and a time-dependent improvement in latency (mean ±SEM; $F(3, 132=41.60$, $p<0.0001$). Tukey's post-hoc test indicated a difference between sleep disruption brain-injured mice compared to no disruption sham on post-injury day 7 (*, $p<0.05$).

There was no significant difference in latency on the rotarod task between the sleep disruption brain-injured mice and no disruption brain-injured mice ($F(1,24)=0.5033$, $p=0.4849$). **(B)** A two-way ANOVA showed a significant injury-dependent effect on distance traveled (mean ±SEM; $F(3, 44)=4.009$, $p=0.0132$) and a time-dependent improvement in distance traveled (mean ±SEM; $F(3, 132)=34.61$, $p<0.0001$). Tukey's post-hoc test indicated a difference between no disruption sham compared to both injury groups (no disruption and sleep disruption) on post-injury day 5 and 7 (*, $p<0.05$). There was no significant difference in distance traveled on the rotarod task between the sleep disruption brain-injured mice and no disruption brain-injured mice ($F(1,24)=0.2592$, $p=0.6154$). (no disruption sham n=12, no disruption injury n=13, sleep disruption sham n=10, sleep disruption injury n=13).

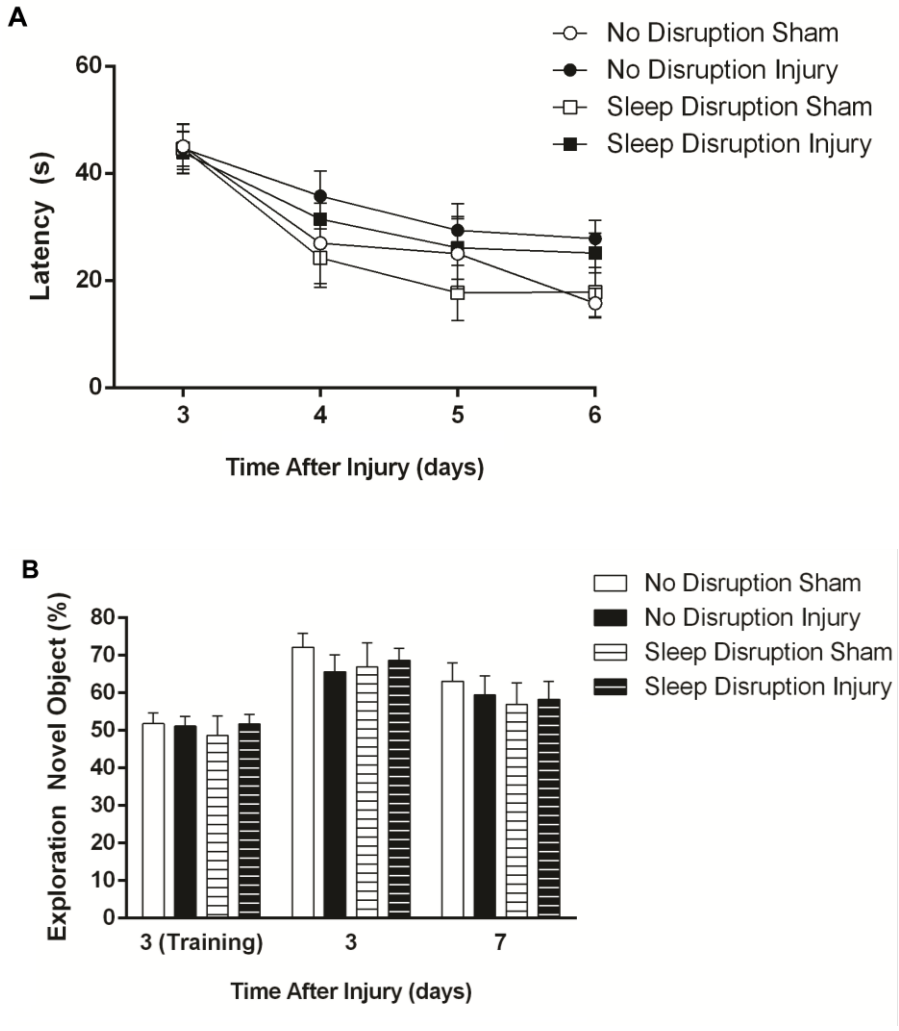


Figure 4.5 Acute sleep disruption following diffuse TBI did not alter cognitive performance on the Morris water maze (MWM) or novel object recognition (NOR) tasks.

(A) A two-way ANOVA showed a significant time-dependent improvement in latency to the platform (mean ±SEM; $F(3, 96)=17.40$, $p<0.0001$). There was no significant injury-dependent effect or sleep disruption effect on cognitive performance at the selected acute post-injury time points. (no disruption sham

n=6, no disruption injury n=12, sleep disruption sham n=6, sleep disruption injury n=12). **(B)** There was no significant injury-dependent effect or sleep disruption effect on cognitive performance at the selected acute post-injury time points. All groups showed a time-dependent learning of the familiar object (mean ±SEM; $F(2,64)=15.61$, $p<0.001$). There was no significant injury effect or disruption effect. (no disruption sham n=12, no disruption injury n=13, sleep disruption sham n=10, sleep disruption injury n=13).

Preface to Chapter Five

In the previous chapters, we showed an injury-induced increase in immediate post-traumatic sleep and demonstrated disrupting this post-traumatic sleep does not worsen functional outcome. The experiments in Chapter Five investigated the effect of acute administration of over-the-counter analgesics on neurological function, acute sleep profiles, and cortical cytokine levels after experimental diffuse TBI in the mouse. A one-time dose was given to replicate clinical settings in which a person sustains a concussion and treats immediate pain as a symptom of injury. Overall, we showed immediate pharmacological intervention did not attenuate or exacerbate TBI-induced functional deficits, but altered sleep profiles. These data may inform clinical recommendations for the at-home treated mildly concussed patient. A more comprehensive outline of the effects of drug intervention on TBI has been included in the Appendix.

Chapter Five: Acute over-the-counter pharmacological intervention does not adversely affect behavioral outcome following diffuse traumatic brain injury in the mouse

Summary

Survivors of mild TBI often do not seek medical attention and may self-treat symptoms of concussion, including post-traumatic headache, by taking over-the-counter (OTC) analgesics. Common OTC analgesic medicines have varying anti-inflammatory properties which could impact acute functional outcomes thought to be related to inflammation, including neurological function

and post-traumatic sleep. Administering one dose of OTC analgesics immediately following experimental brain injury mimics the at-home treated population of concussed patients and may accelerate the understanding of the relationship between brain injury and OTC pharmacological intervention. In the current study, we investigate the effect of acute administration of OTC analgesics on neurological function, acute sleep profiles, and cortical cytokine levels after experimental diffuse TBI in the mouse.

Adult, male C57BL/6 mice were injured using a midline fluid percussion mFPI injury model of concussion (6-10 min righting reflex time for brain-injured mice). Experimental groups included mFPI paired with either ibuprofen (60mg/kg, i.p.; n=16), acetaminophen (40mg/kg, i.p.; n=9), or vehicle (15% ethanol (v/v) in 0.9% saline; n=13) and sham injury paired OTC medicine or vehicle (n=7-10 per group). At 24 hours after injury, functional outcome was assessed using the rotarod task and a modified neurological severity score (NSS), and then related back to acute sleep profiles. Following behavior assessment, cortical cytokine levels were measured by multiplex ELISA. In the diffuse brain-injured mouse, immediate pharmacological intervention did not attenuate or exacerbate TBI-induced functional deficits, but altered sleep profiles. Cortical cytokine levels were not affected by injury or treatment at 24 hours post-injury. These data indicate acute administration of OTC analgesics did not exacerbate or attenuate brain-injury deficits which may inform clinical recommendations for the at-home treated mildly concussed patient.

Introduction

TBI is a major cause of death and disability throughout the world (Langlois et al. 2006; Reilly 2007; Roozenbeek et al. 2013). In the United States between 2002 and 2006, the Centers for Disease Control and Prevention estimated 52,000 deaths, 275,000 hospitalizations, and 1,365,000 emergency department visits resulting from TBI each year (Faul et al. 2010b). It is also estimated that as high as 42% of TBIs are not included in these statistics because 1.2-4.3 million survivors of mild TBI annually do not seek medical attention (Setnik and Bazarian 2007) and likely self-medicate.

Headache is among the most frequently reported symptoms following diffuse brain injury in both adolescents (Butler 2013) and adults (Keidel and Diener 1997; Nicholson and Martelli 2004; Lew et al. 2006; Theeler et al. 2013). Clinical recommendations for treating headache after mild TBI suggest analgesics (e.g. acetaminophen) and warn against non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin and ibuprofen), because of risk for intracranial bleeding (Maiese 2008). Despite the large proportion of mild TBI survivors not seeking medical attention (Setnik and Bazarian 2007) who likely self-medicate for post-traumatic headache, the role of over-the counter pain relief medicines in the course of brain injury is not completely understood.

The mechanical forces of TBI initiate a cascade of secondary injury processes, including inflammation, which continue for days to weeks following injury (Werner and Engelhard 2007). In conflicting studies, cerebral inflammation has been shown to contribute to either beneficial or deleterious effects after

traumatic insult (for review, see (Morganti-Kossmann et al. 2002)). TBI triggers a cascade of inflammation-mediating cytokines (Morganti-Kossmann et al. 2001; Frugier et al. 2010; Semple et al. 2010; Ziebell and Morganti-Kossmann 2010), which can elicit a range of responses including cell differentiation, immune activation, and cell death (Allan and Rothwell 2001). For the present study, mFPI experimental model in the mouse induces multifocal neuropathology with translational application to mild diffuse TBI, or concussion. Principally in the first day after mFPI in mice, we have reported significantly increased levels of pro-inflammatory cytokine IL-1β in the cortex (Rowe et al. 2013c) along with acute neurological impairments manifested within one hour of injury (Rowe et al. 2013a). In diffuse TBI, the effects of clinically relevant acute pharmacological inhibition of inflammation on functional outcome are not yet understood.

Secondary injury processes initiated by traumatic brain injury, including inflammation, are tractable therapeutic targets. Inflammation in the wake of TBI is, in part, mediated by the conversion of membrane-released arachidonic acid into pro-inflammatory prostaglandins by cyclooxygenase-2 (COX-2) (Dash et al. 2000). NSAIDs are widely available over-the-counter drugs used to treat acute pain and inflammation, with mechanisms of action to block COX-1 and/or COX-2, thereby slowing the production of prostaglandins (Vane 1971). Acetaminophen, on the other hand, is presented as an analgesic with actions on cannabinoid receptors (Ottani et al. 2006; Dani et al. 2007), without inflammatory properties. Previous studies suggest anti-inflammatory drugs improve outcome following brain injury as early as 72 hours post-injury (Gopez et al. 2005; Ng et al. 2012;

Thau-Zuchman et al. 2012; Chio et al. 2013; Gatson et al. 2013). Treatment with the highly specific COX-2 inhibitor DFU [5,5-dimethyl-3(3-fluorophenyl)-4(4-methylsulfonyl)phenyl-2(^{5}H)-furanone], administered daily for three days following lateral cortical impact in rats attenuated injury-induced prostaglandin production in the brain and improved functional recovery measured by the Morris water maze and neuroscore at 72 hours post-injury (Gopez et al. 2005). Carprofen, a COX-2 inhibitor, administered daily for seven days following closed head injury (CHI) in mice, also improved functional recovery (Thau-Zuchman et al. 2012). Recovery of function measured by the NSS, however, was not present until 72 hours post-injury (Thau-Zuchman et al. 2012). Treatment with anti-inflammatory minocycline for fourteen days following CHI in mice resulted in improved NSS scores starting at 72 hours post-injury, with improvements lasting through day 7 (Ng et al. 2012). These studies suggest that inhibiting inflammation after mild to severe TBI can improve functional recovery; however, there is evidence to suggest that treatment with ibuprofen over an extended timeframe may worsen cognitive outcome. Rats which were continuously treated with ibuprofen for four months following lateral fluid percussion injury performed significantly worse in the Morris Water Maze than non-treated brain-injured rats (Browne et al. 2006b). Taken together, previous reports indicate that repeated doses of OTC analgesics, depending on the timeframe, may be beneficial or detrimental to recovery from TBI. The acute nature of neurological impairments induced by the mFPI model necessitates acute behavioral analysis to assess the effects of pharmacological intervention (Rowe et al. 2013a). The current study delivers

ibuprofen and acetaminophen to determine if a single treatment with common over-the-counter (OTC) analgesics after diffuse TBI promotes recovery or worsens behavioral outcome.

Several pro-inflammatory cytokines, including IL-1β, also have been characterized as sleep regulatory substances (SRSs) (Krueger and Majde 1995; Krueger et al. 2007). Cytokines with dual roles as SRSs can modulate sleep-wake behavior by acting on sleep circuits of the brain (Krueger et al. 2001a; Krueger et al. 2007). Exogenous IL-1β increases non-REM sleep in rodent models and humans (Tobler et al. 1984; Dinarello 1991; Opp et al. 1991). Inhibiting endogenous IL-1 through the administration of IL-1 antagonists or IL-1 soluble receptors has been shown to inhibit sleep (Opp and Krueger 1994; Takahashi et al. 1996; Fang et al. 1998). In mFPI in the mouse, increased IL-1β occurred concomitantly with a period of increased post-traumatic sleep (Rowe et al. 2013c). A recent study demonstrated that a single dose of ibuprofen administered two hours after TBI significantly lowered IL-1β levels in the brain 24 hours after closed head injury (Keshavarzi et al. 2012). Together, these studies highlight the dynamic relationship between sleep and inflammation in the context of TBI. To this end, inflammation may contribute to acute post-traumatic sleep (Rowe et al. 2013c).

The current study investigates the effects of acetaminophen and ibuprofen—two common analgesic drugs with different anti-inflammatory mechanisms—on neurological function, acute sleep profiles, and cortical cytokine levels after diffuse TBI in the mouse. We hypothesize acute pharmacological

inhibition of injury-induced inflammation will lead to a decrease in inflammatory cytokines and acute post-traumatic sleep, possibly altering functional outcome.

Materials and Methods

Animals

Male C57BL/6 mice (Harlan Laboratories, Inc., Indianapolis, IN) were used for all experiments (n=57). Mice were housed in a 12 h light/12h dark cycle at a constant temperature (23°C ± 2° C) with food and water available *ad libitum* according to the Association for Assessment and Accreditation of Laboratory Animal Care International. Mice were acclimated to their environment following shipment for at least three days prior to any experiments. After surgery, mice were evaluated daily for post-operative care by a physical examination and documentation of each animal's condition. Animal care was approved by the Institutional Animal Care and Use Committees at St. Joseph's Hospital and Medical Center (Phoenix, AZ).

Housing

All mice used in this study were singly housed. Mice were housed in standard individually ventilated cages or housed in the non-invasive sleep-monitoring cage system (Signal Solutions, Lexington, KY).

Midline Fluid Percussion Injury (mFPI)

Mice (20-24g) were subjected to midline fluid percussion injury (mFPI) consistent with methods previously described (Lifshitz 2008). Group sizes are indicated in the results section and figure legends for individual studies. Mice

were anesthetized using 5% isoflurane in 100% oxygen for five minutes and the head of the mouse was placed in a stereotaxic frame with continuously delivered isoflurane at 2.5% via nosecone. While anesthetized, body temperature was maintained using a Deltaphase® isothermal heating pad (Braintree Scientific Inc., Braintree, MA). A midline incision was made exposing bregma and lambda, and fascia was removed from the surface of the skull. A trephine (3 mm outer diameter) was used for the craniotomy, centered on the sagittal suture between bregma and lambda without disruption of the dura. An injury cap prepared from the female portion of a Luer-Loc needle hub was fixed over the craniotomy using cyanoacrylate gel and methyl-methacrylate (Hygenic Corp., Akron, OH). The incision was sutured at the anterior and posterior edges and topical Lidocaine ointment was applied. The injury cap was closed using a Luer-Loc cap and mice were placed in a heated recovery cage and monitored until ambulatory before being returned to their sleep cage.

For injury induction 24 hours post-surgery, mice were re-anesthetized with 5% isoflurane delivered for five minutes. The cap was removed from the injury-hub assembly and the dura was visually inspected through the hub to make sure it was intact with no debris. The hub was then filled with normal saline and attached to a tube connected to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). An injury of moderate severity (1.4 atm) was administered by releasing the pendulum onto the fluid-filled cylinder. Sham-injured mice underwent the same procedure except the pendulum was not released. Mice were monitored for the

presence of a forearm fencing response and righting reflex times were recorded for the injured mice as indicators of injury severity (Hosseini and Lifshitz 2009). The righting reflex time is the total time from the initial impact until the mouse spontaneously rights itself from a supine position. The fencing response is a tonic posturing characterized by extension and flexion of opposite arms that has been validated as an overt indicator of injury severity (Hosseini and Lifshitz 2009). The injury hub was removed and the brain was inspected for uniform herniation and integrity of the dura. The dura was intact in all mice, none were excluded as technical failures. The incision was cleaned using saline and closed using sutures. Moderate brain-injured mice had righting reflex recovery times greater than six minutes and a positive fencing response. Sham injured mice recovered a righting reflex within 20 seconds. After spontaneously righting, mice were placed in a heated recovery cage and monitored until ambulatory (approximately 5 to 15 minutes) before being returned to their cage. Adequate measures were taken to minimize pain or discomfort.

Pharmacological Intervention

All mice received either vehicle or drug treatment immediately following induction of injury or sham. Drugs were administered intraperitoneally in 100µl of sterile vehicle solution of normal saline and 15% (v/v) ethanol. Drug-treated mice received either ibuprofen (60 mg/kg; Sigma-Aldrich, St. Louis, MO) or acetaminophen (40 mg/kg; Sigma-Aldrich, St. Louis, MO). These doses were chosen with respect to clinically relevant doses. Dose translations from human to mice were based on body surface area (Reagan-Shaw et al. 2008) and were

maintained within the maximum daily dose recommended by the United States Federal Drug Administration (www.fda.gov). Both drugs were compared to the same vehicle-treated control group treated with normal saline and 15% (v/v) ethanol.

Behavioral Testing

Rotarod. Sensorimotor function was assessed using the Economex Rotarod system from Columbus Instruments (Columbus, OH). Mice were pre-trained for three consecutive days. The first two days were acclimation (60 sec at 4 RPM for 3 trials) and on day three baseline scores were collected using the test day procedures (see below). For the test at 24 hours post-injury, mice were placed on the rod with a starting speed of 4 RPM, and rod rotation speed was continuously increased over 5 minutes up to a max speed of 28 RPM, as previously published (Bachstetter et al. 2013). The trial ended when the mouse fell from the rod or 5 minutes elapsed. Two trials were performed at each time point. Data are presented (average of two trials) as latency to fall in seconds and total distance traveled in centimeters. Improvement in performance is presented as the difference in each mouse's baseline score and test day score, where positive numbers indicate improvement in the task.

