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RECOVERY OF SENSORIMOTOR FUNCTION IN RATS FOLLOWING ACUTE RAPID EYE MOVEMENT SLEEP DEPRIVATION AND CONTROLLED CORTICAL IMPACT

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Bachelor of Arts in Psychology

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May, 2008

submitted in partial fulfillment of requirements for the degree

MASTER OF ARTS IN PSYCHOLOGY

at the

CLEVELAND STATE UNIVERSITY

December, 2010

This thesis has been approved

for the Department of PSYCHOLOGY

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DEDICATION

This project is dedicated to my family: my husband, Jeff, who believed in me even when I didn't believe in myself and my kids for the countless hours I spent away from them working on this project; my parents and mother in law for watching the kids when I would be at work late. Thank you and I couldn't have done it without the support of everyone!

ACKNOWLEDGEMENT

The author would like to thanks the persons who assisted in and supported the onset and completion of this project. In particular:

Dr. Sherry Ball for all of her guidance and assistance, without which this project would not have been possible; Abby Kyser, Lisa Franklin and Brett Hanzlicek for their hours of training and help with getting this project off the ground; Vera O'Brien for the surgical support, tissue sectioning and countless hours of microscopy in a small dark room; Amber Chenoweth, Katy Bryan, and Ronald Riechers for the psychological support, energy, and support to keep me motivated and progressing! Steven Soehnlen for taking on the task of answering the questions left unanswered from my project. A special thank you to Drs. Stephen Slane and Michael Hammonds for their help and assistance as members of my thesis committee.

RECOVERY OF SENSORIMOTOR FUNCTION IN RATS FOLLOWING ACUTE RAPID EYE MOVEMENT SLEEP DEPRIVATION AND TRAUMATIC BRAIN INJURY FOLLOWING CONTROLLED CORTICAL IMPACT

JAIME LYNN SHUSTER

ABSTRACT

Traumatic brain injury (TBI) resulting from bomb blasts and explosions is common among military personnel. The effects of Rapid Eye Movement (REM) sleep deprivation on the sensorimotor behavior and physiological mechanisms related to TBI are unknown. Thirty-two Long Evans rats were randomly assigned to REM sleep deprivation (RSD) with controlled cortical impact (CCI), social isolation (SI) with CCI, or normal housing (NH) with CCI or Sham. Two behavioral tasks [beam walk and bilateral tactile adhesive removal somatosensory (BTARS)] testing motor and sensory function were used to investigate recovery of function. Brain tissue was analyzed using Cresyl Violet stain (cell bodies), GFAP (astrocytes) and Fluoro Jade-B (dying cells) labeling. Results indicated that 24 hour RSD immediately prior to CCI impaired recovery of sensorimotor function when tested on the adhesive removal task. Recovery of sensorimotor function as a result of 24 hour RSD immediately prior to CCI was not significantly impaired when tested on the balance beam walk task. Results also indicated that sleep deprivation seemed to intensify inflammation, lesion size and neuron loss when compared to non sleep deprived animals.

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ABBREVIATIONS/ACRONYMS

AAALAC	Association for Assessment and Accreditation for Laboratory Animal Care International
BTARS	Bilateral Tactile Adhesive Removal Somatosensory
CCI	Controlled Cortical Impact
FJB	Fluoro Jade-B
GFAP	Glial Fibrillary Acidic Protein
IACUC	Institutional Animal Care and Use Committee
NHCCI	Normal Housing Controlled Cortical Impact
REM	Rapid Eye Movement
RSD	Rapid Eye Movement Sleep Deprivation
SI	Social Isolation
TBI	Traumatic Brain Injury
VA	Veterans Affairs

Key words:

Traumatic Brain Injury (TBI), Sleep deprivation, Sensorimotor, Controlled Cortical Impact (CCI)