Neurological Severity Score (NSS). Post-traumatic neurological impairments were assessed at 24 hours post-injury using an 8-point NSS paradigm adapted from those previously used in experimental models of TBI (Chen et al. 1996; Semple et al. 2010; Pleasant et al. 2011; Ziebell et al. 2011). One point was given for failure on an individual task, and no points were given if a mouse

completed a task successfully. Mice were observed for hind limb flexion, startle reflex, and seeking behavior (presence of these behaviors was considered successful task completion). Mice traversed in sequence, 3, 2, and 1 centimeter beams. The beams were elevated and mice were given 1 minute to travel 30 centimeters on the beams. The task was scored as a success if the mouse traveled 30 centimeters with normal forelimb and hindlimb position (forelimb/hindlimb did not hang from the beam). Mice were also required to balance on a 0.5 centimeter beam and a 0.5 centimeter round rod for 3 seconds in a stationary position with front paws between hind paws. Non-parametric data are presented as a composite score ranging from 0 to 8 representing performance on all tasks combined. High final NSS scores were indicative of task failure and interpreted as neurological impairment.

Sleep Recordings

The non-invasive sleep cage system (Signal Solutions, Lexington, KY) consisted of 16 separate units that could simultaneously monitor sleep and wake states, as previously published (Rowe et al. 2013c). Each cage unit housed a single mouse inside 18 x 18 centimeter walled compartments with attached food and water compartments (Donohue et al. 2008). The cages had open bottoms resting on Polyvinylidine Difluoride (PVDF) sensors serving as the cage floor (Donohue et al. 2008). The non-invasive high-throughput PVDF sensors were coupled to an input differential amplifier and pressure signals were generated and classified by an algorithm (see below) as motions consistent with either wake activity or the inactivity and regular breathing movements associated with sleep

(Donohue et al. 2008). Briefly, sleep was characterized primarily by periodic (3 Hz) and regular amplitude signals recorded from the PVDF sensors, typical of respiration from a still mouse. In contrast, signals characteristic of wake were both the absence of characteristic sleep signals and higher amplitude, irregular spiking associated with volitional movements. The piezoelectric signals in two second epochs were classified by a linear discriminant classifier algorithm based on frequency and amplitude to assign a binary label of “sleep” or “wake” (Donohue et al. 2008). Mice sleep in a polycyclic manner (more than 40 sleep episodes per hour) (McShane et al. 2010). For experimental studies, mouse sleep was quantified as the minutes spent sleeping per hour, presented as a percentage for each hour. Sleep activity data were binned over specified time periods (e.g. 1 hour) to calculate the average of percent sleep.

Tissue preparation and cytokine quantification

At 24 hours post-injury mice were given an overdose of sodium pentobarbital and transcardially perfused with ice cold phosphate buffered saline (PBS). Mice were decapitated and the brains were dissected on ice. Cortical biopsies (2mm diameter x 2mm thickness) were taken and snap frozen in methanol cooled over dry ice then stored at -80°C. The protein levels of a panel of inflammation-related cytokines were measured by Quansys Biosciences Mouse Cytokine IR Q-Plex assay (Quansys Biosciences, Logan, UT), according to manufacturer protocol. Cortical biopsies were bead-homogenized using a Precellys 24 in 200 µl of ice-cold Tris-buffered lysis solution supplemented with protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Mini Tablet,

Roche Diagnostics, Mannheim, Germany). The cortical homogenate was centrifuged at 3000 RCF for 20 minutes at 4°C in a microcentrifuge. The resulting supernatant (25µl) was loaded per well of the Q-Plex plate, and cytokine levels were determined by Q-Plex assay. Cytokine levels in the cortex were normalized to the total amount of protein in the sample, as determined by BCA Protein Assay (Thermo Scientific, Rockford, IL).

Statistical Analysis

Data are shown as mean ± SEM and analyzed using statistical software (GraphPad-Prism 6). For analysis of behavior and sleep, uninjured shams from all drug treatment groups were combined and used as a single control (see results). Differences in rotarod performance following TBI were determined by one-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test. Non-parametric NSS data were analyzed by Kruskal-Wallis ANOVA, followed by Dunn's comparison post-hoc test (see results). Percent sleep was analyzed using a repeated measure two-way ANOVA followed by Sidak's multiple comparisons test when appropriate. Differences in cytokine concentrations were analyzed by two-way ANOVA. Statistical significance was assigned when $p<0.05$.

Results

It was not anticipated that drug treatment would change functional outcome or mean percent sleep in the uninjured sham mice. Statistical analysis confirmed no significant change in rotatrod performance, neurological severity score, or mean percent sleep between any sham treatment groups. Vehicle-

treated, ibuprofen-treated, and acetaminophen-treated shams were combined into a single control. As anticipated, anti-inflammatory treatment altered cytokine levels in sham treatment groups; cytokine data were analyzed without combining shams.

Diffuse TBI reduced motor performance on the rotarod task regardless of pharmacological intervention.

To assess motor function we used the rotarod task as previously published (Bachstetter et al. 2013). Across groups, there was a significant effect on latency to stay on the rotarod ($F(3, 53)=3.688$, $p=0.0174$; Figure 5.1A; sham n=27, vehicle-treated injury n=10, ibuprofen-treated injury n=12, acetaminophen-treated injury n=8). Rotarod latency was significantly reduced in vehicle-treated and ibuprofen-treated brain-injured mice compared to sham mice at 24 hours post-injury (Figure 5.1A). There was no significant latency reduction in acetaminophen-treated brain-injured mice compared to shams (Figure 5.1A). Further analysis of rotarod performance confirmed the latency data with distance traveled, showing similar significant effects on distance traveled ($F(3, 53)=3.909$, $p=0.0135$; Figure 5.1B). Distance traveled was significantly reduced in both vehicle and ibuprofen-treated brain-injured mice compared to uninjured sham. There was no difference in distance traveled by acetaminophen-treated brain-injured mice compared to shams. To compensate for trial-based learning, improvement in motor performance was analyzed. Latencies (Figure 5.1C) and distances (Figure 5.1D) of each mouse at 24 hours post-injury were compared to their individual baseline scores at training. Brain-injured mice treated with vehicle

and ibuprofen showed significantly less improvement in latency to stay on the rod compared to the improvement of uninjured shams ($F(3, 53)=4.553$, $p=0.0065$; Figure 5.1C). Acetaminophen-treated brain-injured mice did not show a difference in improvement compared to uninjured shams (Figure 5.1C). All brain-injured mice, regardless of treatment, showed significantly less improvement in distance traveled compared to shams ($F(3, 53)=6.017$, $p=0.0013$; Figure 5.1D). Overall, diffuse brain injury reduced motor performance measured on the rotarod task, without an effect of post-injury pharmacological treatment.

Diffuse TBI resulted in neurological impairments regardless of pharmacological intervention.

All brain-injured mice showed significant neurological impairments measured by the neurological severity score (NSS) compared to uninjured shams, regardless of pharmacological intervention ($KW(4, 57)=27.37$, $p<0.001$; Figure 5.2; sham n=27, vehicle- treated injury n=10, ibuprofen-treated injury n=12, acetaminophen-treated injury n=8). At 24 hours post-injury all brain-injured groups had significantly higher NSS scores compared to uninjured shams. There was no significant effect of post-injury pharmacological treatment.

Pharmacological intervention led to increased post-traumatic sleep following diffuse brain injury.

Sleep-wake activity was recorded non-invasively immediately following diffuse brain injury or sham injury. Initial analysis focused on the first six hours, as post-traumatic sleep was increased in our previous reports (Rowe, 2013). Acetaminophen- treated brain-injured mice had significant increases in sleep

during the first six hours post-injury compared to uninjured shams (mean ±SEM; $F(1, 29)=11.98$, $p=0.0017$; Figure 5.3C). There was no significant increase in the vehicle-treated ($F(1, 30)=0.8886$, $p=0.3534$; Figure 5.3A) or ibuprofen-treated ($F(1, 30)=1.853$, $p=0.1835$; Figure 5.3B) groups compared to shams. Further analysis of sleep focused on the dark cycle following injury (the time the nocturnal mice should be awake), to evaluate the mouse equivalent of 'human daytime sleepiness'. There was a significant increase in ibuprofen-treated brain-injured mice ($F(1, 30)=4.540$, $p=0.0410$; Figure 5.4B) compared to uninjured shams. While the acetaminophen-treated brain-injured group showed an increase in mean percent sleep compared to sham, this increase failed to reach statistical significance ($F(1, 29)=3.939$, $p=0.0567$; Figure 5.4C). Unexpectedly, the vehicle-treated mice had a significant decrease in sleep ($F(1, 30)=4.455$, $p=0.0432$; Figure 5.4A) following brain injury compared to uninjured shams. Overall, brain-injured mice had increased mean percent sleep during the dark cycle following drug treatment.

Inflammation-related interleukin cytokines were not increased in the cortex at 24 hours post-injury.

Upon brain dissection, no differences in hemorrhage or gross pathology were noted between groups. A panel of inflammatory related interleukin cytokines was evaluated at 24 hours post-injury in whole cortex. There was no statistically significant increase or decrease in protein concentrations of cytokines in the cortex of brain-injured mice compared to uninjured shams (Table 5.1). Cytokines evaluated included: IL-1α ($F(1, 23)=1.996$, $p=0.1711$), IL-1β ($F(1,$

23)=0.01040, p=0.9197), IL-2 (F(1, 23)=0.2045, p=0.6554), IL-6 (F(1, 23)=0.3326, p=0.5697), IL-10 (F(1, 23)=0.3581, p=0.5554) and IL-12 (F(1, 23)=0.1672, p=0.6864). IFNγ, IL-4, and TNFα levels were also evaluated, but concentrations were undetectable.

Discussion

In the diffuse brain-injured mouse, immediate pharmacological intervention with over-the-counter analgesics did not adversely affect sensorimotor or neurological outcome. A single, clinically relevant dose of ibuprofen or acetaminophen was hypothesized to reduce early inflammation and prevent (or delay) the increased injury-induced sleep necessary for recovery, thereby leading to a worsened functional outcome. In the current study, we show immediate treatment with ibuprofen or acetaminophen increased sleep within the first 24 hours of injury, without impacting TBI-induced functional deficits measured by the rotarod and neurological severity score (NSS). We also show drug treatment did not alter expression of cortical cytokines at 24 hour post-injury.

Given the majority of human TBI encompasses mild to moderate diffuse brain injury for which self-medication may be the primary treatment, the current study sought to investigate the clinical situation in which a survivor of mild TBI self-treats with a single dose of an OTC analgesic medicine. The most frequent symptom after TBI is post-traumatic headache TBI (Theeler et al. 2013), making ibuprofen and acetaminophen principal choices for self-medication. Administering one dose of over-the-counter (OTC) analgesics immediately following brain injury

mimics the at-home treated population of concussed patients and may accelerate the understanding of the relationship between brain injury and OTC pharmacological intervention. Administering ibuprofen, an NSAID and COX inhibitor, in opposition to administering acetaminophen, an analgesic with weak anti-inflammatory properties, allowed for the investigation of inflammation inhibition on brain injury-induced deficits.

While clinical and experimental data suggest the chronic over-production of pro-inflammatory cytokines contributes to the progression of pathology in TBI (Schmidt et al. 2005; Lloyd et al. 2008; Cao et al. 2012), the role of immediate inflammation is less clear. Inflammation is critical to the repair process and health of the organism, however, inflammation that is excessive or prolonged can exacerbate damage after the primary injury (Bachstetter et al. 2013). Previous reports have shown that multiple doses of analgesics can alter not only functional outcome but also cellular mechanisms following experimental TBI, see review (Rowe et al. 2013b). In this study, a single dose of ibuprofen or acetaminophen given at the time of injury did not attenuate or exacerbate injury-induced sensorimotor or neurological deficits measured 24 hours post-injury. Previous studies suggest anti-inflammatory drugs can improve outcome following brain injury as early as 72 hours post-injury (Gopez et al. 2005; Ng et al. 2012; Thau-Zuchman et al. 2012; Chio et al. 2013; Gatson et al. 2013). Treatment with the highly specific COX-2 inhibitor DFU [5,5-dimethyl-3(3-fluorophenyl)-4(4-methylsulfonyl)phenyl-2(^{5}H)-furanone], following lateral cortical impact in rats attenuated injury-induced prostaglandin production in the brain and improved

functional recovery measured by the Morris water maze and neuroscore at 72 hours post-injury (Gopez et al. 2005). Carprofen, a COX-2 inhibitor, administered following closed head injury (CHI) in mice, also improved functional recovery (Thau-Zuchman et al. 2012). Recovery of function measured by the NSS, however, was not present until 72 hours post-injury (Thau-Zuchman et al. 2012). Treatment with anti-inflammatory minocycline following CHI in mice resulted in improved NSS scores starting at 72 hours post-injury, with improvements lasting through day 7 (Ng et al. 2012). These studies suggest that inhibiting inflammation can improve functional recovery. While the administration of analgesics has been primarily shown to positively influence functional outcome, these studies have incorporated multiple dosing strategies either before or after TBI. While the results are experimentally valid, they do not address the situation faced by a mildly concussed individual not seeking medical attention. In this scenario, an individual would likely self-treat prominent symptoms, including headache, with OTC analgesics immediately post-injury. Experimentally, it would be expected that a single dose of OTC analgesics would have less profound effects upon outcome than a more aggressive dosing strategy.

In the current study, we found that a single dose of OTC analgesics did not attenuate or exacerbate TBI induced functional deficits. Sensorimotor deficits measured by the Rotarod task were present in brain-injured groups compared to uninjured shams regardless of drug treatment at the time of injury. Similarly, brain-injured groups had neurological deficits measured by a modified NSS compared to uninjured shams regardless of drug treatment. Multiple studies have

shown analgesics to provide neuroprotection from TBI when administered continually, such that a single clinically relevant dose of OTC analgesics does not affect the pathophysiological and molecular cascades induced by diffuse brain injury. In this way, any initial inhibition of inflammation provided by a single analgesic dose may not prevent the development of neurological deficits by 24 hours post-injury. It is also possible that the route of drug administration used in this study reduced the bioavailability of the compounds. Alternate administration routes could increase the bioavailability of the drugs, and should be considered for future studies, recognizing the reduced clinical applicability. Overall, this study shows one dose of OTC analgesics given immediately following injury does not alter functional outcome. Given that the OTC analgesics administered in the current study did not worsen behavioral outcome, they may be safe for the clinical treatment of post-traumatic symptoms. It is of note, though, that some anti-inflammatory drugs, including ibuprofen, are not indicated for clinical use after TBI due to their anti-coagulant effects increasing the possibility of intracranial bleeding (Maiese 2008).

Using our injury model, we have previously demonstrated an injury-induced increase in inflammatory cytokines which have dual roles as SRSs (Bachstetter et al. 2013; Rowe et al. 2013c) as well as an increase in post-traumatic sleep starting at the time of injury and persisting for the first six hours (Rowe et al. 2013c). In the current study, we show a significant increase in post-traumatic sleep over the first six hours following injury in the acetaminophen-treated brain-injured mice compared to the uninjured shams. This increase in

sleep is not present in the ibuprofen-treated brain-injured mice. Ibuprofen, an NSAID, is used to treat both pain and inflammation (Wyatt et al. 2012). Ibuprofen may inhibit the injury-induced immediate increase of SRS cytokines, thereby preventing the increase of post-traumatic sleep in the first six hours post-injury. Acetaminophen, an analgesic with weak anti-inflammatory properties, would not inhibit the upregulation of pro-inflammatory cytokines as seen by an increase in inflammation-induced sleep. However, the study design precluded measurements of cytokines prior to 24 hours. Unexpectedly, the vehicle-treated brain-injured mice did not have an injury-induced increase in post-traumatic sleep as previously published by our lab (Rowe et al. 2013c). It is possible that the vehicle itself or stress from the injections contributed to this unexpected sleep profile. It is possible that using low doses of ethanol as the vehicle contributed to decreases in mean percent sleep. Clinical studies indicate low doses of ethanol have contributed to decreases in total sleep (Roehrs et al. 1999; Geoghegan et al. 2012).

Further analysis of sleep profiles examined mean percent sleep during the dark cycle following injury, a time when nocturnal mice are usually awake. Disturbances during this period would be compared to excessive daytime sleepiness in humans, which is a commonly reported clinically after TBI (Castriotta et al. 2007; Verma et al. 2007; Baumann 2012). Data indicate that vehicle-treated brain-injured mice slept significantly less than uninjured shams during this time. In contrast, increases in mean percent sleep were observed in both ibuprofen and acetaminophen-treated brain-injured mice compared to

uninjured shams, although mean percent sleep in acetaminophen treated mice failed to reach significance (p=0.0567). It is possible increasing the number of mice in each group would reduce variability and this increase would reach significance. This drug-injury interaction resulting in increased dark cycle sleep models excessive daytime sleepiness not seen in vehicle-treated brain-injured mice.

Inflammatory cytokines such as IL-1β, IL-6, and TNF-α promote sleep (Krueger et al. 1995; Fang et al. 1998; Krueger et al. 2001a; Yasuda et al. 2005; Krueger 2008). Our experimental model of concussion has shown elevated levels of pro-inflammatory cytokines peaking between three and nine hours post-injury (Bachstetter et al. 2013; Rowe et al. 2013c). In the current study, we measured a panel of inflammation related interleukins at 24 hours post-injury to investigate the presence or absence of inflammation at the time behavioral testing was completed. As expected, based on previous temporal associations of injury-induced cytokine levels in our injury model, there were no significant changes in cytokine levels at 24 hours post-injury in any brain-injured group compared to uninjured shams. Inflammation mediates behavioral, biochemical, and systemic changes which collectively make up the acute phase response, characterized by fever and changes in metabolism and sleep patterns (Gabay and Kushner 1999). Our study measured cytokines identified as key regulators of the acute phase response including IL-1β, IL-6, IL-8, and TNFα (Gabay and Kushner 1999). In line with previous findings (Keshavarzi et al. 2012), there were no significant changes in these cytokines at 24 hours post-injury, suggesting that acute

inflammation following experimental diffuse brain injury has resolved, which may or may not emerge at later time points.

Overall, immediate pharmacological intervention following brain injury did not adversely impact functional outcome as indicated by performance on the rotarod and NSS task. Further investigation is needed to determine if multiple doses of over-the-counter analgesics attenuate injury-induced deficits. It is possible that chronic treatment may impact the course of recovery following TBI. Ibuprofen administered chronically over a four month period to rats subjected to FPI led to a decline in cognitive function, as measured by the Morris water maze (Browne et al. 2006b). Future studies should extend the functional evaluation beyond 24 hours post-injury. It is possible that the single dose given in this study may have improved or worsened functional outcome at later post-injury time points.

Conclusion

In the diffuse brain-injured mouse, immediate pharmacological intervention altered sleep profiles, but did not attenuate or exacerbate TBI-induced functional deficits. This study is significantly limited by the single 24 hour time point. Our previous studies document a rapid increase in inflammatory cytokines; however, this study only investigates inflammation-related interleukin cytokine levels at 24 hours after injury. At this time point it is not possible to conclude whether the anti-inflammatory drug affected the expression of inflammatory cytokines or not. Future studies should measure cytokine levels at earlier time points (i.e. 6, 9 hours) in order to identify the relationship between the

tested anti-inflammatory drugs and their effect on inflammatory cytokine. Furthermore, inflammation at later time points, including the 24 hour point, should be evaluated using the presence of microglia activation. This would be a more a useful measure of inflammation that extends beyond the initial cytokine surge. While the one-time drug dose offers clinical relevance, it is possible chronic administration would have improved functional outcome. There may also be a more optimal one time dosing of the OTC drugs that was overlooked in the preparation of this study. Previous work in a mouse model of TBI suggests a lower one-time dose following TBI was beneficial to neurological outcome (Hall 1985). We conclude that inhibition of immediate injury-induced inflammation may prevent an early increase in post-traumatic sleep but does not adversely affect functional outcome. Further investigation is needed to examine the role of immediate post-traumatic sleep on recovery following TBI.

Chapter Five is in review in the following manuscript:
Harrison JL, Rowe RK, O'Hara BF, Adelson PD, and Lifshitz J. (2013). Over-the-counter pharmacological intervention does not adversely affect outcome following diffuse traumatic brain injury in the mouse.

Chapter Five: Figures

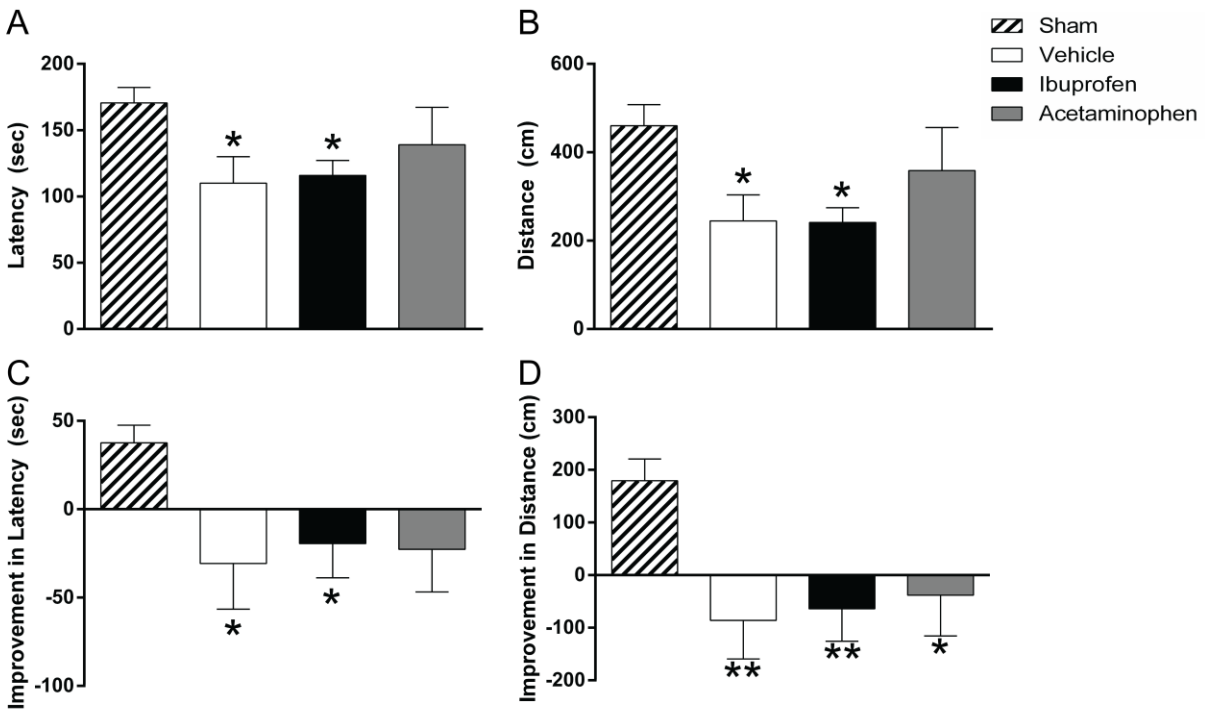


Figure 5.1 No adverse effects of pharmacological intervention on injury-induced motor deficits on the rotarod task.

(A) Injury significantly impaired motor performance as indicated by reduced latency to stay on the rotarod (mean ±SEM; $F(3, 53)=3.688$, $p=0.0174$), with significant differences between vehicle-treated and ibuprofen-treated brain-injured mice compared to uninjured shams at 24 hours post-injury. There was no significant difference between acetaminophen-treated brain-injured mice compared to uninjured shams. **(B)** Reduced distance traveled on the rotarod also indicated a significant injury-induced impairment in motor function (mean ±SEM; $F(3, 53)=3.909$, $p=0.0135$). There was a significant difference between

vehicle-treated and ibuprofen-treated brain-injured mice compared to uninjured shams at 24 hours post-injury. There was no difference in distance traveled by acetaminophen-treated brain-injured mice compared to uninjured shams. **(C)** Injury significantly impaired improvement in latency to stay on the rotarod from baseline (mean ±SEM; $F(3, 53)=4.553$, $p=0.0065$) indicated by a difference between vehicle-treated and ibuprofen-treated brain-injured mice compared to uninjured shams at 24 hours post-injury. **(D)** Injury also significantly impaired improvement in distance traveled (mean ±SEM; $F(3, 53)=6.017$, $p=0.0013$) between vehicle-treated, ibuprofen-treated, and acetaminophen-treated brain-injured mice compared to uninjured shams at 24 hours post-injury. (sham n=27, vehicle-treated injury n=10, ibuprofen-treated injury n=12, acetaminophen-treated injury n=8; *, $p<0.05$; **, $p<0.01$).

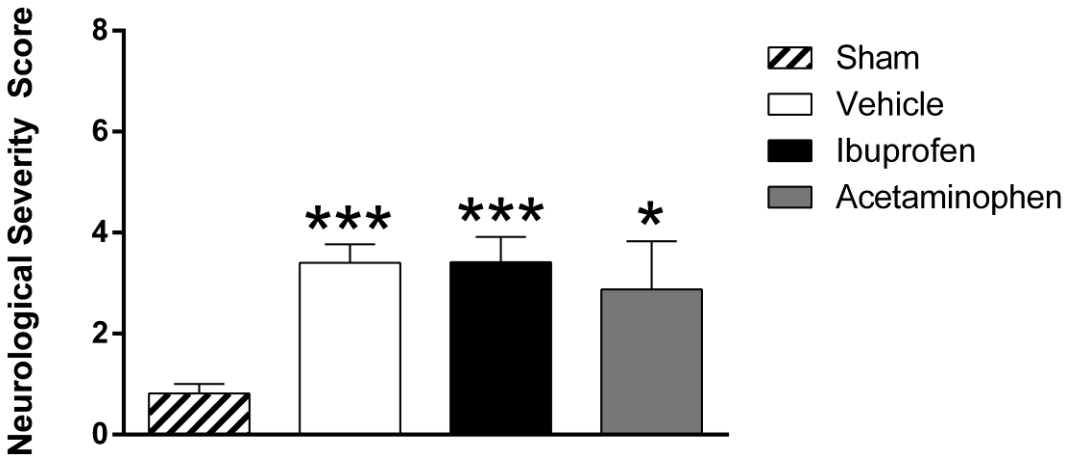


Figure 5.2 No adverse effects of pharmacological intervention on injury-induced neurological impairments.

Significant neurological impairments were detected between groups, as measured by modified neurological severity score (mean ±SEM; KW(4, 57)=27.37, $p<0.001$). Dunn's multiple comparisons test indicated vehicle-treated, ibuprofen-treated, and acetaminophen-treated brain-injured mice showed significantly higher NSS scores compared to uninjured shams 24 hours post-injury. There were no significant changes in function between any brain-injured groups regardless of treatment. (sham n=27, vehicle-treated injury n=10, ibuprofen-treated injury n=12, acetaminophen-treated injury n=8; *, $p<0.05$; ***, $p<0.001$).

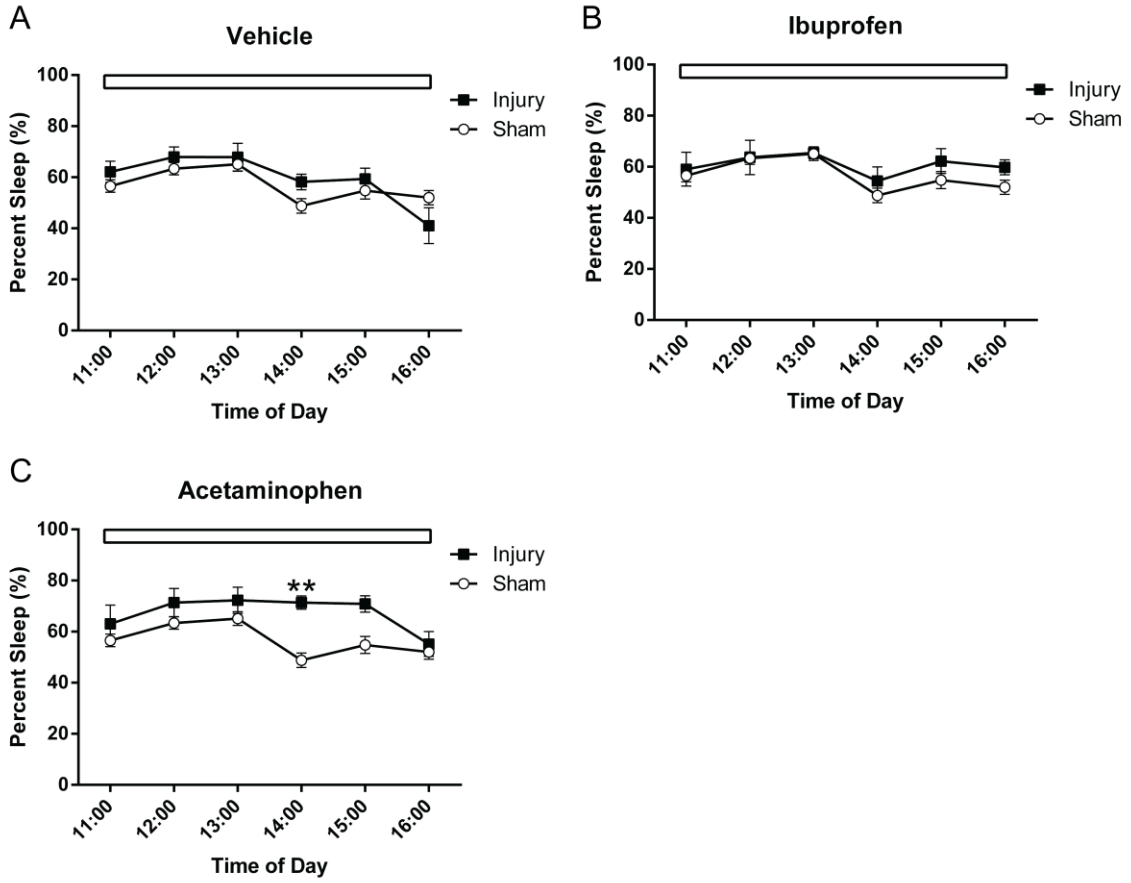


Figure 5.3 Acetaminophen-treated mice slept significantly more than uninjured mice in the six hours immediately following diffuse TBI.

In the first six hours post-injury no significant change was detected in percent sleep between **(A)** vehicle-treated brain-injured (mean ±SEM; $F(1, 30)=0.8886$, $p=0.3534$) as well as **(B)** ibuprofen-treated brain-injured mice (mean ±SEM; $F(1, 30)=1.853$, $p=0.1835$) compared to uninjured sham groups. **(C)** Acetaminophen-treated brain-injured mice slept significantly more compared to uninjured shams (mean ±SEM; $F(1, 29)=11.98$, $p=0.0017$). Additionally, the difference in percent sleep between injured and uninjured mice was detected at 14:00. (sham n=26,

vehicle-treated injury n=6, ibuprofen-treated injury n=6, acetaminophen-treated injury n=5; **, $p<0.01$)

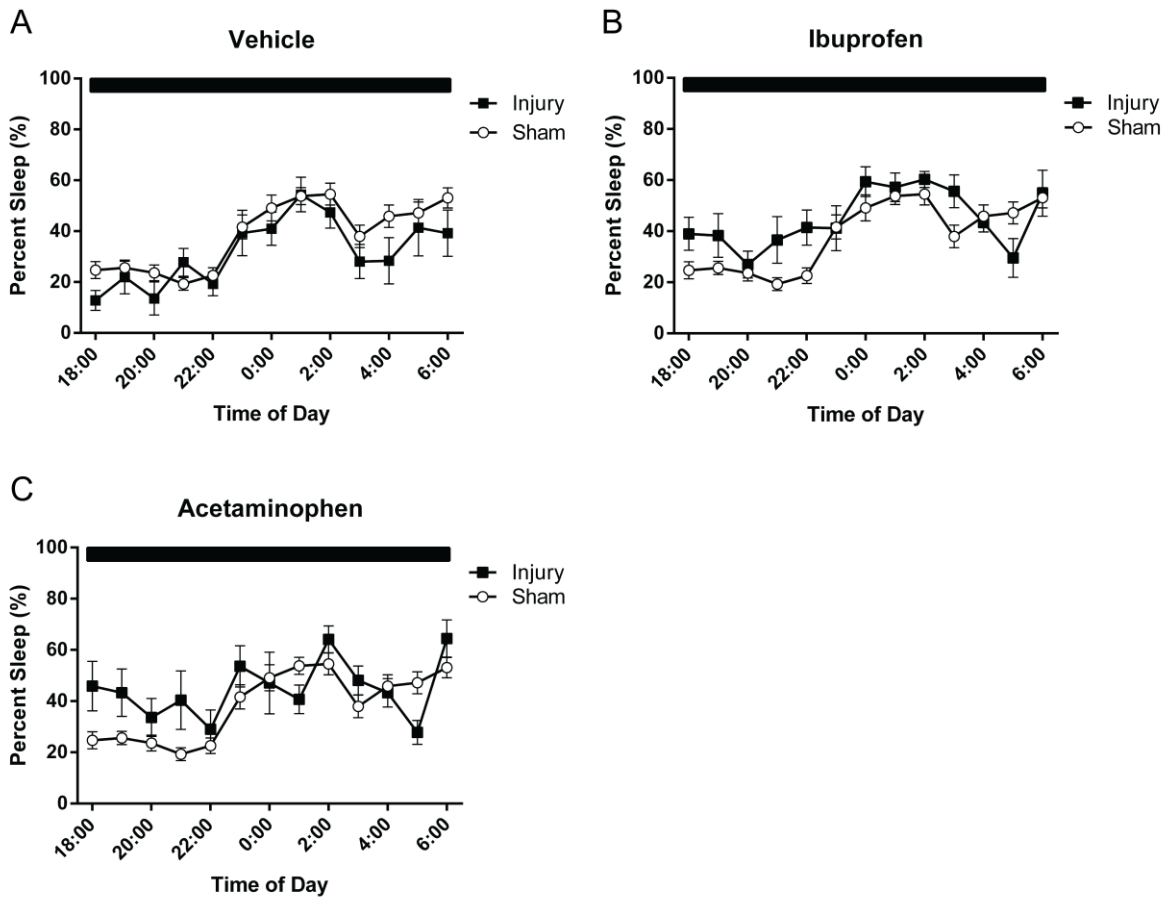


Figure 5.4 Vehicle-treated mice slept significantly less and ibuprofen-treated mice slept significantly more during the first dark-cycle post-injury compared to uninjured shams.

(A) In the first dark cycle post-injury there was a significant decrease in percent sleep between vehicle-treated brain-injured mice (mean ±SEM; $F(1, 30)=4.455$, $p=0.0432$) compared to uninjured shams. **(B)** Ibuprofen-treated brain-injured mice had a significant increase in mean percent sleep (mean ±SEM; $F(1, 30)=4.540$, $p=0.0410$) compared to uninjured shams. **(C)** Increases in mean percent sleep of acetaminophen-treated brain-injured mice compared to uninjured shams failed to reach significance (mean ±SEM; $F(1, 29)=3.939$,

p=0.0567). (sham n=26, vehicle-treated injury n=6, ibuprofen-treated injury n=6, acetaminophen-treated injury n=5).

Treatment	IL-1α	IL-1β	IL-2	IL-6	IL-10	IL-12
Vehicle Sham	134.65 ±18.73	432.93 ±106.84	173.42 ±37.77	201.85 ±95.00	46.97 ±4.92	40.43 ±4.30
Vehicle Injured	151.68 ±35.24	299.37 ±81.64	100.18 ±31.31	232.21 ±30.40	41.77 ±6.31	34.23 ±5.26
Ibuprofen Sham	111.54 ±12.28	315.70 ±89.79	130.70 ±26.19	120.85 ±82.82	41.36 ±3.46	35.64 ±2.96
Ibuprofen Injured	158.82 ±47.05	372.59 ±145.99	129.45 ±34.28	353.24 ±291.66	46.89 ±2.91	40.25 ±2.45
Acetaminophen Sham	96.57 ±12.01	370.85 ±38.18	130.67 ±21.80	107.92 ±45.07	39.79 ±4.56	34.43 ±4.03
Acetaminophen Injured	123.37 ±14.87	470.77 ±39.65	168.92 ±39.53	40.14 ±40.14	46.24 ±5.35	40.07 ±4.96

Table 5.1 Inflammation-related interleukin cytokines were not altered in the cortex at 24 hours post-injury.

Data are presented as concentration levels (mean ±SEM; pg/ml/mg). No injury-induced increases or decreases in cortical interleukins were detected 24 hours post injury. There were also no changes in cortical cytokine levels among vehicle and drug treated groups. **IL-1α** ($F(1, 23)=1.996$, $p=0.1711$), **IL-1β** ($F(1, 23)=0.01040$, $p=0.9197$), **IL-2** ($F(1, 23)=0.2045$, $p=0.6554$), **IL-6** ($F(1, 23)=0.3326$, $p=0.5697$), **IL-10** ($F(1, 23)=0.3581$, $p=0.5554$), **IL-12** ($F(1, 23)=0.1672$, $p=0.6864$). IFNγ, IL-4, and TNFα levels were evaluated, but found to be undetectable. (vehicle sham n=5, acetaminophen sham n=5, ibuprofen sham n=5, vehicle- treated injury n=4, ibuprofen-treated injury n=5, acetaminophen-treated injury n=5).

Chapter Six: Overall Conclusions

The physiological consequences of TBI often develop into neurological impairments which impact outcome and quality of life of survivors. While acute sleep disturbances are among one of the most commonly reported clinical neurological impairments following TBI, there is a lack of investigations into the duration, manipulation, and implications of this post-traumatic sleep. The work of this thesis was designed as a first step in identifying the role of post-traumatic sleep in recovery from diffuse brain injury. Data from these studies outline an animal model of diffuse TBI which produces an increase in acute sleep coinciding with the frequently reported symptom of excessive sleepiness post-concussion in man. Further, the studies included in this thesis manipulated aspects of post-traumatic sleep in order to identify its contribution to the post-injury recovery process. Combined, this creates a framework of preliminary studies on which future work in the field of sleep and TBI can be built.

In summary, we have shown diffuse brain injury promotes acute post-traumatic sleep in the mouse, and the secondary injury related inflammatory processes coincide with this increase. Second, we determined the injury-induced increase in sleep was present during the first week post-injury, but was not maintained thereafter using this injury model. Third, disrupting acute post-traumatic sleep following diffuse TBI did not worsen functional outcome. Fourth, immediate pharmacological intervention altered sleep profiles, but did not attenuate or exacerbate TBI-induced functional deficits.