CHAPTER 1

INTRODUCTION

1.1 Traumatic Brain Injury

Approximately 1.5 million Americans each year experience some form of traumatic brain injury (TBI) and approximately 1.1 million of these are treated and released from a hospital or trauma center (Rutland-Brown, Langlois, & Thomas, 2006; Rutland-Brown, Langlois, Thomas, & Xi, 2006). TBI is defined as: "a nondegenerative, noncongenital insult to the brain from an external mechanical force, possibly leading to permanent or temporary impairment of cognitive, physical and psychosocial functions, with an associated diminished or altered state of consciousness," (Dawodu, 2009, p. 1). Approximately 50,000 deaths occur each year as a result of TBI (Dawodu, 2009; Langlois, Rutland-Brown, & Wald, 2006). Some form of TBI in combat is experienced by 150,000 to 300,000 returning US soldiers from Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF) (Centers for Disease Control (CDC), 2010; Emery, 2007; Warden 2006). Of this group, 44% experienced mild TBI, while 56% were afflicted with moderate to severe TBI (French & Parkinson, 2008; Warden, 2006). TBI related costs are estimated to be over $10 billion a year in the United States with an average lifetime cost per person of nearly $2 million/ patient (Dawodu, 2009; Guerrero, Thurman, & Sniezek, 2000).

TBI is generally caused by motor vehicle accidents, falls, assaults and blast injuries (Langlois et al., 2006). The largest group affected is between the ages of fifteen and twenty four. However, the groups at greatest risk for sustaining a TBI are for adults over the age of eighty and children under the age of nine (Katz & Deluca, 1992; Rutland-Brown, Langlois, Thomas & Xi, 2006; U.S. Department of Health and Human Services (USDHHS), 2004).

TBI can be categorized on several dimensions. The direct impact on the brain can cause compression, shear or tearing of the affected tissue, and can be considered focal or diffuse as well as coup (direct impact) and/or countrecoup (bounce back; (Adams, 1975; Guerrero, et al., 2000; Katz & Deluca, 1992). TBI includes both primary and secondary injury processes. The primary injury process involves mechanical effects that occur at the time of injury resulting in massive initial cell death. The secondary injury includes post effects such as recurring and recovery from cell death, that correlate with long term mental and physical deficits (French et al., 2008; Laurer & McIntosh, 1999; Sutton et al., 1993).

The level of damage and location of impact determine the severity and symptoms. Mild TBI patients may have lost consciousness and tend to suffer from headaches, vomiting, nausea, dizziness, lack of motor coordination, difficulty balancing and changes in sleep patterns. Difficulty adjusting to mood and/or behavioral changes, memory, concentration, attention or thinking, and confusion are common cognitive and emotional symptoms (NINDS, 2008; Kushner, 1998; Katz et al., 1992). Although some patients show memory improvements within 6 months to 2 years of the brain injury (French et al., 2008; Lannoo, Colardyns, Jannes, & De Soete, 2001), other patients continue to

experience deficits at 10 years post-injury (Laurer & McIntosh, 1999; Zec et al., 2001). Memory shortfalls most likely occur because TBI leads to widespread axonal injury (Ommaya, Goldsmith & Thibault, 2002) in which the temporal and frontal lobes were found to be the most vulnerable cortical locations (Adams, 1975; Kennedy et al., 2007; Laurer & McIntosh, 1999). Moderate to severe TBI patients may suffer from social judgment deficits, problems with sustained attention, processing speed and executive functioning in addition to mild level symptoms (Kokiko & Hamm, 2007; Lombardi, 2008). These difficulties can make post-injury life particularly difficult with regard to activities of daily living, such as maintaining employment and sleep regulation (Busch, McBride, Curtiss, & Vanderploeg, 2005; Kim, 2002; McDonald, Flanagan, Rollins, & Kinch, 2003; Ponsford, Draper, & Schonberger, 2008; Stone, Baron-Cohen, & Knight, 1998).

Well recognized TBI deficits in humans are motor or sensorimotor impairments (Fujimoto, Longhi, Saatman, & McIntosh, 2004; Piot-Grosjean, Wahl, Gobbo & Stutzmann, 2001). According to Wiley and colleagues (1996), rats have reduced sensorimotor reactivity after TBI (Fujimoto, et al., 2004; Wiley et al., 1996). A motor impairment reduces the speed of rats traversing a beam post-injury (Fujimoto, et al., 2004; Piot-Grosjean, et al., 2001; Smith, et al., 2007). Reduced interlimb coordination, decreased gait velocity and stride length have also been noted after TBI (Neumann, et al., 2009).