Cumulatively, our studies indicate experimental diffuse brain injury increased sleep immediately following injury, extending through the first week post-injury. Data showed the injury-induced increase in sleep is not a primary event. Analyzing post-traumatic sleep in five minute intervals showed the increase in mean percent sleep over the first-hour post-injury was time dependent. This supports the conclusion that injury-induced cellular cascades contribute to the overall sleep increase. If the primary impact solely contributed to post-traumatic sleep, then an immediate increase in post-traumatic sleep to a maximum level would have been observed.

Secondary injury responses were temporally associated with the observed increase in post-traumatic sleep. IL-1β increased immediately following injury and returned to baseline by twelve hours. We also show activation of microglia at six hours post-injury. Activated microglia can contribute to the production of IL-1β, which in turn acts locally to affect neuronal assemblies, altering their functional status and acting on sleep regulatory circuits (Krueger et al. 2007). Our injury model induces an activation of microglia for the first seven days post-injury (Ziebell et al. 2012). It is likely that activated microglia act as effectors of circuit disruption, extended beyond local circuits to sleep regulatory circuits contributing to an increase in sleep (Krueger and Majde 1995; Krueger et al. 2001a), which did not persist in our model.

Evidence of microglia activation during the first week post-injury (Ziebell et al. 2012) supports the hypothesis that microglia signaling processes may contribute to the overall increase in sleep during the first week post-injury which

is not extended into weeks two through five. Chapter Three concludes our injury model does not successfully produce chronic sleep disturbances documented by clinical studies. This discordance of mouse data with clinical data suggests this mouse model does not adequately reflect the human condition. It is possible that using an animal that does not exhibit polycyclic sleep pattern, may be more appropriate to model the chronic sleep disturbance reported by TBI survivors. It is also possible a more severe experimental brain injury may be necessary to induce secondary damage that develops into sleep disturbances at chronic time points.

The present body of work also demonstrated disruption of immediate post-traumatic sleep does not worsen injury-induced motor or cognitive deficits. It is likely that both rodents and humans can recover from transient sleep disruption after brain injury without significant functional consequence. It is possible that sleep disruption prior to injury of longer durations could contribute to a worsened outcome. The results from this dissertation suggest an increase in immediate post-traumatic sleep, extending throughout the first week post-injury, is a natural response to brain injury. In light of the progress toward understanding the role of post-traumatic sleep in the recovery from TBI, further experimentation is required to determine the cellular benefit or detriment, if any, of acute post-traumatic sleep. While sleep may aid in recovery, from Chapter Four we conclude disruption of immediate post-traumatic sleep does not worsen outcome in our injury model.

Limitations of Chapter Four include the lack of injury induced cognitive deficits. Following diffuse brain injury mice did not show a cognitive deficit within the 7 day time point. It is possible that our injury model induces circuit disruption and that the reorganization of these circuits at later time points (i.e. 28 days) post-injury may produce measurable injury-induced cognitive deficits. Following diffuse brain injury with our model, minimal damage to the hippocampus contributes to a lack in cognitive deficit at 7 days. The limited cognitive deficit prevents us from determining if disrupting post-traumatic sleep worsened cognitive outcome. Cognitive behavior needs to be evaluated at multiple time points extending chronically to investigate the possibility of late onset cognitive deficits with our injury model. Establishing a timeline of the development of cognitive deficits following diffuse brain injury would facilitate future studies in order to draw a conclusion whether sleep disruption after TBI worsens cognitive outcome following diffuse injury.

The studies presented in this dissertation also showed a single dose of analgesics administered following TBI is not sufficient to prevent the development of injury-induced deficits, nor did the administration exacerbate impairments. Following a mild concussion, OTC drugs are taken to relieve headache associated with the injury. These studies focus on the individuals relieving immediate pain as opposed to survivors self-medicating to treat chronic headaches associated with TBI. Drug administration did contribute to changes in sleep profiles, with acetaminophen-treated brain-injured mice sleeping significantly more than uninjured shams over the first six hours post-injury.

Ibuprofen-treated and vehicle treated brain-injured mice did not have an increase in sleep compared to uninjured shams over the first six hours post-injury. These differences in acute post-traumatic sleep did not contribute to differences in functional outcome measures which are in line with our findings from Chapter Four which suggest disrupting sleep in the first six hours does not contribute to a worsened outcome. We conclude from Chapter Five that a single dose of OTC analgesics does not affect outcome following experimental diffuse brain injury.

In the Chapter Five the study is significantly limited by the single 24 hour time point. Chronic dosing may have a different effect on outcome. The 24 hour time point is also limiting in the conclusions that can be draw from the inflammatory cytokines. Our previous studies document a rapid increase in inflammatory cytokines; however, this study only investigates inflammation-related interleukin cytokine levels at 24 hours after injury. At this time point it is not possible to conclude whether the anti-inflammatory drug affected the expression of inflammatory cytokines or not. Future studies should measure cytokine levels at earlier time points (i.e. 6, 9 hours) in order to identify the relationship between the tested anti-inflammatory drugs and their effect on inflammatory cytokine. Furthermore, inflammation at later time points, including the 24 hour point, should be evaluated using the presence of microglia activation. This would be a more a useful measure of inflammation that extends beyond the initial cytokine surge.

In summary, the studies presented in this dissertation have made progress towards our goal of informing clinical recommendations for the at-home

treated population of mildly concussed individuals. With a lack of research on TBI and acute sleep, it is imperative that an animal model is established in which scientific questions can be investigated. The studies from this thesis outline an injury model that accurately models the acute increase in sleep reported by TBI survivors. The studies of this thesis will help future investigators test hypotheses in the field of sleep and TBI.

Future Experiments

Throughout these experiments some questions have been answered, at the same time we are now positioned with several exciting opportunities for future investigations. Potential future experiments were recommended in the discussion sections for each chapter; however, there are additional studies that could further the advancement of post-traumatic sleep following diffuse brain injury. Foremost would be the extension of time points to investigate if immediate sleep disruption has implications in functional outcome at chronic time points; here our behavioral assessments were restricted to the first week post-injury. Likewise, extending the behavioral evaluation in drug studies, as well as increasing the dosing regimen, would more fully evaluate pharmacological interventions following diffuse TBI.

Our current studies indicate interleukin-1, a pro-inflammatory cytokine with dual roles as a sleep regulatory substance, is part of the acute inflammatory response following TBI. In order to further investigate the mechanistic link between inflammation and post-traumatic sleep, future experiments should focus on the interleukin-1 receptor antagonist (IL-1ra), which modulates inflammatory

cascades by blocking the binding of IL-1 to its signaling receptor. If the transgenic overexpression of IL-1ra prevents the injury-induced increase in IL-1 (an SRS), then it is hypothesized mice would sleep less after TBI in comparison to wild type brain-injured mice.

Additionally, adding a second injury in the six hour window where acute post-traumatic sleep occurs would allow further evaluation of vulnerable time periods post-injury. Studies could investigate repetitive head injury that occurs within the first six hours compared to a second insult after the first six hours. Clinical studies would allow for further analysis of post-traumatic sleep following TBI. Monitoring the sleep of patients admitted to the emergency room with a TBI, through electroencephalography recordings, may provide clinical insight for injury-induced sleep. Sleep measurements could be evaluated and conclusions could be drawn based on injury severity and sleep duration as well as on the overall impact sleep has on injury prognosis. If sleep is differentially promoted (duration, stage etc.) between injury severities, evaluation of immediate post-traumatic sleep could become a potential diagnostic tool for TBI.

Controversy exists in both the medical and lay communities regarding opposing suggestions for individuals not being allowed to sleep or frequently awoken following injury. The controversy is fueled by an absence of peer-reviewed biomedical literature on the topic of acute post-traumatic sleep. The data from this thesis begin to dispel the controversies regarding sleep and brain injury. Collectively, these data suggest sleep is part of the natural recovery process and self-medicating with acetaminophen or ibuprofen and falling asleep

immediately following a mild concussion is an appropriate at-home treatment plan.

Appendix

Anesthetics and analgesics in experimental traumatic brain injury: Selection based on experimental objectives

Summary

The use of animal modeling in traumatic brain injury (TBI) research is justified by the lack of sufficiently comprehensive in vitro and computer modeling that incorporates all components of the neurovascular unit. Valid animal modeling of TBI requires accurate replication of both the mechanical forces and secondary injury conditions observed in human patients. Regulatory requirements for animal modeling emphasize the administration of appropriate anesthetics and analgesics unless withholding these drugs is scientifically justified. The objective of this review is to present scientific justification for standardizing the use of anesthetics and analgesics, within a study, when modeling TBI in order to preserve study validity. Evidence for the interference of anesthetics and analgesics in the natural course of brain injury calls for consistent consideration of pain management regimens when conducting TBI research. Anesthetics administered at the time of or shortly after induction of brain injury can alter cognitive, motor, and histological outcomes following TBI. A consistent anesthesia protocol based on experimental objectives within each individual study is imperative when conducting TBI studies to control for the confounding effects of anesthesia on outcome parameters. Experimental studies

that replicate the clinical condition are essential to gain further understanding and evaluate possible treatments for TBI. However, with animal models of TBI it is essential that investigators assure a uniform drug delivery protocol that minimizes confounding variables, while minimizing pain and suffering.

Introduction

TBI is a serious health epidemic contributing to death and permanent disability. The United States Centers for Disease Control and Prevention estimate that 1.7 million individuals sustain a TBI annually in the United States alone (Faul et al. 2010a). There is currently no pharmacological treatment for individuals who suffer the lifelong neurological morbidities associated with TBI and efforts to lower the high incidence are primarily preventive. Animal models have been designed to parallel pathological processes in humans to reproduce a consistent injury (O'Connor et al. 2003). The validity of TBI animal models to the clinical condition makes them powerful tools in evaluating post-traumatic morbidity and testing rational therapeutic interventions after brain injury. When using research animals, the consideration of animal welfare is vital and pain and distress should be minimized. However, the introduction of anesthetics during experimental TBI and the use of analgesics for post-traumatic pain management can interfere with post-injury processes and functional outcomes. In this overview, we discuss how the use of anesthetic and analgesic compounds can affect evaluation of functional and histopathological outcomes following experimental TBI and how their selection or exclusion should be established based on experimental objectives.

Need for TBI models

Federal research regulations require that all reasonable alternatives which could adequately answer the proposed question be explored before considering a study that incorporates animals. The complex neurovascular responses after TBI require investigations that involve the immune, circulatory, and central nervous systems of live animals. The long term consequences of diffuse TBI include a host of emotional, cognitive, and sensory deficits that can degrade quality of life. Specific aspects of brain injury, such as cell death, have been successfully modeled with *in vitro* neural injury (Morrison et al. 1998; Geddes et al. 2003). However, *in vitro* models cannot be sustained over chronic time points to evaluate injury progression, and lack the complex interactions among systems that characterize TBI neuropathy. Additionally, current computer models cannot reproduce the complicated pathophysiology of TBI. A wide range of well-accepted animal models are available for neurotrauma investigation (see table A.1), and the use of whole animal models is justified for TBI research and deemed appropriate for conduct of pre-clinical studies (Chen et al. 2008). Therefore, neurotrauma research necessitates live animal models of human TBI, which must be employed within the existing animal welfare regulatory environment.

Experimental Animal Models of TBI

Traumatic brain injury is a complex process characterized by two pathological phases: cellular injury resulting from a primary mechanical impact and the ensuing secondary injury mediated by pathological processes (Werner

and Engelhard 2007). A range of experimental models of TBI are used in research differing in primary injury mechanisms. Biomechanical mechanisms of TBI can be classified as either focal or diffuse injury (see table A.1). Each model can be used to answer specific scientific questions. The majority of experimental brain injury research relates to mild and moderate human injuries, which do not involve coma. For this reason, rodents are rarely kept in drug induced sedation or coma after injury. All brain injury models have been carried out beyond six months to evaluate histopathology, behavior, and biochemistry.

Animal Welfare in TBI Research

The privilege of conducting research on living animals demands high levels of responsibility. The use of animals in research must be justified from an ethical cost-benefit perspective where animal pain, morbidity, and mortality must be outweighed or balanced by the potential benefits of the experimental findings to human or animal health, advancement of knowledge, or good of society. Investigators are obligated to minimize the amount of pain and distress in animals utilized for research. Withholding anesthetics, analgesics, or tranquilizers can only be allowed if it is scientifically justified by investigators and subsequently approved by an institutional animal care and use committee (IACUC). Studies in which animals are subjected to painful or stressful conditions without the use of anesthetics, analgesics, or tranquilizers are classified by the USDA and Animal Welfare Act as Category E. Category E studies must show that less painful or stressful alternatives are not available, or that less painful/stressful endpoints cannot be reasonably substituted. Since human TBI occurs in the absence of

anesthetics and analgesics, which can confound outcomes (see below), scientifically well-justified, complete, and accurate studies using in vivo models of TBI can be classified as Category E procedures in which drugs can be withheld.

Pain and TBI

By its nature, TBI is associated with acute and chronic pain both in animal models and man (Walker 2004; Nampiaparampil 2008). The initial trauma elicits somatic pain, and secondary injury mechanisms contribute to visceral and neuropathic pain (Walker 2004). Measures to reduce nociception and distress should be implemented before, during, and following surgical procedures (deep, general anesthesia) and scientific objectives must be balanced against the use of anesthetics and analgesics in experimental animal models of TBI to protect animal welfare and prevent unnecessary suffering. Administration of anesthetics and analgesics can both positively and negatively influence post-injury processes and elicit functional changes in animal models of TBI, thus, these drugs should be selected based on experimental objectives. Consistent control of anesthetics and analgesics (drug choice and dose) throughout a study will help alleviate confounding factors that may influence post-injury outcome measures. The standardization of pain management within a study will also permit cross study comparisons. Pharmacological pain management immediately following experimental TBI in animal models can influence post-injury outcomes (see below), therefore selection should be based on IACUC approved experimental objectives. This review is intended to aggregate examples of research-based instances of anesthetic and analgesic interference on TBI outcome measures.

Anesthetics in TBI Research

Anesthetics induce a variety of reversible effects depending on the concentrations at which they are delivered (Campagna et al. 2003). At lower concentrations inhaled anesthetics induce euphoria, amnesia, and hyperreflexia, but as concentrations increase anesthetics can lead to deep sedation and diminished somatic and autonomic responses to stimuli (Campagna et al. 2003). Inhaled anesthetics target both the spinal cord and the brain with differing mechanisms of action. The amnesic actions of inhaled anesthetics are mediated within the brain, depressing blood flow and glucose metabolism, with greater levels of depression in regions such as the thalamus and midbrain reticular formation (Campagna et al. 2003). Most inhaled anesthetics alter receptor-mediated synaptic signaling and consequently synaptic transmission (Franks 2008). Absence of movement in response to painful stimuli can be mediated by anesthetic action on the spinal cord, whereas general anesthesia results from supraspinal sites of action (Eger 1984; Campagna et al. 2003; Antognini et al. 2005). Local anesthetics are used for topical pain management by reversibly inhibiting sodium currents and thereby nerve impulses (Strichartz 1976).

When using animal models to replicate clinical TBI, anesthetics are routinely administered during surgical procedures to minimize pain and discomfort as well as provide immobilization. However, anesthetics used during the induction of experimental TBI can interfere with histopathological and functional outcomes. Commonly used anesthetics can provide varying degrees of neuroprotection, suggesting that the choice of anesthetics used in TBI models

can greatly alter post-injury outcome (Statler et al. 2006b). For example, isoflurane, an inhaled anesthetic commonly used in veterinary medicine, has been shown to facilitate functional outcome in comparison to other anesthetics following the controlled cortical impact (CCI) model of TBI in rats, possibly through modulation of excitotoxicity or an increase of cerebral blood flow (Statler et al. 2000). Rats anesthetized with isoflurane and subjected to CCI performed better on motor tests (beam balance, beam walking) as well as cognitive tests (Morris Water Maze) compared to rats anesthetized intravenously with fentanyl (Statler et al. 2000). Isoflurane also attenuated hippocampal damage, specifically to the CA1 region when compared to fentanyl (Statler et al. 2000). A follow-up comprehensive study evaluated cognitive outcome following CCI in the rat with the administration of several anesthetics and provided support that different anesthetics in experimental TBI critically influence outcome (Statler et al. 2006a). In all cases, animals treated with anesthetics differed from untreated animals. Isoflurane-treated rats exhibited the most improved latency times in the Morris Water Maze compared to rats injected with fentanyl or diazepam, which trended toward longer latencies to locate the platform (Statler et al. 2006a). The analysis of cresyl violet staining to investigate neuronal survival in brain tissue following CCI provided evidence that different anesthetics protect against cell death to varying extents. Again, isoflurane treatment demonstrated the most neuroprotection against hippocampal cell death, whereas rats treated with ketamine following CCI demonstrated the least protection against hippocampal cell death (Statler et al. 2006a). Isoflurane modulates excitotoxicity by reducing

the release of glutamate (Patel et al. 1995) as well as blocking NMDA receptors (Bickler et al. 1994) which may mechanistically contribute to neuroprotection (Statler et al. 2006a).

Although preconditioning with isoflurane has produced beneficial effects in animal models of TBI (Statler et al. 2000; Statler et al. 2003; Statler et al. 2006a; Statler et al. 2006b), Hertle et al. recently published data suggesting deep sedation using isoflurane negatively influences neurological outcome in a rat model of TBI (Hertle et al. 2012). Rats preconditioned with isoflurane and subjected to CCI demonstrated increased cortical damage assessed by apoptotic cell markers and a worsened neurological outcome measured by a standardized inclined plane test (Hertle et al. 2012). Investigating the cerebral protective effects of anesthetics used in combination with hypothermia following diffuse TBI has shown that propofol may be a better treatment for head injury than isoflurane (Kahveci et al. 2001). Propofol, a general injectable anesthetic similar to the inhalant isoflurane (both of which modulate GABA receptors (Kahveci et al. 2001; Krasowski et al. 2001)), used in combination with moderate hypothermia in rats subjected to diffuse impact-acceleration TBI significantly lowered intracranial pressure (ICP) and significantly raised cerebral perfusion pressure (CPP) compared to isoflurane combined with hypothermia (Kahveci et al. 2001). Investigation of ICP and CPP after diffuse impact-acceleration brain injury in rats has shown that sevoflurane, a general inhalational anesthetic, raised ICP and caused a more pronounced decrease in CPP than observed with isoflurane treated animals (Goren et al. 2001). The authors proposed that different

cerebrovasodilatatory effects of each anesthetic can account for the effects in ICP (Goren et al. 2001). Similarly, administration of anesthetics themselves can affect physiological conditions which influence outcome. Anesthetic delivery can affect normal ventilation and carbon dioxide levels which consequently alter ICP, CPP, and cerebral blood flow (CBF) (Reinert et al. 2003; Asgari et al. 2011; Haubrich et al. 2011).

TBI initiates a cascade of secondary injury processes that define the disease. Studies including but not limited to the ones above have shown that administration of anesthetics shortly after experimental TBI can act on these secondary injury mechanisms to mitigate or possibly exacerbate damage. The administration of propofol in conjunction with the closed head injury model in rats alters the synthesis of nitric oxide, ultimately reducing lipid peroxidation (Ozturk et al. 2008). Yurdakoc et al. has shown similarly that, isoflurane given after closed head trauma may protect against lipid peroxidation (Yurdakoc et al. 2008). Altering lipid peroxidation, a secondary injury mechanism characteristic of TBI, provides evidence that specific anesthetics may have neuroprotective effects on head injuries through pathophysiological pathways and may change the natural course of brain injury in animal models.

Anesthetic compounds which act as antagonists at the N-methyl-D-aspartate (NMDA) receptor may also exhibit neuroprotective properties (McIntosh et al. 1990; Jevtovic-Todorovic et al. 1998; Kawaguchi et al. 2005) that improve outcome from TBI (Smith et al. 1993). Rats subjected to fluid percussion injury and administered ketamine, an injectable non-competitive NMDA receptor

antagonist, showed preserved memory function (Smith et al. 1993). However, intraperitoneal injections of ketamine given to rats before CCI did not prevent TBI induced intracerebral cytokine production and had minimal effect on the early local inflammatory response (Ward et al. 2011). Thus, ketamine can alter the course of brain injury through mechanisms other than inhibition of inflammation.

The administration of anesthetics during TBI modeling interferes with neurological outcome. Anesthetics can be neuroprotective, making them beneficial treatments for TBI, however, contradicting studies conclude that anesthetic administration may worsen outcome. For this reason, the use of anesthetics in TBI modeling should be standardized within studies to provide consistent timing and dosage. Compounds should be selected based on specific experimental objectives for each study in accordance with local and federal regulations. It is suggested that anesthetics be carefully controlled in studies exploring the natural course of brain injury to preserve validity.

Analgesics in TBI Research

Ethical standards have been developed and enforced to protect the well-being of animals used for biomedical research. This includes proper pain management following surgical procedures. However, post-surgical analgesics have been shown to be neuroprotective and may confound the evaluation of post-injury outcome measures (McIntosh et al. 1994; Raghupathi and McIntosh 1998). Conversely, chronic administration of analgesics following TBI has led to worsened outcomes (Browne et al. 2006a). Comprehensive dosing paradigms of common analgesic drugs have not been determined in all experimental TBI

models. It is also not well known the degree of interference from drugs, whether subtle or substantial, on molecular, functional and behavioral outcomes. This inconsistency supports the claim that analgesics must be carefully considered and tested before being administered following TBI in order to maintain a valid and reproducible animal model.

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly administered to mitigate inflammation and accompanying pain. During the inflammatory process, cyclooxygenase (COX) enzymes facilitate the conversion of arachidonic acid into inflammation-mediating prostaglandins (Choi et al. 2009; Strauss 2010). NSAIDs act to reduce prostaglandin synthesis by inhibiting the production of COX-1 and COX-2 (Simmons et al. 2004). NSAIDs and specific COX-2 inhibitors are effective in suppressing inflammation, possibly by interfering with secondary injury mechanisms.

As an apparent contradiction to the acute neuroprotective actions of some NSAIDs, their chronic administration following TBI can negatively influence functional outcome (Browne et al. 2006a). Ibuprofen administered chronically over a 4 month period to rats subjected to fluid percussion injury led to a worsening in cognitive function measured by the Morris Water Maze (Browne et al. 2006a). These results show the complexity of analgesic administration post-injury and lend caution to the selection and duration of analgesics in animal models of TBI.

Carprofen, an NSAID, administered to mice after a closed head injury (CHI) results in improvements in neurobehavioral function (Thau-Zuchman et al. 2012). Mice receiving carprofen following TBI exhibited a more rapid and pronounced improvement of neurological deficits measured with the Neurological Severity Score when compared to mice receiving vehicle treatment (Thau-Zuchman et al. 2012). In addition, rats receiving nimesulide, a COX-2 inhibitor, after a diffuse TBI using the impact acceleration model showed improvement in functional and motor deficits (Cernak et al. 2002). Treatment with nimesulide improved mean escape latencies over the post-injury assessment period in comparison to vehicle-treated controls using the Barnes circular maze to assess spatial reference memory. Motor deficits, measured using the rotarod test, were also attenuated after treatment with nimesulide following TBI (Cernak et al. 2002). Similarly, 5,5-dimethyl-3(3-fluorophenyl)-4(4-methylsulfonyl)phenyl-2(5H)-furanone (DFU), a selective COX-2 inhibitor, has been shown to improve neurological reflexes and memory when administered following cortical impact in the rat, as compared to administration of a vehicle control (Gopez et al. 2005). Thus, analgesics can interfere with the natural course of the injury toward an improved recovery of function.

Endogenous and administered opioids attenuate responses to many painful stimuli in both humans and animals. TBI causes increased release of endogenous opiate receptor agonists which contribute to the trauma-induced secondary injury cascade (Hall et al. 1987; McIntosh et al. 1987a; Zohar et al. 2006). For example, morphine administration immediately following TBI in the

mouse confounds the evaluation of cognitive deficits as measured in the Morris Water Maze task (Zohar et al. 2006), where mice treated with morphine immediately post-injury were protected from long–term but not short term cognitive deficits (Zohar et al. 2006). Morphine has also been shown to reduce behavioral deficits when given to rats just prior to diffuse brain injury induced by fluid percussion (Hayes et al. 1990; Hamm et al. 1993; Lyeth et al. 1993). Morphine given in combination with scopolamine, a muscarinic receptor antagonist, provided even greater protection on motor performance (Lyeth et al. 1993). These results suggest that administering opioids following TBI alters the natural course of brain injury.

Further, the administration of analgesics following TBI not only alters functional outcome, but also alters the pathophysiological course of the injury. Brain-injured mice treated with carprofen had a significant decrease in lesion size post-injury (Thau-Zuchman et al. 2012). Carprofen-treated mice showed increased cell proliferation and gliogenesis in comparison to vehicle-treated controls (Thau-Zuchman et al. 2012). The administration of other analgesics, such as nimesulide, has been shown to alter cellular mechanisms following experimental TBI (Cernak et al. 2002). Rats subjected to an impact acceleration-induced TBI exhibited a marked increase in COX-2 protein expression in the hippocampus (Cernak et al. 2002). Based upon the hypothesis that COX-2 mediates the production of prostanoids (including prostaglandins), inhibiting COX-2 expression with an analgesic should attenuate aspects of secondary injury and neurological deficits following experimental TBI (Cernak et al. 2002).

Administration of other analgesics following TBI in the rat attenuated the amount and intensity of immunoreactive COX-2 in the cortex as compared to vehicle-treated controls (Gopez et al. 2005). Rats administered analgesics after TBI have also shown a decreased number of activated caspase-3-immunoreactive cells in both the injured cortex and hippocampus (Gopez et al. 2005). Thus, the administration of analgesics post-injury can prevent cell death and alter the pathophysiology of experimental TBI. For these reasons, it is imperative to design analgesic protocols for post-operative care that do not inhibit the processes being evaluated following experimental TBI.

While analgesics and anesthetics have great potential to confound the natural of course brain injury, justified pain management in experimental animals remains of utmost concern. Investigators have the obligation to minimize animal pain and distress in their research protocols. Perioperative pain management should be based on experimental objectives in light of the potential confounds on assessment outcomes.

Conclusions

Valid animal modeling of TBI requires accurate replication of both the mechanical forces and secondary injury conditions experienced by human patients. The interference of anesthetics and analgesics in the natural course of brain injury calls for special consideration of pain management drugs when conducting TBI research. Our analysis of these studies indicates that anesthetics administered at the time of or shortly after the impact alter outcome following TBI. To protect animal welfare anesthetics cannot be completely avoided, however, a

consistent (drug and dose) intra-study anesthesia protocol is imperative when conducting TBI studies. Similarly, studies indicate the administration of analgesics to control perioperative pain may alter outcome following TBI. Due to the variability among traumatic brain injury models and experimental outcome measures, uniform recommendations for anesthesia and analgesia are not practical. For this reason, recommendations for specific drug choice and dosing were not included in this review. Investigators are urged to establish a standard of animal care specific to individual experimental objectives based on pilot study data.

Additional Considerations

Differences in outcome among physically similar individuals following equivalent traumatic brain injuries can be particularly noticeable. Minimizing confounds of analgesic and anesthetic drugs are only one step toward conducting valid and reproducible animal modeling of TBI. Differences in weight, sex, age at injury, and genetic background can influence recovery by altering how individuals sustain the mechanical forces and endure the secondary injury processes of brain injury.

The Appendix is published in the following review:
Rowe RK, Harrison JL, Thomas TC, Pauly JR, Adelson PD, and Lifshitz J. (2013). Using anesthetics and analgesics in experimental traumatic brain injury.

Appendix: Tables

Model	Description	Refs
Fluid Percussion Injury (FPI)	Fluid pressure pulse delivers mechanical forces onto exposed brain. Can be performed laterally or centrally. Requires craniectomy.	(McIntosh et al. 1987b; McIntosh et al. 1989; Thompson et al. 2005; Alder et al. 2011)
Controlled Cortical Impact (CCI)	Computer controlled pneumatic piston drives into cortex, creating a focal injury. Requires craniectomy.	(Lighthall 1988; Dixon et al. 1991; Saatman et al. 2006)
Blast Injury	Compressed gas delivers pressure wave, resulting in a diffuse injury. Minimal surgical preparation.	(Cheng et al. 2010; Reneer et al. 2011; Risling and Davidsson 2012)
Impact Acceleration Injury	Weight is dropped onto head supported by foam pad. Scalp incision may be required.	(Heath and Vink 1995; Schmidt et al. 2000; Pandey et al. 2009)
Weight Drop Injury	Weight is dropped onto head supported by metal base. Scalp incision may be required.	(Feeney et al. 1981; Foda and Marmarou 1994; Marmarou et al. 1994; Kilbourne et al. 2009)
Penetrating Injury	Projectile is driven into the brain causing a focal injury. Requires craniectomy.	(Williams et al. 2005; Williams et al. 2006; Plantman et al. 2012)

Table A.1 Traumatic brain injury models.

Summary table of representative animal models of traumatic brain injury including mechanism of injury and degree of surgical invasiveness.

Drug	TBI Model	Behavioral	Histological	Physiological	Refs
Isoflurane	CCI	+	+		(Statler et al. 2000; Statler et al. 2006b)
Isoflurane	CCI			+	(Bickler et al. 1994; Patel et al. 1995; Statler et al. 2006a)
Isoflurane	CCI	-	-		(Hertle et al. 2012)
Isoflurane	WD			+	(Yurdakoc et al. 2008)
Propofol	IA			+	(Kahveci et al. 2001)
Sevoflurane	IA			-	(Goren et al. 2001)
Propofol	WD			+	(Ozturk et al. 2005)
Ketamine	FPI	+			(Smith et al. 1993)
Ketamine	CCI	+	+	-	(Ward et al. 2011)
Carprofen	WD	+	+		(Thau-Zuchman et al. 2012)
Nimesulide	IA	+		+	(Cernak et al. 2002)
DFU	CCI	+		+	(Gopez et al. 2005)
Morphine	WD	+			(Zohar et al. 2003)
Morphine	FPI	+			(Lyeth et al. 1993)
Scopalamine	FPI	+			(Lyeth et al. 1993)
Ibuprofen	FPI	-			(Browne et al. 2006a)

Table A.2 Anesthetic and Analgesic Effects

Summary table of anesthetic and analgesic effects on the natural course of behavioral, histological, and physiological outcomes from experimental brain injury. Experimental objectives assessed using (+) to indicate improvement or positive outcome and (-) to indicate worsening or negative outcome. CCI=Controlled Cortical Impact, WD=Weight Drop, IA=Impact Acceleration, FPI=Fluid Percussion Injury.

References

Albensi BC, Janigro D (2003) Traumatic brain injury and its effects on synaptic plasticity. Brain Inj 17:653-663

Alder J, Fujioka W, Lifshitz J, Crockett DP, Thakker-Varia S (2011) Lateral fluid percussion: model of traumatic brain injury in mice. J Vis Exp

Allan SM, Rothwell NJ (2001) Cytokines and acute neurodegeneration. Nat Rev Neurosci 2:734-744 doi: 10.1038/35094583

Ansari MA, Roberts KN, Scheff SW (2008) Oxidative stress and modification of synaptic proteins in hippocampus after traumatic brain injury. Free radical biology & medicine 45:443-452

Antognini JF, Barter L, Carstens E (2005) Overview movement as an index of anesthetic depth in humans and experimental animals. Comparative medicine 55:413-418

Aoyama N, Lee SM, Moro N, Hovda DA, Sutton RL (2008) Duration of ATP reduction affects extent of CA1 cell death in rat models of fluid percussion injury combined with secondary ischemia. Brain Res 1230:310-319

Arciniegas DB, Topkoff J, Silver JM (2000) Neuropsychiatric aspects of traumatic brain injury. Curr Treat Options Neurol 2:169-186

Asgari S, Bergsneider M, Hamilton R, Vespa P, Hu X (2011) Consistent changes in intracranial pressure waveform morphology induced by acute hypercapnic cerebral vasodilatation. Neurocritical care 15:55-62

Asikainen M, Deboer T, Porkka-Heiskanen T, Stenberg D, Tobler I (1995) Sleep deprivation increases brain serotonin turnover in the Djungarian hamster. Neuroscience letters 198:21-24

Ayalon L, Borodkin K, Dishon L, Kanety H, Dagan Y (2007) Circadian rhythm sleep disorders following mild traumatic brain injury. Neurology 68:1136-1140

Bachstetter AD, Rowe RK, Kaneko M, Goulding D, Lifshitz J, Van Eldik LJ (2013) The p38alpha MAPK regulates microglial responsiveness to diffuse traumatic brain injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 33:6143-6153

Bachstetter AD, Xing B, de Almeida L, Dimayuga ER, Watterson DM, Van Eldik LJ (2011) Microglial p38alpha MAPK is a key regulator of proinflammatory cytokine up-regulation induced by toll-like receptor (TLR) ligands or beta-amyloid (Abeta). J Neuroinflammation 8:79

Banks S, Dinges DF (2007) Behavioral and physiological consequences of sleep restriction. J Clin Sleep Med 3:519-528

Baumann CR (2012) Traumatic brain injury and disturbed sleep and wakefulness. Neuromolecular medicine

Baumann CR, Stocker R, Imhof HG, Trentz O, Hersberger M, Mignot E, Bassetti CL (2005) Hypocretin-1 (orexin A) deficiency in acute traumatic brain injury. Neurology 65:147-149

Baumann CR, Werth E, Stocker R, Ludwig S, Bassetti CL (2007) Sleep-wake disturbances 6 months after traumatic brain injury: a prospective study. Brain 130:1873-1883

Beaulieu-Bonneau S, Morin CM (2012) Sleepiness and fatigue following traumatic brain injury. Sleep Med 13:598-605

Benington JH, Heller HC (1995) Restoration of brain energy metabolism as the function of sleep. Progress in neurobiology 45:347-360

Bhalerao SU, Geurtjens C, Thomas GR, Kitamura CR, Zhou C, Marlborough M (2013) Understanding the neuropsychiatric consequences associated with significant traumatic brain injury. Brain Inj 27:767-774
Bickler PE, Buck LT, Hansen BM (1994) Effects of isoflurane and hypothermia on glutamate receptor-mediated calcium influx in brain slices. Anesthesiology 81:1461-1469
Billiard M, Podesta C (2013) Recurrent hypersomnia following traumatic brain injury. Sleep Med 14:462-465
Bjork L, Andersson J, Ceska M, Andersson U (1992) Endotoxin and Staphylococcus aureus enterotoxin A induce different patterns of cytokines. Cytokine 4:513-519
Bonnet MH (1985) Effect of sleep disruption on sleep, performance, and mood. Sleep 8:11-19
Bonnet MH (1986) Performance and sleepiness following moderate sleep disruption and slow wave sleep deprivation. Physiol Behav 37:915-918
Boone DR, Sell SL, Micci MA, et al. (2012) Traumatic brain injury-induced dysregulation of the circadian clock. PLoS One 7:e46204
Borbely AA, Achermann P (1999) Sleep homeostasis and models of sleep regulation. Journal of biological rhythms 14:557-568
Brown RE, Basheer R, McKenna JT, Strecker RE, McCarley RW (2012) Control of sleep and wakefulness. Physiol Rev 92:1087-1187
Browne KD, Iwata A, Putt ME, Smith DH (2006a) Chronic ibuprofen administration worsens cognitive outcome following traumatic brain injury in rats. Experimental neurology 201:301-307
Browne KD, Iwata A, Putt ME, Smith DH (2006b) Chronic ibuprofen administration worsens cognitive outcome following traumatic brain injury in rats. Exp Neurol 201:301-307
Butler IJ (2013) Postconcussion syndrome after mild traumatic brain injury in children and adolescents requires further detailed study. JAMA Neurol 70:636-637 doi: 10.1001/jamaneurol.2013.2801
Cam E, Gao B, Imbach L, Hodor A, Bassetti CL (2013) Sleep deprivation before stroke is neuroprotective: A pre-ischemic conditioning related to sleep rebound. Exp Neurol
Campagna JA, Miller KW, Forman SA (2003) Mechanisms of actions of inhaled anesthetics. N Engl J Med 348:2110-2124
Cao T, Thomas TC, Ziebell JM, Pauly JR, Lifshitz J (2012) Morphological and genetic activation of microglia after diffuse traumatic brain injury in the rat. Neuroscience 225:65-75
Castriotta RJ, Murthy JN (2011) Sleep disorders in patients with traumatic brain injury: a review. CNS Drugs 25:175-185
Castriotta RJ, Wilde MC, Lai JM, Atanasov S, Masel BE, Kuna ST (2007) Prevalence and consequences of sleep disorders in traumatic brain injury. J Clin Sleep Med 3:349-356
Cernak I, O'Connor C, Vink R (2002) Inhibition of cyclooxygenase 2 by nimesulide improves cognitive outcome more than motor outcome following diffuse traumatic brain injury in rats. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale 147:193-199
Chen J, Xu XM, Xu ZC (2008) Animal Models of Acute Neurological Injuries. Humana Press
Chen Y, Constantini S, Trembovler V, Weinstock M, Shohami E (1996) An experimental model of closed head injury in mice: pathophysiology, histopathology, and cognitive deficits. J Neurotrauma 13:557-568