The severity and location of injury, as well as impact velocity affects the recovery rate after TBI (Smith, et al., 2007; Soblosky, Colgin, Chorney-Lane, Davidson, & Carey, 1997). Recovery can be spontaneous, vary over time, and can be positively influenced by

factors such as environmental enrichment, physical exercise, pharmacology and behavioral task practice (Fujimoto, et al., 2004; Piot-Grosjean, et al., 2001; Soblosky, et al., 1997; Smith, et al., 2007). According to Soblosky and colleagues (1997), the recovery period will have 2 phases: (1) initial rapid recovery the first few days post-injury and (2) slower recovery over the following weeks. The rate of recovery is variable depending on the injury mechanics sustained. Compensatory motor movement learning can also take place both acutely and chronically without task specific practice even though task specific practice enhances the recovery rate post TBI (Piot-Grosjean, et al., 2001; Soblosky, et al, 1997; Smith, et al., 2007).

1.2 Different Models of TBI

The brain of a rodent is similar to the human brain (Gennarelli, 1994; Kokiko-Cochran, Michaels, & Hamm, 2008; Laurer & McIntosh, 1999). Therefore, animal models can be utilized to study TBI. Several types of brain injury animal models have been developed in recent years. A frequently used model is the fluid percussion injury (FPI) model. The FPI model consists of a fluid filled pulse applied to the intact dura through a craniotomy by means of a pendulum striking a saline filled Plexiglas cylinder over the craniotomy site (Dixon et al., 1987; Kokiko-Cochran et al., 2008; Lighthall, Dixon, & Anderson, 1989). However, it is difficult to reproduce this injury due to its diffuse nature. According to Cernak (2005) there has also been evidence of some contralateral and ipsilateral damage beyond the originally intended area.

The weight drop model, (Marmarou et al., 1994), is a closed head impact acceleration injury procedure in which a column of brass weights is dropped from a designated height (Clausen & Hillered, 2005; Foda & Marmarou, 1994). This model is

easy to use and is cost effective, but produces inconsistent injuries (Clausen & Hillered, 2005).

To combat the diffuse injury area issue of the FPI model and inconsistencies of the weight drop method, the Controlled Cortical Impact (CCI) method was developed (Dixon, Clifton, Lighthall, Yaghmai, & Hayes, 1991; Lighthall, 1988; Lighthall et al., 1989). The injury is delivered through a craniotomy to the intact dura by an air driven piston causing deformation to the cortex. The CCI method produces a more focused injury compared to the FPI model and is more reproducible as compared to the weight drop method (Cernak, 2005; Dixon et al., 1991). The CCI model is more appropriate when used to analyze resulting neurological deficits and mechanisms underlying neuronal cell death (Brody et al., 2007; Cernak, 2005; Hoffman, Fulop & Stein, 1994).

1.3 Sleep Characteristics

Sleep is categorized into two major types: Non Rapid Eye Movement (NREM) and Rapid Eye Movement (REM). NREM sleep has characteristics that include slow regular heart rate and breathing, low body temperature and muscle tone maintenance, as well as low frequency brain waves with a relatively high voltage on electroencephalographic (EEG) recordings (Amici et al., 2008). NREM sleep appears to be a phase in which energy is conserved and in which both the central nervous system and other systems are able to recover from the activity of the previous episode of wakefulness or to prepare for the next episode (McEwen, 2006).

REM sleep has distinct characteristics that include low muscle tone, rapid eye movement and a rapid frequency low voltage EEG pattern. Breathing and heart rates as well as body temperature are irregular during periods of REM sleep (Amici et al., 2008). During REM sleep, information obtained during wakefulness appears to be reprocessed

and integrated into existing neural templates (McEwen, 2006). Restorative processes take place in the brain during REM sleep. Prolonged continuous wakefulness causes impairments in hippocampal long term synaptic plasticity and hippocampus-dependent memory formation (McDermott et al., 2003).

There are different forms of sleep loss: sleep reduction (less sleep than normal), sleep fragmentation (interrupted sleep), and total sleep loss. All may lead to similar effects on sleepiness, psychomotor performance and hormonal disturbances (Boonstra, Stins, Daffertshofer, & Beek, 2007). Frey and colleagues (2007), stated that it is very common for people in society today to forgo one night of sleep and this short term sleep deprivation has significant yet small effects on inflammatory markers in healthy young adults (Frey, Fleshner, & Wright, 2007). REM sleep deprivation increased errors of commission and omission as well as reaction time variability in studies utilizing both the Continuous Performance and Psychomotor Vigilance Tests in healthy adults (Dinges & Kribbs, 1991).

The prefrontal cortex, usually the most active area of the brain in rested individuals, becomes more active as a person remains awake for long periods of time (Keshavan, et al., 1995). This region restores during the first stage of sleep, giving a person the ability to feel somewhat refreshed after only a short nap (McEwen, 2006). The length of the first stage of the sleep cycle is somewhat dependent upon how long the person had previously been awake. The longer the period of wakefulness, the longer the brain remains in the first stage of sleep. When the brain enters into the REM stage of sleep, the prefrontal cortex begins to actively process information once more. Since different regions of the brain rest during different stages of the sleep cycle, sleep should

not be cut short. If the brain remains in a wakeful state for too long, it begins to shut down for periods of microsleep. This microsleep phase is essentially several seconds of actual sleep. Microsleep generally happens directly before performance failure occurs (Keshavan, et al., 1995).

1.4 Sleep Deprivation Techniques

Several techniques have been established to induce sleep deprivation in animals. The gentle handling technique can be carried out by either touching the animal with a soft paintbrush (Modirrousta, Mainville & Jones, 2007; Rao et al, 2007), light tapping (Murillo-Rodriguez, Liu, Blanco-Centurion, & Shiromani, 2008) or by picking up the animal every time it displays a sleeping posture (Schwierin, Borbely, & Tobler, 1999). The gentle handling technique is extremely stressful to the animal and provides somewhat variable results since it is unclear by visual observation which cycle of sleep the animal is actually engaged in (REM or NREM). Novel objects in the animals' environment can induce wakefulness (Vyazovskiy, Cirelli, Tononi & Tobler, 2008) by engaging the animal. However, sometimes the animal would already be engaged in REM sleep and not wake up to notice a novel object in its immediate area (Halassa et al., 2009).

The rotating drum or rotating wheel is another sleep deprivation technique that is widely used (Roman, Van der Borght, Leemburg, Van der Zee, & Meerlo, 2005). This technique utilizes a yoked control with a subject in an adjacent cage. When the experimental animal starts to sleep, the disk is rotated which forces both animals to wake up and walk in the opposite directions. As long as the animal walks for the set amount of time, he will not fall into the water below the platform (Kim, Laposky, Bergmann, & Turek, 2007; Novati et al, 2008). The drawback is that the experimental animals fall into

the water more often than their controls and stay there because they are too tired to continue.

Similar to the rotating wheel or drum is the treadmill sleep deprivation technique. Guzman-Marin and colleagues (2008) used EEG to detect when the animal entered REM sleep and immediately started the treadmill to induce walking in the animal (Guzman-Marin, Bashir, Suntsova, Szymusiak & McGinty, 2007). They also used a yoked control animal that engaged in the same activity as the experimental animal. McKenna and colleagues (2007) utilized the treadmill technique but without the EEG component. They simply started the treadmill roughly every 2 minutes for either 6 or 24 hours. This may be extremely stressful to the animal.

A simpler yet reliable approach to inducing REM sleep deprivation (RSD) in rats is known as the “flower pot technique” or disc over water method, and was originally developed in 1963 for cats (Jouvet, Vimont, Delorme, & Jouvet, 1964). In this technique, the animal sits on an inverted flower pot surrounded by water. They can obtain slow wave sleep by sitting or crouching; however, when they enter REM sleep, their muscular tone diminishes and causes them to fall into the water which wakes them up (Mendelson, Guthrie, Frederick, & Wyatt, 1974). The advantages of this technique are that it is simple, reliable and allows for a large number of animals to be tested at the same time (Mendelson et al, 1974). There are variations to this technique which include housing multiple animals in the same platform housing with multiple platforms (Allard, Tizabi, Shaffery, & Manaye, 2007; Machado, Suchecki, & Turfik, 2006) as well as housing alone with one platform (Jouvet et al., 1963; Mendelson et al., 1974). Since rodents are social animals, housing separately increases their stress level, which is why the multiple

platforms multiple subjects containment system was originally devised (Biswas, Mishra, & Mallick, 2006; Mirescu, Peters, Noiman, & Gould, 2006; Murison, Ursin, Coover, Lien, & Ursin, 1982). There are also various time points for housing in such an apparatus which range from 4 hours to 10 days (Cirelli, Faraguna, & Tononi, 2006; Koban, Sita, Le, & Hoffman, 2008). To ensure the well being and minimize stress levels of the animals, in this study, the single platform method was utilized to induce RSD for 24 hours.