Cheng J, Gu J, Ma Y, Yang T, Kuang Y, Li B, Kang J (2010) Development of a rat model for studying blast-induced traumatic brain injury. J Neurol Sci 294:23-28
Chensue SW, Terebuh PD, Remick DG, Scales WE, Kunkel SL (1991) In vivo biologic and immunohistochemical analysis of interleukin-1 alpha, beta and tumor necrosis factor during experimental endotoxemia. Kinetics, Kupffer cell expression, and glucocorticoid effects. The American journal of pathology 138:395-402
Chikahisa S, Sei H (2011) The role of ATP in sleep regulation. Frontiers in neurology 2:87
Chio CC, Chang CH, Wang CC, et al. (2013) Etanercept attenuates traumatic brain injury in rats by reducing early microglial expression of tumor necrosis factor-alpha. BMC Neurosci 14:33
Choi SH, Aid S, Bosetti F (2009) The distinct roles of cyclooxygenase-1 and -2 in neuroinflammation: implications for translational research. Trends Pharmacol Sci 30:174-181
Chokroverty S (2010) Overview of sleep & sleep disorders. Indian J Med Res 131:126-140
Cirelli C, Tononi G (2000) Gene expression in the brain across the sleep-waking cycle. Brain Res 885:303-321
Clark BD, Bedrosian I, Schindler R, Cominelli F, Cannon JG, Shaw AR, Dinarello CA (1991) Detection of interleukin 1 alpha and 1 beta in rabbit tissues during endotoxemia using sensitive radioimmunoassays. J Appl Physiol 71:2412-2418
Cohen HB, Dement WC (1965) Sleep: changes in threshold to electroconvulsive shock in rats after deprivation of "paradoxical" phase. Science 150:1318-1319
Cohen M, Oksenberg A, Snir D, Stern MJ, Groswasser Z (1992) Temporally related changes of sleep complaints in traumatic brain injured patients. J Neurol Neurosurg Psychiatry 55:313-315
Cohen S, Doyle WJ, Alper CM, Janicki-Deverts D, Turner RB (2009) Sleep habits and susceptibility to the common cold. Arch Intern Med 169:62-67
Crane AT, Fink KD, Smith JS (2012) The effects of acute voluntary wheel running on recovery of function following medial frontal cortical contusions in rats. Restorative neurology and neuroscience 30:325-333
Curry P, Viernes D, Sharma D (2011) Perioperative management of traumatic brain injury. International journal of critical illness and injury science 1:27-35
D'Ambrosio R, Fairbanks JP, Fender JS, Born DE, Doyle DL, Miller JW (2004) Post-traumatic epilepsy following fluid percussion injury in the rat. Brain 127:304-314
Dalgard CL, Cole JT, Kean WS, et al. (2012) The cytokine temporal profile in rat cortex after controlled cortical impact. Front Mol Neurosci 5:6
Dani M, Guindon J, Lambert C, Beaulieu P (2007) The local antinociceptive effects of paracetamol in neuropathic pain are mediated by cannabinoid receptors. Eur J Pharmacol 573:214-215 doi: 10.1016/j.ejphar.2007.07.012
Dash PK, Mach SA, Moore AN (2000) Regional expression and role of cyclooxygenase-2 following experimental traumatic brain injury. J Neurotrauma 17:69-81
Davalos D, Grutzendler J, Yang G, et al. (2005) ATP mediates rapid microglial response to local brain injury in vivo. Nature neuroscience 8:752-758
Dean PJ, O'Neill D, Sterr A (2012) Post-concussion syndrome: prevalence after mild traumatic brain injury in comparison with a sample without head injury. Brain Inj 26:14-26
Dinarello CA (1991) Interleukin-1 and interleukin-1 antagonism. Blood 77:1627-1652

Dixon CE, Clifton GL, Lighthall JW, Yaghmai AA, Hayes RL (1991) A controlled cortical impact model of traumatic brain injury in the rat. Journal of neuroscience methods 39:253-262

Dixon CE, Lyeth BG, Povlishock JT, et al. (1987) A fluid percussion model of experimental brain injury in the rat. J Neurosurg 67:110-119

Donohue KD, Medonza DC, Crane ER, O'Hara BF (2008) Assessment of a non-invasive high-throughput classifier for behaviours associated with sleep and wake in mice. Biomed Eng Online 7:14

Dworak M, McCarley RW, Kim T, Kalinchuk AV, Basheer R (2010) Sleep and brain energy levels: ATP changes during sleep. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:9007-9016 doi: 10.1523/JNEUROSCI.1423-10.2010

Eger EI, 2nd (1984) The pharmacology of isoflurane. British journal of anaesthesia 56 Suppl 1:71S-99S

Eiland MM, Ramanathan L, Gulyani S, Gilliland M, Bergmann BM, Rechtschaffen A, Siegel JM (2002) Increases in amino-cupric-silver staining of the supraoptic nucleus after sleep deprivation. Brain Res 945:1-8

Ennaceur A, Aggleton JP (1997) The effects of neurotoxic lesions of the perirhinal cortex combined to fornix transection on object recognition memory in the rat. Behavioural brain research 88:181-193

Everson CA, Laatsch CD, Hogg N (2005) Antioxidant defense responses to sleep loss and sleep recovery. Am J Physiol Regul Integr Comp Physiol 288:R374-383

Fan L, Young PR, Barone FC, Feuerstein GZ, Smith DH, McIntosh TK (1995) Experimental brain injury induces expression of interleukin-1 beta mRNA in the rat brain. Brain Res Mol Brain Res 30:125-130

Fang J, Wang Y, Krueger JM (1998) Effects of interleukin-1 beta on sleep are mediated by the type I receptor. Am J Physiol 274:R655-660

Farkas O, Povlishock JT (2007) Cellular and subcellular change evoked by diffuse traumatic brain injury: a complex web of change extending far beyond focal damage. Prog Brain Res 161:43-59

Faul M XL, Wald MM, Coronado VG (2010) Traumatic brain injury in the United States: emergency department visits, hospitalizations and deaths 2002-2006. In: Centers for Disease Control and Prevention NCfIPaC (ed), Atlanta, GA

Faul M, Xu L, Wald MM, Coronado V, Dellinger AM (2010a) Traumatic Brain Injury in the United States: National Estimates of Prevalence and Incidence, 2002-2006. Injury Prevention 16:A268-A268

Faul M, Xu L, Wald MM, Coronado VG (2010b) Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations and Deaths 2002 – 2006. In: Centers for Disease Control and Prevention NCfIPaC (ed), Atlanta, GA

Feeney DM, Boyeson MG, Linn RT, Murray HM, Dail WG (1981) Responses to cortical injury: I. Methodology and local effects of contusions in the rat. Brain research 211:67-77

Flores AE, Flores JE, Deshpande H, et al. (2007) Pattern recognition of sleep in rodents using piezoelectric signals generated by gross body movements. IEEE Trans Biomed Eng 54:225-233

Foda MA, Marmarou A (1994) A new model of diffuse brain injury in rats. Part II: Morphological characterization. J Neurosurg 80:301-313

Fox GB, Fan L, Levasseur RA, Faden AI (1998) Sustained sensory/motor and cognitive deficits with neuronal apoptosis following controlled cortical impact brain injury in the mouse. J Neurotrauma 15:599-614

Franks NP (2008) General anaesthesia: from molecular targets to neuronal pathways of sleep and arousal. Nat Rev Neurosci 9:370-386
Frieboes RM, Muller U, Murck H, von Cramon DY, Holsboer F, Steiger A (1999) Nocturnal hormone secretion and the sleep EEG in patients several months after traumatic brain injury. The Journal of neuropsychiatry and clinical neurosciences 11:354-360
Frugier T, Morganti-Kossmann MC, O'Reilly D, McLean CA (2010) In situ detection of inflammatory mediators in post mortem human brain tissue after traumatic injury. J Neurotrauma 27:497-507
Gabay C, Kushner I (1999) Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 340:448-454
Gao B, Cam E, Jaeger H, Zunzunegui C, Sarnthein J, Bassetti CL (2010) Sleep disruption aggravates focal cerebral ischemia in the rat. Sleep 33:879-887
Gatson JW, Liu MM, Abdelfattah K, Wigginton JG, Smith S, Wolf S, Minei JP (2013) Resveratrol decreases inflammation in the brain of mice with mild traumatic brain injury. The journal of trauma and acute care surgery 74:470-474; discussion 474-475
Geddes DM, LaPlaca MC, Cargill RS, 2nd (2003) Susceptibility of hippocampal neurons to mechanically induced injury. Experimental neurology 184:420-427
Gentleman D (1999) Improving outcome after traumatic brain injury--progress and challenges. Br Med Bull 55:910-926
Geoghegan P, O'Donovan MT, Lawlor BA (2012) Investigation of the effects of alcohol on sleep using actigraphy. Alcohol Alcohol 47:538-544 doi: 10.1093/alcalc/ags054
Gopalakrishnan A, Ji LL, Cirelli C (2004) Sleep deprivation and cellular responses to oxidative stress. Sleep 27:27-35
Gopez JJ, Yue H, Vasudevan R, et al. (2005) Cyclooxygenase-2-specific inhibitor improves functional outcomes, provides neuroprotection, and reduces inflammation in a rat model of traumatic brain injury. Neurosurgery 56:590-604
Goren S, Kahveci N, Alkan T, Goren B, Korfali E (2001) The effects of sevoflurane and isoflurane on intracranial pressure and cerebral perfusion pressure after diffuse brain injury in rats. J Neurosurg Anesthesiol 13:113-119
Greer JE, Hanell A, McGinn MJ, Povlishock JT (2013) Mild traumatic brain injury in the mouse induces axotomy primarily within the axon initial segment. Acta Neuropathol
Greer JE, McGinn MJ, Povlishock JT (2011) Diffuse traumatic axonal injury in the mouse induces atrophy, c-Jun activation, and axonal outgrowth in the axotomized neuronal population. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:5089-5105
Greer JE, Povlishock JT, Jacobs KM (2012) Electrophysiological abnormalities in both axotomized and nonaxotomized pyramidal neurons following mild traumatic brain injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:6682-6687
Griesbach GS (2011) Exercise after traumatic brain injury: is it a double-edged sword? PM R 3:S64-72
Griesbach GS, Tio DL, Vincelli J, McArthur DL, Taylor AN (2012) Differential effects of voluntary and forced exercise on stress responses after traumatic brain injury. J Neurotrauma 29:1426-1433
Hagewoud R, Havekes R, Novati A, Keijser JN, Van der Zee EA, Meerlo P (2010) Sleep deprivation impairs spatial working memory and reduces hippocampal AMPA receptor phosphorylation. J Sleep Res 19:280-288

Hall ED (1985) Beneficial effects of acute intravenous ibuprofen on neurologic recovery of head-injured mice: comparison of cyclooxygenase inhibition with inhibition of thromboxane A2 synthetase or 5-lipoxygenase. Cent Nerv Syst Trauma 2:75-83

Hall ED, Wolf DL, Althaus JS, Von Voigtlander PF (1987) Beneficial effects of the kappa opioid receptor agonist U-50488H in experimental acute brain and spinal cord injury. Brain research 435:174-180

Hall KD, Lifshitz J (2010) Diffuse traumatic brain injury initially attenuates and later expands activation of the rat somatosensory whisker circuit concomitant with neuroplastic responses. Brain Res 1323:161-173

Hamm RJ (2001) Neurobehavioral assessment of outcome following traumatic brain injury in rats: an evaluation of selected measures. J Neurotrauma 18:1207-1216

Hamm RJ, O'Dell DM, Pike BR, Lyeth BG (1993) Cognitive impairment following traumatic brain injury: the effect of pre- and post-injury administration of scopolamine and MK-801. Brain Res Cogn Brain Res 1:223-226

Han X, Tong J, Zhang J, et al. (2011) Imipramine treatment improves cognitive outcome associated with enhanced hippocampal neurogenesis after traumatic brain injury in mice. J Neurotrauma 28:995-1007

Haubrich C, Steiner L, Kasprowicz M, et al. (2011) Short-term moderate hypocapnia augments detection of optimal cerebral perfusion pressure. Journal of neurotrauma 28:1133-1137

Hayes RL, Lyeth BG, Jenkins LW, Zimmerman R, Mcintosh TK, Clifton GL, Young HF (1990) Possible Protective Effect of Endogenous Opioids in Traumatic Brain Injury. J Neurosurg 72:252-261

Headrick JP, Bendall MR, Faden AI, Vink R (1994) Dissociation of adenosine levels from bioenergetic state in experimental brain trauma: potential role in secondary injury. J Cereb Blood Flow Metab 14:853-861

Heath DL, Vink R (1995) Impact acceleration-induced severe diffuse axonal injury in rats: characterization of phosphate metabolism and neurologic outcome. Journal of neurotrauma 12:1027-1034

Helmy A, Carpenter KL, Menon DK, Pickard JD, Hutchinson PJ (2011) The cytokine response to human traumatic brain injury: temporal profiles and evidence for cerebral parenchymal production. J Cereb Blood Flow Metab 31:658-670

Hertle D, Beynon C, Zweckberger K, et al. (2012) Influence of isoflurane on neuronal death and outcome in a rat model of traumatic brain injury. Acta Neurochir Suppl 114:383-386

Hosseini AH, Lifshitz J (2009) Brain injury forces of moderate magnitude elicit the fencing response. Med Sci Sports Exerc 41:1687-1697

Huber R, Deboer T, Tobler I (2000) Effects of sleep deprivation on sleep and sleep EEG in three mouse strains: empirical data and simulations. Brain research 857:8-19

Huber R, Ghilardi MF, Massimini M, Tononi G (2004) Local sleep and learning. Nature 430:78-81

Jacobsen KR, Kalliokoski O, Teilmann AC, Hau J, Abelson KS (2012) The effect of isoflurane anaesthesia and vasectomy on circulating corticosterone and ACTH in BALB/c mice. Gen Comp Endocrinol 179:406-413 doi: 10.1016/j.ygcen.2012.09.012

Jevtovic-Todorovic V, Todorovic SM, Mennerick S, et al. (1998) Nitrous oxide (laughing gas) is an NMDA antagonist, neuroprotectant and neurotoxin. Nat Med 4:460-463

Jin X, Ishii H, Bai Z, Itokazu T, Yamashita T (2012) Temporal changes in cell marker expression and cellular infiltration in a controlled cortical impact model in adult male C57BL/6 mice. PLoS One 7:e41892

Kahveci FS, Kahveci N, Alkan T, Goren B, Korfali E, Ozluk K (2001) Propofol versus isoflurane anesthesia under hypothermic conditions: effects on intracranial pressure and local cerebral blood flow after diffuse traumatic brain injury in the rat. Surg Neurol 56:206-214

Kalinchuk AV, Urrila AS, Alanko L, et al. (2003) Local energy depletion in the basal forebrain increases sleep. Eur J Neurosci 17:863-869

Kaufman Y, Tzischinsky O, Epstein R, Etzioni A, Lavie P, Pillar G (2001) Long-term sleep disturbances in adolescents after minor head injury. Pediatr Neurol 24:129-134

Kawaguchi M, Furuya H, Patel PM (2005) Neuroprotective effects of anesthetic agents. J Anesth 19:150-156

Keidel M, Diener HC (1997) [Post-traumatic headache]. Nervenarzt 68:769-777

Kelley BJ, Farkas O, Lifshitz J, Povlishock JT (2006) Traumatic axonal injury in the perisomatic domain triggers ultrarapid secondary axotomy and Wallerian degeneration. Exp Neurol 198:350-360

Kempf J, Werth E, Kaiser PR, Bassetti CL, Baumann CR (2010) Sleep-wake disturbances 3 years after traumatic brain injury. J Neurol Neurosurg Psychiatry 81:1402-1405

Keshavarzi Z, Khaksari M, Razmi Z, Soltani Hekmat A, Naderi V, Rostami S (2012) The effects of cyclooxygenase inhibitors on the brain inflammatory response following traumatic brain injury in rats. Iran J Basic Med Sci 15:1102-1105

Kharatishvili I, Nissinen JP, McIntosh TK, Pitkanen A (2006) A model of posttraumatic epilepsy induced by lateral fluid-percussion brain injury in rats. Neuroscience 140:685-697

Khoury S, Chouchou F, Amzica F, Giguere JF, Denis R, Rouleau GA, Lavigne GJ (2013) Rapid EEG activity during sleep dominates in mild traumatic brain injury patients with acute pain. J Neurotrauma 30:633-641

Kilbourne M, Kuehn R, Tosun C, et al. (2009) Novel model of frontal impact closed head injury in the rat. Journal of neurotrauma 26:2233-2243

Krasowski MD, Jenkins A, Flood P, Kung AY, Hopfinger AJ, Harrison NL (2001) General anesthetic potencies of a series of propofol analogs correlate with potency for potentiation of gamma-aminobutyric acid (GABA) current at the GABA(A) receptor but not with lipid solubility. The Journal of pharmacology and experimental therapeutics 297:338-351

Krueger JM (2008) The Role of Cytokines in Sleep Regulation. Current Pharmaceutical Design 14:3408-3416

Krueger JM, Majde JA (1995) Cytokines and sleep. International Archives of Allergy and Immunology 106:97-100

Krueger JM, Obal F, Fang JD, Kubota T, Taishi P (2001a) The role of cytokines in physiological sleep regulation. Role of Neural Plasticity in Chemical Intolerance 933:211-221

Krueger JM, Obal F, Jr., Fang J (1999) Why we sleep: a theoretical view of sleep function. Sleep Med Rev 3:119-129

Krueger JM, Obal FJ, Fang J, Kubota T, Taishi P (2001b) The role of cytokines in physiological sleep regulation. Ann N Y Acad Sci 933:211-221

Krueger JM, Rector DM, Churchill L (2007) Sleep and cytokines. Sleep Med Clin 2:161-169

Krueger JM, Takahashi S, Kapas L, et al. (1995) Cytokines in sleep regulation. Advances in Neuroimmunology 5:171-188

Langlois JA, Rutland-Brown W, Wald MM (2006) The epidemiology and impact of traumatic brain injury: a brief overview. J Head Trauma Rehabil 21:375-378

Laurer HL, Bareyre FM, Lee VM, et al. (2001) Mild head injury increasing the brain's vulnerability to a second concussive impact. J Neurosurg 95:859-870

Lay CC, Davis MF, Chen-Bee CH, Frostig RD (2010) Mild sensory stimulation completely protects the adult rodent cortex from ischemic stroke. PLoS One 5:e11270

Lew HL, Lin PH, Fuh JL, Wang SJ, Clark DJ, Walker WC (2006) Characteristics and treatment of headache after traumatic brain injury: a focused review. Am J Phys Med Rehabil 85:619-627 doi: 10.1097/01.phm.0000223235.09931.c0

Lifshitz J (2008) Fluid Percussion Injury In: J Chen ZX, X-M Xu and J Zhang (ed) Animal Models of Acute Neurological Injuries. The Humana Press Inc., Totowa, NJ

Lifshitz J, Friberg H, Neumar RW, et al. (2003) Structural and functional damage sustained by mitochondria after traumatic brain injury in the rat: evidence for differentially sensitive populations in the cortex and hippocampus. J Cereb Blood Flow Metab 23:219-231

Lifshitz J, Kelley BJ, Povlishock JT (2007) Perisomatic thalamic axotomy after diffuse traumatic brain injury is associated with atrophy rather than cell death. J Neuropathol Exp Neurol 66:218-229

Lifshitz J, Lisembee AM (2012) Neurodegeneration in the somatosensory cortex after experimental diffuse brain injury. Brain Struct Funct 217:49-61

Lighthall JW (1988) Controlled cortical impact: a new experimental brain injury model. Journal of neurotrauma 5:1-15