Sustained attention and focus, primarily located in the prefrontal cortex, is affected by both sleep deprivation and TBI (Bloomfield, Espie, & Evans, 2010). Bloomfield and colleagues (2010) believe that since attention and focus are detrimentally affected during periods of sleep deprivation and separately post TBI, that when combined, the sleep disturbance would further exacerbate exhibited TBI deficits. Focus and attention mechanisms are disturbed during sleep deprivation and TBI. This may cause sensorimotor function to be affected as well since attention and focus are required to elicit intact sensorimotor function (Piot-Grosjean, et al., 2001). Insomniacs and TBI affected patients experience dizziness and basic motor impairment such as balance problems (Zollman, Cyborski, & Duraski, 2010). However, to date, in the literature there has not been an animal model study of the influence of prior sleep deprivation on TBI. This gap in the literature was investigated and addressed in this study.

1.5 Cellular Mechanisms

All of the underlying cellular mechanisms following TBI are not fully understood. It is known that post TBI symptoms include: inflammation, edema, apoptosis, necrosis, neuron death, and excitotoxity (Kokiko, Murashov, & Hoane, 2006; Werner & Engelhard, 2007). Although understanding all of the underlying cellular mechanisms is

important, this study focused on a specific facet of cellular mechanisms. According to Stanimirovic and Satoh (2000), inflammatory cells are known to be a major source of post-injury toxins that result in a feed forward process of inflammation induced cell death (Stanimirovic & Satoh, 2000). During the natural healing process, the clearing of dead or dying cell debris is routine, however it may induce further stress to the already compromised regions of the injured brain during the feed forward cascade of inflammation (Williams, Wei, Dave & Tortella, 2007). Williams and colleagues (2007) also claim that astrocytes are more resistant to damage than neurons following toxic insult. The relationship between neurons and astrocytes appears to play an important role for the repair of the injured central nervous system (Lenzlinger, Morganti-Kossmann, Laurer, & McIntosh, 2001). During the excitotoxicity phase of the body's response to trauma, high levels of glutamate, calcium and cytokines are released which also contribute to tissue damage (Pettus, Wright, Stein & Hoffman, 2005). Besides this destructive process, neuroprotective events in repair and regeneration also take place and it is this delicate balance that plays an important role in cell survival (Lenzlinger et al., 2001).

It is this gap in the literature regarding the association between the implications of sleep deprivation prior to TBI that requires further investigation. Without the knowledge of these underlying mechanisms and their effects on subsequent behavior, rehabilitative treatments are difficult to outline for those affected.

1.6 Research Question

The purpose of this study was to investigate how prior sleep deprivation influences the response of the brain to TBI because some of the neurological processes occurring during sleep deprivation parallel those in TBI. The hypothesis was that sleep

deprivation, even for 24 hours, negatively affects the brain and its ability to function and recover from TBI. Animals were REM sleep deprived for 24 hours via the flower pot technique and subsequently underwent surgery via the controlled cortical impact (CCI) method, followed by behavioral and tissue testing and analysis. The balance beam and bilateral tactile adhesive removal task (BTARS) served as sensorimotor behavioral measures while the tissue was analyzed with three types of stains: Cresyl Violet (quantify lesion area), Fluoro Jade-B (degenerating cells), and Glial Fibrillary Acidic Protein (GFAP astrocytes and Bergmann glial cells).