Lloyd E, Somera-Molina K, Van Eldik LJ, Watterson DM, Wainwright MS (2008) Suppression of acute proinflammatory cytokine and chemokine upregulation by post-injury administration of a novel small molecule improves long-term neurologic outcome in a mouse model of traumatic brain injury. J Neuroinflammation 5:28

Longhi L, Watson DJ, Saatman KE, et al. (2004) Ex vivo gene therapy using targeted engraftment of NGF-expressing human NT2N neurons attenuates cognitive deficits following traumatic brain injury in mice. J Neurotrauma 21:1723-1736

Lyeth BG, Liu S, Hamm RJ (1993) Combined scopolamine and morphine treatment of traumatic brain injury in the rat. Brain research 617:69-75

Machado RB, Hipolide DC, Benedito-Silva AA, Tufik S (2004) Sleep deprivation induced by the modified multiple platform technique: quantification of sleep loss and recovery. Brain Res 1004:45-51

Madsen PL, Vorstrup S (1991) Cerebral blood flow and metabolism during sleep. Cerebrovasc Brain Metab Rev 3:281-296

Maiese K (2008) Head Injuries: Diagnosis and Treatment. In: The Merck Manual Home Health Handbook, vol 2013. Merck Sharp & Dohme Corp., Whitehouse Station, NJ

Maquet P (1995) Sleep function(s) and cerebral metabolism. Behavioural brain research 69:75-83

Marmarou A, Foda MA, van den Brink W, Campbell J, Kita H, Demetriadou K (1994) A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics. J Neurosurg 80:291-300

Martinez-Vargas M, Estrada Rojo F, Tabla-Ramon E, et al. (2012) Sleep deprivation has a neuroprotective role in a traumatic brain injury of the rat. Neuroscience letters 529:118-122

Masel BE, DeWitt DS (2010) Traumatic brain injury: a disease process, not an event. J Neurotrauma 27:1529-1540

Mathias JL, Alvaro PK (2012) Prevalence of sleep disturbances, disorders, and problems following traumatic brain injury: a meta-analysis. Sleep Med 13:898-905

McCoy JG, Strecker RE (2011) The cognitive cost of sleep lost. Neurobiol Learn Mem 96:564-582

McEwen BS (2008) Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. Eur J Pharmacol 583:174-185

McGinn MJ, Kelley BJ, Akinyi L, et al. (2009) Biochemical, structural, and biomarker evidence for calpain-mediated cytoskeletal change after diffuse brain injury uncomplicated by contusion. J Neuropathol Exp Neurol 68:241-249

McIntosh TK, Fernyak S, Yamakami I, Faden AI (1994) Central and systemic kappa-opioid agonists exacerbate neurobehavioral response to brain injury in rats. The American journal of physiology 267:R665-672

McIntosh TK, Hayes RL, DeWitt DS, Agura V, Faden AI (1987a) Endogenous opioids may mediate secondary damage after experimental brain injury. The American journal of physiology 253:E565-574

McIntosh TK, Noble L, Andrews B, Faden AI (1987b) Traumatic brain injury in the rat: characterization of a midline fluid-percussion model. Cent Nerv Syst Trauma 4:119-134

McIntosh TK, Vink R, Noble L, Yamakami I, Fernyak S, Soares H, Faden AL (1989) Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model. Neuroscience 28:233-244

McIntosh TK, Vink R, Soares H, Hayes R, Simon R (1990) Effect of noncompetitive blockade of n-methyl-d-aspartate receptors on the neurochemical sequelae of experimental brain injury. J Neurochem 55:1170-1179

McNamara KC, Lisembee AM, Lifshitz J (2010) The whisker nuisance task identifies a late-onset, persistent sensory sensitivity in diffuse brain-injured rats. J Neurotrauma 27:695-706

McShane BB, Galante RJ, Jensen ST, Naidoo N, Pack AI, Wyner A (2010) Characterization of the bout durations of sleep and wakefulness. Journal of neuroscience methods 193:321-333 doi: 10.1016/j.jneumeth.2010.08.024

Meerlo P, Mistlberger RE, Jacobs BL, Heller HC, McGinty D (2009) New neurons in the adult brain: the role of sleep and consequences of sleep loss. Sleep Med Rev 13:187-194

Morganti-Kossmann MC, Rancan M, Otto VI, Stahel PF, Kossmann T (2001) Role of cerebral inflammation after traumatic brain injury: a revisited concept. Shock 16:165-177

Morganti-Kossmann MC, Rancan M, Stahel PF, Kossmann T (2002) Inflammatory response in acute traumatic brain injury: a double-edged sword. Curr Opin Crit Care 8:101-105

Morrison B, 3rd, Saatman KE, Meaney DF, McIntosh TK (1998) In vitro central nervous system models of mechanically induced trauma: a review. Journal of neurotrauma 15:911-928

Murai H, Pierce JE, Raghupathi R, et al. (1998) Twofold overexpression of human beta-amyloid precursor proteins in transgenic mice does not affect the neuromotor, cognitive, or neurodegenerative sequelae following experimental brain injury. The Journal of comparative neurology 392:428-438

Nakamura M, Saatman KE, Galvin JE, Scherbel U, Raghupathi R, Trojanowski JQ, McIntosh TK (1999) Increased vulnerability of NFH-LacZ transgenic mouse to

traumatic brain injury-induced behavioral deficits and cortical damage. J Cereb Blood Flow Metab 19:762-770
Nampiaparampil DE (2008) Prevalence of chronic pain after traumatic brain injury: a systematic review. JAMA : the journal of the American Medical Association 300:711-719
Ng SY, Semple BD, Morganti-Kossmann MC, Bye N (2012) Attenuation of microglial activation with minocycline is not associated with changes in neurogenesis after focal traumatic brain injury in adult mice. J Neurotrauma 29:1410-1425
Nicholson K, Martelli MF (2004) The problem of pain. J Head Trauma Rehabil 19:2-9
Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308:1314-1318
O'Connor CA, Cernak I, Vink R (2003) Interaction between anesthesia, gender, and functional outcome task following diffuse traumatic brain injury in rats. Journal of neurotrauma 20:533-541
O'Hara BF, Young KA, Watson FL, Heller HC, Kilduff TS (1993) Immediate early gene expression in brain during sleep deprivation: preliminary observations. Sleep 16:1-7
Opp MR, Krueger JM (1994) Anti-interleukin-1 beta reduces sleep and sleep rebound after sleep deprivation in rats. Am J Physiol 266:R688-695
Opp MR, Obal F, Jr., Krueger JM (1991) Interleukin 1 alters rat sleep: temporal and dose-related effects. Am J Physiol 260:R52-58
Orff HJ, Ayalon L, Drummond SP (2009) Traumatic brain injury and sleep disturbance: a review of current research. J Head Trauma Rehabil 24:155-165
Oswald I (1980) Sleep as restorative process: human clues. Prog Brain Res 53:279-288
Ottani A, Leone S, Sandrini M, Ferrari A, Bertolini A (2006) The analgesic activity of paracetamol is prevented by the blockade of cannabinoid CB1 receptors. Eur J Pharmacol 531:280-281 doi: 10.1016/j.ejphar.2005.12.015
Ouellet MC, Morin CM (2006) Subjective and objective measures of insomnia in the context of traumatic brain injury: a preliminary study. Sleep Med 7:486-497
Ozturk E, Demirbilek S, Kadir But A, Saricicek V, Gulec M, Akyol O, Ozcan Ersoy M (2005) Antioxidant properties of propofol and erythropoietin after closed head injury in rats. Progress in neuro-psychopharmacology & biological psychiatry 29:922-927
Ozturk E, Demirbilek S, Koroglu A, et al. (2008) Propofol and erythropoietin antioxidant properties in rat brain injured tissue. Progress in neuro-psychopharmacology & biological psychiatry 32:81-86
Pandey DK, Yadav SK, Mahesh R, Rajkumar R (2009) Depression-like and anxiety-like behavioural aftermaths of impact accelerated traumatic brain injury in rats: a model of comorbid depression and anxiety? Behav Brain Res 205:436-442
Patel PM, Drummond JC, Cole DJ, Goskowicz RL (1995) Isoflurane reduces ischemia-induced glutamate release in rats subjected to forebrain ischemia. Anesthesiology 82:996-1003
Patti CL, Zanin KA, Sanday L, et al. (2010) Effects of sleep deprivation on memory in mice: role of state-dependent learning. Sleep 33:1669-1679
Paul T, Lemmer B (2007) Disturbance of circadian rhythms in analgosedated intensive care unit patients with and without craniocerebral injury. Chronobiol Int 24:45-61
Plantman S, Ng KC, Lu J, Davidsson J, Risling M (2012) Characterization of a novel rat model of penetrating traumatic brain injury. Journal of neurotrauma 29:1219-1232
Pleasant JM, Carlson SW, Mao H, Scheff SW, Yang KH, Saatman KE (2011) Rate of neurodegeneration in the mouse controlled cortical impact model is influenced by

impactor tip shape: implications for mechanistic and therapeutic studies. J Neurotrauma 28:2245-2262
Pleines UE, Morganti-Kossmann MC, Rancan M, Joller H, Trentz O, Kossmann T (2001) S-100 beta reflects the extent of injury and outcome, whereas neuronal specific enolase is a better indicator of neuroinflammation in patients with severe traumatic brain injury. J Neurotrauma 18:491-498
Povlishock JT, Katz DI (2005) Update of neuropathology and neurological recovery after traumatic brain injury. J Head Trauma Rehabil 20:76-94
Prigatano GP, Stahl ML, Orr WC, Zeiner HK (1982) Sleep and dreaming disturbances in closed head injury patients. J Neurol Neurosurg Psychiatry 45:78-80
Prins M, Greco T, Alexander D, Giza CC (2013) The pathophysiology of traumatic brain injury at a glance. Dis Model Mech
Prins ML, Hovda DA (2001) Mapping cerebral glucose metabolism during spatial learning: interactions of development and traumatic brain injury. J Neurotrauma 18:31-46
Pull I, McIlwain H (1972) Adenine derivatives as neurohumoral agents in the brain. The quantities liberated on excitation of superfused cerebral tissues. Biochem J 130:975-981
Raghupathi R, McIntosh TK (1998) Pharmacotherapy for traumatic brain injury: a review. Proc West Pharmacol Soc 41:241-246
Ramanathan L, Gulyani S, Nienhuis R, Siegel JM (2002) Sleep deprivation decreases superoxide dismutase activity in rat hippocampus and brainstem. Neuroreport 13:1387-1390
Rao V, Rollings P (2002) Sleep disturbances following traumatic brain injury. Curr Treat Options Neurol 4:77-87
Reagan-Shaw S, Nihal M, Ahmad N (2008) Dose translation from animal to human studies revisited. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 22:659-661
Reilly P (2007) The impact of neurotrauma on society: an international perspective. Prog Brain Res 161:3-9 doi: 10.1016/S0079-6123(06)61001-7
Reimund E (1994) The free radical flux theory of sleep. Med Hypotheses 43:231-233
Reinert M, Barth A, Rothen HU, Schaller B, Takala J, Seiler RW (2003) Effects of cerebral perfusion pressure and increased fraction of inspired oxygen on brain tissue oxygen, lactate and glucose in patients with severe head injury. Acta neurochirurgica 145:341-349; discussion 349-350
Reneer DV, Hisel RD, Hoffman JM, Kryscio RJ, Lusk BT, Geddes JW (2011) A multi-mode shock tube for investigation of blast-induced traumatic brain injury. Journal of neurotrauma 28:95-104
Risling M, Davidsson J (2012) Experimental animal models for studies on the mechanisms of blast-induced neurotrauma. Front Neurol 3:30
Roehrs T, Papineau K, Rosenthal L, Roth T (1999) Ethanol as a hypnotic in insomniacs: self administration and effects on sleep and mood. Neuropsychopharmacology 20:279-286 doi: 10.1016/S0893-133X(98)00068-2
Roozenbeek B, Maas AI, Menon DK (2013) Changing patterns in the epidemiology of traumatic brain injury. Nat Rev Neurol 9:231-236
Rowe RK, Harrison JL, O'Hara BF, Lifshitz J (2013a) Recovery of neurological function despite immediate sleep disruption following diffuse brain injury in the mouse: clinical relevance to medically untreated concussion Sleep
Rowe RK, Harrison JL, Thomas TC, Pauly JR, Adelson PD, Lifshitz J (2013b) Using anesthetics and analgesics in experimental traumatic brain injury. Lab Anim (NY) 42:286-291

Rowe RK, Striz M, Bachstetter AD, Van Eldik LJ, Donohue KD, O'Hara BF, Lifshitz J (2013c) Diffuse Brain Injury Induces Acute Post-Traumatic Sleep. PLoS One
Saatman KE, Feeko KJ, Pape RL, Raghupathi R (2006) Differential behavioral and histopathological responses to graded cortical impact injury in mice. Journal of neurotrauma 23:1241-1253
Sapolsky RM, Pulsinelli WA (1985) Glucocorticoids potentiate ischemic injury to neurons: therapeutic implications. Science 229:1397-1400
Scharf MT, Naidoo N, Zimmerman JE, Pack AI (2008) The energy hypothesis of sleep revisited. Progress in neurobiology 86:264-280
Schmidt OI, Heyde CE, Ertel W, Stahel PF (2005) Closed head injury--an inflammatory disease? Brain research. Brain research reviews 48:388-399
Schmidt RH, Scholten KJ, Maughan PH (2000) Cognitive impairment and synaptosomal choline uptake in rats following impact acceleration injury. Journal of neurotrauma 17:1129-1139
Schoch KM, Evans HN, Brelsfoard JM, Madathil SK, Takano J, Saido TC, Saatman KE (2012) Calpastatin overexpression limits calpain-mediated proteolysis and behavioral deficits following traumatic brain injury. Exp Neurol 236:371-382
Semple BD, Bye N, Rancan M, Ziebell JM, Morganti-Kossmann MC (2010) Role of CCL2 (MCP-1) in traumatic brain injury (TBI): evidence from severe TBI patients and CCL2-/- mice. J Cereb Blood Flow Metab 30:769-782
Setnik L, Bazarian JJ (2007) The characteristics of patients who do not seek medical treatment for traumatic brain injury. Brain Inj 21:1-9
Sheth BR, Janvelyan D, Khan M (2008) Practice makes imperfect: restorative effects of sleep on motor learning. PLoS One 3:e3190
Siegel JM (2005) Clues to the functions of mammalian sleep. Nature 437:1264-1271
Signoretti S, Marmarou A, Tavazzi B, Lazzarino G, Beaumont A, Vagnozzi R (2001) N-Acetylaspartate reduction as a measure of injury severity and mitochondrial dysfunction following diffuse traumatic brain injury. Journal of neurotrauma 18:977-991
Silva LF, Hoffmann MS, Gerbatin R, et al. (2013) Treadmill exercise protects against pentylenetetrazol-induced seizures and oxidative stress after traumatic brain injury. J Neurotrauma
Simmons DL, Botting RM, Hla T (2004) Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol Rev 56:387-437
Singleton RH, Zhu J, Stone JR, Povlishock JT (2002) Traumatically induced axotomy adjacent to the soma does not result in acute neuronal death. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:791-802
Smith DH, Nakamura M, McIntosh TK, et al. (1998) Brain trauma induces massive hippocampal neuron death linked to a surge in beta-amyloid levels in mice overexpressing mutant amyloid precursor protein. The American journal of pathology 153:1005-1010
Smith DH, Okiyama K, Gennarelli TA, McIntosh TK (1993) Magnesium and ketamine attenuate cognitive dysfunction following experimental brain injury. Neuroscience letters 157:211-214
Smith DH, Soares HD, Pierce JS, et al. (1995) A model of parasagittal controlled cortical impact in the mouse: cognitive and histopathologic effects. J Neurotrauma 12:169-178
Statler KD, Alexander H, Vagni V, Dixon CE, Clark RS, Jenkins L, Kochanek PM (2006a) Comparison of seven anesthetic agents on outcome after experimental traumatic brain injury in adult, male rats. Journal of neurotrauma 23:97-108

Statler KD, Alexander H, Vagni V, et al. (2006b) Isoflurane exerts neuroprotective actions at or near the time of severe traumatic brain injury. Brain research 1076:216-224

Statler KD, Janesko KL, Melick JA, Clark RS, Jenkins LW, Kochanek PM (2003) Hyperglycolysis is exacerbated after traumatic brain injury with fentanyl vs. isoflurane anesthesia in rats. Brain research 994:37-43

Statler KD, Kochanek PM, Dixon CE, et al. (2000) Isoflurane improves long-term neurologic outcome versus fentanyl after traumatic brain injury in rats. Journal of neurotrauma 17:1179-1189

Stepanski E, Lamphere J, Badia P, Zorick F, Roth T (1984) Sleep fragmentation and daytime sleepiness. Sleep 7:18-26

Stepanski EJ (2002) The effect of sleep fragmentation on daytime function. Sleep 25:268-276

Strauss KI (2010) COX2 inhibitors for acquired brain injuries: is the time ripe? Crit Care Med 38:723-724

Strichartz G (1976) Molecular mechanisms of nerve block by local anesthetics. Anesthesiology 45:421-441

Takahashi S, Kapas L, Fang J, Seyer JM, Wang Y, Krueger JM (1996) An interleukin-1 receptor fragment inhibits spontaneous sleep and muramyl dipeptide-induced sleep in rabbits. Am J Physiol 271:R101-108

Tham SW, Palermo TM, Vavilala MS, et al. (2012) The longitudinal course, risk factors, and impact of sleep disturbances in children with traumatic brain injury. J Neurotrauma 29:154-161

Thau-Zuchman O, Shohami E, Alexandrovich AG, Trembovler V, Leker RR (2012) The anti-inflammatory drug carprofen improves long-term outcome and induces gliogenesis after traumatic brain injury. J Neurotrauma 29:375-384

Theeler B, Lucas S, Riechers RG, 2nd, Ruff RL (2013) Post-traumatic headaches in civilians and military personnel: a comparative, clinical review. Headache 53:881-900 doi: 10.1111/head.12123

Thompson HJ, Lifshitz J, Marklund N, Grady MS, Graham DI, Hovda DA, McIntosh TK (2005) Lateral fluid percussion brain injury: a 15-year review and evaluation. Journal of neurotrauma 22:42-75

Tobler I, Borbely AA, Schwyzer M, Fontana A (1984) Interleukin-1 derived from astrocytes enhances slow wave activity in sleep EEG of the rat. Eur J Pharmacol 104:191-192

Tononi G, Cirelli C (2003) Sleep and synaptic homeostasis: a hypothesis. Brain Res Bull 62:143-150

Tononi G, Cirelli C (2006) Sleep function and synaptic homeostasis. Sleep Med Rev 10:49-62

Toth LA (1995) Sleep, sleep deprivation and infectious disease: studies in animals. Advances in Neuroimmunology 5:79-92

Toth LA, Krueger JM (1988) Alteration of sleep in rabbits by Staphylococcus aureus infection. Infect Immun 56:1785-1791

Toth LA, Tolley EA, Krueger JM (1993) Sleep as a prognostic indicator during infectious disease in rabbits. Proc Soc Exp Biol Med 203:179-192

Vane JR (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 231:232-235

Verma A, Anand V, Verma NP (2007) Sleep disorders in chronic traumatic brain injury. J Clin Sleep Med 3:357-362

Wakabayashi G, Gelfand JA, Burke JF, Thompson RC, Dinarello CA (1991) A specific receptor antagonist for interleukin 1 prevents Escherichia coli-induced shock in rabbits. FASEB J 5:338-343
Walker JM, Berger RJ (1980) Sleep as an adaptation for energy conservation functionally related to hibernation and shallow torpor. Prog Brain Res 53:255-278
Walker MP, Stickgold R, Alsop D, Gaab N, Schlaug G (2005) Sleep-dependent motor memory plasticity in the human brain. Neuroscience 133:911-917
Walker WC (2004) Pain pathoetiology after TBI: neural and nonneural mechanisms. J Head Trauma Rehabil 19:72-81
Walsh CM, Booth V, Poe GR (2011) Spatial and reversal learning in the Morris water maze are largely resistant to six hours of REM sleep deprivation following training. Learning & memory 18:422-434
Wang KK, Larner SF, Robinson G, Hayes RL (2006) Neuroprotection targets after traumatic brain injury. Curr Opin Neurol 19:514-519
Ward JL, Harting MT, Cox CS, Jr., Mercer DW (2011) Effects of ketamine on endotoxin and traumatic brain injury induced cytokine production in the rat. The Journal of trauma 70:1471-1479
Werner C, Engelhard K (2007) Pathophysiology of traumatic brain injury. Br J Anaesth 99:4-9
Williams AJ, Hartings JA, Lu XC, Rolli ML, Dave JR, Tortella FC (2005) Characterization of a new rat model of penetrating ballistic brain injury. Journal of neurotrauma 22:313-331
Williams AJ, Hartings JA, Lu XC, Rolli ML, Tortella FC (2006) Penetrating ballistic-like brain injury in the rat: differential time courses of hemorrhage, cell death, inflammation, and remote degeneration. Journal of neurotrauma 23:1828-1846
Willie JT, Lim MM, Bennett RE, Azarion AA, Schwetye KE, Brody DL (2012) Controlled cortical impact traumatic brain injury acutely disrupts wakefulness and extracellular orexin dynamics as determined by intracerebral microdialysis in mice. J Neurotrauma 29:1908-1921
Wiseman-Hakes C, Murray B, Moineddin R, Rochon E, Cullen N, Gargaro J, Colantonio A (2013) Evaluating the impact of treatment for sleep/wake disorders on recovery of cognition and communication in adults with chronic TBI. Brain Inj
Wisor JP, Schmidt MA, Clegern WC (2011) Evidence for neuroinflammatory and microglial changes in the cerebral response to sleep loss. Sleep 34:261-272
Wyatt JE, Pettit WL, Harirforoosh S (2012) Pharmacogenetics of nonsteroidal anti-inflammatory drugs. Pharmacogenomics J 12:462-467
Xie L, Kang H, Xu Q, et al. (2013) Sleep drives metabolite clearance from the adult brain. Science 342:373-377 doi: 10.1126/science.1241224
Yang SH, Gustafson J, Gangidine M, et al. (2013) A murine model of mild traumatic brain injury exhibiting cognitive and motor deficits. J Surg Res
Yang SR, Sun H, Huang ZL, Yao MH, Qu WM (2012a) Repeated sleep restriction in adolescent rats altered sleep patterns and impaired spatial learning/memory ability. Sleep 35:849-859
Yang X, He Z, Zhang Q, et al. (2012b) Pre-Ischemic Treadmill Training for Prevention of Ischemic Brain Injury via Regulation of Glutamate and Its Transporter GLT-1. International journal of molecular sciences 13:9447-9459
Yasuda T, Yoshida H, Garcia-Garcia F, Kay D, Krueger JM (2005) Interleukin-1beta has a role in cerebral cortical state-dependent electroencephalographic slow-wave activity. Sleep 28:177-184

Yeates KO, Taylor HG, Wade SL, Drotar D, Stancin T, Minich N (2002) A prospective study of short- and long-term neuropsychological outcomes after traumatic brain injury in children. Neuropsychology 16:514-523

Yurdakoc A, Gunday I, Memis D (2008) Effects of halothane, isoflurane, and sevoflurane on lipid peroxidation following experimental closed head trauma in rats. Acta Anaesthesiol Scand 52:658-663

Zepelin H, Siegel JM, Tobler I (2005) Principles and Practice of Sleep Medicine. In: Kryger MH, Roth T, Derment WC (eds). Elsevier Saunders, Philadelphia

Zhang F, Wu Y, Jia J, Hu YS (2010) Pre-ischemic treadmill training induces tolerance to brain ischemia: involvement of glutamate and ERK1/2. Molecules 15:5246-5257

Ziebell JM, Bye N, Semple BD, Kossmann T, Morganti-Kossmann MC (2011) Attenuated neurological deficit, cell death and lesion volume in Fas-mutant mice is associated with altered neuroinflammation following traumatic brain injury. Brain Res 1414:94-105

Ziebell JM, Morganti-Kossmann MC (2010) Involvement of pro- and anti-inflammatory cytokines and chemokines in the pathophysiology of traumatic brain injury. Neurotherapeutics 7:22-30

Ziebell JM, Taylor SE, Cao T, Harrison JL, Lifshitz J (2012) Rod microglia: elongation, alignment, and coupling to form trains across the somatosensory cortex after experimental diffuse brain injury. J Neuroinflammation 9:247

Zohar O, Getslev V, Miller AL, Schreiber S, Pick CG (2006) Morphine protects for head trauma induced cognitive deficits in mice. Neuroscience letters 394:239-242

Zohar O, Rubovitch V, Milman A, Schreiber S, Pick CG (2011) Behavioral consequences of minimal traumatic brain injury in mice. Acta Neurobiol Exp (Wars) 71:36-45

Zohar O, Schreiber S, Getslev V, Schwartz JP, Mullins PG, Pick CG (2003) Closed-head minimal traumatic brain injury produces long-term cognitive deficits in mice. Neuroscience 118:949-955

Zunzunegui C, Gao B, Cam E, Hodor A, Bassetti CL (2011) Sleep disturbance impairs stroke recovery in the rat. Sleep 34:1261-1269

Rachel Kathleen Rowe

Curriculum Vita

Born: Somerset, Kentucky
Citizenship: USA
Office Address: Phoenix Children's Hospital Neurotrauma Research Laboratory
Translational Genomics Research Institute (TGen)
Phoenix, Arizona

EDUCATION

2009-present	Pre-Doctoral Candidate, Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Lexington, Kentucky 40536
2009-2010	Graduate Student, Integrated Biomedical Sciences Program University of Kentucky College of Medicine, Lexington, Kentucky 40536
2004-2007	B.S., Pre-Medical Sciences, Eastern Kentucky University, Richmond, Kentucky 40475; *summa cum laude*

RESEARCH EXPERIENCE

2012-present — Graduate Student at Phoenix Children's Hospital
University of Arizona College of Medicine, Phoenix, AZ
Mentor: J. Lifshitz, Ph.D.
Project: Investigating post-traumatic sleep following diffuse traumatic brain injury using a midline fluid percussion injury model in mice, including cognitive, behavioral, and histopathological outcome measures.

2010-2012 — Graduate Student in the Dept. of Anatomy and Neurobiology
University of Kentucky, Lexington, KY
Mentor: J. Lifshitz, Ph.D.
Project: Investigating post-traumatic sleep following diffuse traumatic brain injury using a midline fluid percussion injury model in mice.

2009-2010 — Graduate Student in the Integrated Biomedical Sciences program
University of Kentucky, Lexington KY Rotations:

- J. Lifshitz, Ph.D., Department of Anatomy and Neurobiology. Examined neurodegeneration in sleep centers in the brain and began preliminary studies on post-

traumatic sleep.

- John D'Orazio, M.D., Ph.D., Markey Cancer Center. Studied melanin metabolism and UV radiation damage on genetically modified mice with tyrosinase modifications.
- Bruce O'Hara, Ph.D., Biology Department. Participated in classroom seminars on sleep and circadian rhythms, and examined sleep from both a behavioral and neurological aspect.

2007 Student Research Assistant in the Department of Chemistry.
Eastern Kentucky University, Richmond, KY
Mentor: Eric Dueno, Ph.D
Project: Worked in an organic chemistry lab and tested the antimicrobial properties of novel quinoxalines against *Streptococcus mutans*.

2007 Student Research Assistant in the Department of Biology.
Mentor: Guenter Schuster, Ph.D.
Project: Traveled to Ambergris Caye, Belize, and snorkeled coral reef systems charting biodiversity of fish species in specific regions.

AWARDS AND RECOGNITION

2013 Poster Presentation Finalist, 31st Annual National Neurotrauma Symposium, Nashville, TN

2010-2013 NIH Research Supplement to Promote Diversity in Health-Related Research Fellowship

2007 B.S., *Summa cum laude*, Eastern Kentucky University

2007 College of Arts and Science Graduation Speaker, Eastern Kentucky University

2007 Kentucky Institute for International Studies, granted admission into program to study abroad in Europe

2006 Professional Education Preparation Program, offered through the University of Kentucky to students who reside in under-served counties of the state who wish to pursue careers in Medicine

2004-2007 Regent's Scholarship, full academic scholarship from the Eastern Kentucky University Board of Regents

2004-2007 Dean's List, Eastern Kentucky University

2004-2007 President's Award, Eastern Kentucky University

2005	Retention Scholarship, competitively awarded to returning freshman at Eastern Kentucky University
2005	Alumni Scholarship to Eastern Kentucky University
2004	High School Valedictorian, McCreary Central High School
2004	In Memory of Scholarship, awarded based on academic merit
2004	Rotary Club Scholarship, awarded based on essay competition
2004	Rural Electric Cooperative Scholarship, awarded based on academic merit
2004	Highland Telephone Company Scholarship, awarded based on essay competition
2003	Kentucky Governor's Scholar, a summer residential program for outstanding high school students in Kentucky that is competitive on a school, district, regional, and state level
2001-2004	Academic All-State, three varsity sports lettered and maintained 3.5 GPA or above for the entire season

Memberships in Honor Societies:

Who's Who Among American College Students
Delta Iota Epsilon Honor Society, Professional Academic Honor Society among colleges
National Honor Roll, collegiate honor roll
Golden Key International Honor Society, invitation only collegiate honor society
Phi Sigma, Biology Honor Society, a member of the Association of College Honor Societies

PROFESSIONAL MEMBERSHIPS

2011-	Society for Neuroscience
2010-	Women in Neurotrauma Research
2009-	National Neurotrauma Society member
2009-2012	Bluegrass Society for Neuroscience member

SEMINARS

2013	Department of Neurosurgery "Effects of Diffuse Traumatic Brain Injury on Sleep in the Mouse" College of Medicine, Texas A&M Health Science Center Scott & White Hospital Central Texas Veterans Health Care System, Temple, Texas
2012	Department of Anatomy and Neurobiology Seminar Series- "Effects of Diffuse Traumatic Brain Injury on Sleep in the Mouse" University of Kentucky College of Medicine, Lexington, KY
2012	Phoenix Children's Hospital Neurology Resident Seminar Series- "Diffuse Traumatic Brain Injury Induces Acute Post-Traumatic Sleep" Phoenix, AZ
2011	Department of Anatomy and Neurobiology Seminar Series- "Post-traumatic Sleep in the Diffuse Brain Injured Mouse" University of Kentucky College of Medicine, Lexington, KY
2011	Qualifying Examination Seminar- "Inflammation and Sleep Correlation Following Diffuse Traumatic Brain Injury in the Mouse" University of Kentucky College of Medicine, Lexington, KY
2007	Chemistry Department Seminar Series- "Antimicrobial Properties of Novel Quinoxalines" Eastern Kentucky University, Richmond, KY

TEACHING EXPERIENCE

2013	Teacher (Village Leader) – Science, Technology, Engineering, Math (STEM) Education Program, Arizona State University. Taught an 8 week course on Anatomy to 5-8 grades.
2009-2012	Teaching Assistant: ANA 109, ANA 110 – *Nursing Anatomy*
2010	Training Instructor – Spinal Cord and Brian Injury Research Center, University of Kentucky. Trained faculty members from Ohio State University on how to perform midline craniotomies and conduct midline fluid percussion injuries in mice.

UNIVERSITY/COMMUNITY SERVICE

2011	Athens Elementary School volunteer for Arts and Science Day
2011	Dance Blue Volunteer, University of Kentucky Dance Marathon to raise money for UK Children's Cancer Clinic
2010	Faith Feeds Volunteer, outreach program in Lexington, KY, to reduce local hunger through food preparation and delivery
2007-2009	Tree of Hope Volunteer, distribution of Christmas presents to less fortunate
2007-2009	Polar Bear Plunge participant, fundraiser for the Special Olympics
2007	University of Kentucky Children's Hospital Volunteer
2007-2008	University of Kentucky Hospital Volunteer, through pre-medical program I have over 200 volunteer hours in the Emergency Room and Labor and Delivery
2007	Cardinal Hill Rehabilitation Hospital Volunteer, Lexington, KY
2005-2010	Children of the Cumberlands Advocate, Oneida, TN, Work with local businesses to raise money for abused children
2005-2010	Relay for Life, Oneida, TN, Work with local businesses to raise money for cancer research and participate in the overnight walk for cancer awareness
2005-2010	St. Jude's Hospital, Organize an annual fundraiser to donate to pediatric cancer research

PUBLICATIONS

Rowe, R.K., J. Harrison, T.C. Thomas, J.R. Pauly, P.D. Adelson and J. Lifshitz. (2013) "Anesthetics and analgesics in experimental traumatic brain injury: Selection based on experimental objectives." *Lab Animal* 42:286-291

A.D. Bachstetter, **R.K. Rowe**, M. Kaneko, D. Goulding, J. Lifshitz, and L.J Van Eldik. (2013) "The p38α MAPK regulates microglial responsiveness to diffuse traumatic brain injury" *J. Neuroscience* 33:6143-6153c

Rowe, R.K., M. Striz, A.D. Bachstetter, L.J. Van Eldik, K.D. Donohue, B.F. O'Hara, and J. Lifshitz. (2013)"Diffuse brain injury induces acute post-traumatic sleep." *PLOS ONE (*accepted*)*

Rowe, R.K., J.L. Harrison, B.F. O'Hara, and J. Lifshitz. (2013) "Recovery of

neurological function despite immediate sleep disruption following diffuse brain injury in the mouse: clinical relevance to medically untreated concussion" *SLEEP* (accepted)

Rowe, R.K., J.L. Harrison, B.F. O'Hara, and J. Lifshitz. (2013) "Diffuse brain injury does not affect chronic sleep patterns in the mouse" *Brain Injury* (in review)

J.L. Harrison, **Rowe, R.K.,** B.F. O'Hara, P.D. Adelson, and J. Lifshitz. (2013) "Acute over-the-counter pharmacological intervention does not adversely affect behavioral outcome following diffuse traumatic brain injury in the mouse" *Experimental Brain Research* (in review)

BOOK CHAPTER

Van Bregt, D, TC Thomas, **RK Rowe**, J Lifshitz. (2011) Morphological assessments of traumatic brain injury. In J Chen, X-M Xu, ZC Xu and JH Zhang *Animal Models of Acute Neurological Injuries II: Injury and Mechanistic Assessments*. Totowa, NJ, The Humana Press Inc.

ABSTRACTS

Rowe, R.K., J. Harrison, B. O'Hara, P.D. Adelson and J. Lifshitz. (2013) "Immediate post-injury sleep disruption does not affect functional outcome following diffuse brain injury in mice." *J. Neurotrauma*

Harrison, J., **R. Rowe**, B. O'Hara, P.D. Adelson, and J. Lifshitz. (2013) "Experimental diffuse brain injury does not impact chronic sleep patterns." *J. Neurotrauma*

Bachstetter, A.D., **R. Rowe**, M. Kaneko, D. Goulding, J. Lifshitz, and L. Van Eldik. (2013) "Microglial responsiveness to diffuse traumatic brain injury: The role of p38α MAPK" *J. Neurotrauma.*

Webster, S.J., A. Bachstetter, M. Watterson, **R. Rowe**, J. Lifshitz, and L. Van Eldik. (2013) "Can targeting the proinflammatory cytokine surge following traumatic brain injury improve pathological outcomes?" *J. Neurotrauma.*

Ellia, T.W., Ziebell, J.M., **R. Rowe**, J. Harrison, P.D. Adelson, and J. Lifshitz. (2013) "Influence of age on rod-microglia formation following diffuse brain injury." *Phoenix Children's Hospital Annual Research Day,* Phoenix, AZ

Goulding, D., A.D. Bachstetter, **R. Rowe**, J. Lifshitz, D.M. Watterson, and L.J. Van Eldik. (2012) "Novel CNS therapeutic suppresses proinflammatory cytokines in a rodent model of diffuse traumatic brain injury". Bluegrass Society for Neuroscience, Lexington, KY

Rowe, R.K., M. Striz, B. O'Hara, and J. Lifshitz. (2012) "Inflammation and sleep correlation following diffuse traumatic brain injury in the mouse." Program Number 254.09. 2012 Neuroscience Meeting Planner. Society for Neuroscience, 2012. Online.

Bachstetter, A.D., D. Watterson, **R. Rowe**, J. Lifshitz, L. Van Eldik. (2012) "Post-injury administration of a novel CNS experimental therapeutic in the rodent fluid percussion model extends the potential range of efficacy to diffuse traumatic brain injury." Program Number 363.08. 2012 Neuroscience Meeting Planner. Society for Neuroscience, 2012. Online.

Rowe, R.K., J. Harrison, M. Striz, B. O'Hara, K. Donohue., and J. Lifshitz. (2012) "Diffuse brain injury increases acute quantitative measures of reparative sleep in the mouse." *J. Neurotrauma*

Harrison, J., **R. Rowe**, B. O'Hara, and J. Lifshitz. (2012) "Immediate post-injury sleep disruption alters the expression of inflammation related genes after diffuse brain injury in mice." *J. Neurotrauma*

Rowe, R.K., M. Striz, B. O'Hara, and J. Lifshitz. (2011) "Effects of Diffuse Traumatic Brain Injury in the Mouse." *J. Neurotrauma,* 28 (6): A-87

Marti, F., A. Bachstetter, **R.K. Rowe,** G. Ellis, L. Van Eldik, and J Lifshitz. (2011) "Systemic Loss of Peripheral Regulatory T-cell Differentiation after Diffuse Brain Injury in the Mouse." *J. Neurotrauma*, 28 (6): A-87

Ellis, G., A.D. Bachstetter, **R. Rowe**, L. Van Eldik, J. Lifshitz, and F. Marti. (2011) "Diffuse traumatic brain injury impairs peripherally induces regulatory T cell differentiation". Autumn Immunology Conference, Chicago, IL, November 18-21, 2011.

Rowe, R., A.D. Bachstetter, D.M. Watterson, J. Lifshitz., and L.J Van Eldik. (2011) "Post-injury administration of a novel CNS therapeutic suppresses proinflammatory cytokines in a rodent model of diffuse traumatic brain injury". Markesbery Symposium on Aging and Dementia, Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY, October 14-15, 2011.