CHAPTER II

METHOD

2.1 Subjects

Thirty two, male, Long Evans Blue Spruce, (to remain consistent with the literature), rats were purchased from Harlan (Indianapolis, IN) at 58-64 days of age, and weighing approximately between 225 and 249 grams. This particular age and weight range was chosen so that once the animals were acclimated to the facilities and received their baseline training measures, they would be the correct age and weight to be consistent with the literature for this type of procedure. According to Goldstein (1993), smaller, younger animals are not as experienced in their motor function and larger older animals tend to walk slower and lose motor control easier; therefore adolescent animals were chosen for these measures. However, by 45-48 days of age, their brains are considered mature. All experimental procedures were reviewed and approved by the Louis Stokes Cleveland Department of Veterans Affairs Medical Center Institutional Animal Care and Use Committee (IACUC). The study was conducted in a facility certified by the American Association for the Accreditation of Laboratory Animal Care on the ethical use of animals and every attempt was made to minimize the number of animals and their suffering. The combination of literature review and power analysis was used to determine the number of animals per group to achieve the best result. A

significance level of $p < 0.05$ was used for all statistical analyses. An *a*-priori sample size calculation was performed for within group differences of at least two standard deviations and was detectable with eight animals per group with at least 80% power. Rats were maintained on a standard 12 hour light/dark cycle with food and water *ad libitum*.

Animals were randomly assigned to one of four groups: 24 hour RSD with CCI (n=8, RSD CCI), 24 hour social isolation with CCI (n=8, SI CCI), normal housing with CCI (n=8, NH CCI), and normal housing with Sham (n=8, Sham). The RSD CCI group represented the combined CCI injury and sleep deprivation model and the SI CCI group was used to account for the environmental effects of the bucket in which the RSD CCI group was housed. The NH CCI group was used to account for the CCI injury without the stress of the sleep deprivation environment and to allow the animal to remain with its cage mates pre and post surgery. The Sham condition was used to mimic the NH CCI group's environmental conditions without the CCI injury or the effects of anesthesia, surgical procedures such as the incision of the scalp and the craniotomy, and post-operative analgesics. All animals were subjected to anesthesia, craniotomy, and post operative analgesics. Baseline testing for the balance beam and BTARS task was completed four days prior to surgery and post-operative sensorimotor performance measures were collected on days four, seven and fourteen post-injury. On day fifteen post-operative, animals were intracardially perfused and brains removed for histological and immunohistochemical analysis.

2.2 Rapid Eye Movement Sleep Deprivation

RSD was carried out by the disk-over-water method also known as the "flower pot" method (Jouvet et al, 1963; Mendelson et al, 1974). A platform was secured to a flower pot base measuring 24 cm in diameter and placed in an opaque plastic container

measuring 30 cm in diameter. There were two different sized platforms utilized in this model: a platform 14 cm in diameter served as the control and a platform 10 cm in diameter served as the experimental platform. The control platform (SI CCI) allowed for both slow wave REM and non-REM sleep and served as the control for the environmental conditions for the experimental group (RSD CCI). All animals were socially isolated in these environments with food and water available *ad libitum* throughout the 24 hour sleep deprivation period. The opaque plastic container was filled with water within 1.0 cm of the platform and allowed to acclimate to room temperature at least 24 hours prior to use. Animals were randomly selected, placed in the apparatus for 24 hours and monitored every four hours as mandated by the VA IACUC. If the animal entered REM sleep, it lost muscle tone, fell off the platform into the water, awoke and climbed back onto the platform. This cycle was repeated throughout the 24 hour time period in the opaque plastic container. At the end of 24 hours, CCI or sham surgery commenced.

2.3 Controlled Cortical Impact (CCI) Surgery

Surgery was performed at 72-78 days of age with the animals weighing between 250 and 315 grams to correlate with the weights needed for the behavioral testing which followed post-operatively and to ensure correct and consistent lesion placement. Animals were anesthetized with a mixture of oxygen and 4% Isoflurane through an anesthesia inhalant apparatus. When the animal became unresponsive (no ocular or pedal reflexes), the head was shaved and scrubbed alternating with Betadine® and 70% EtOH. Anesthesia was monitored continuously and reduced appropriately as the procedure continued. Aseptic surgical technique was maintained throughout the procedure. The animal was placed in a stereotactic device (Stoelting, Wood Dale, IL) with the head held

in place with two ear bars and an incisor bar which maintained consistent orientation. Normal body temperature was maintained with a heating pad during surgery set at 37° Celsius. A midline incision was made in the skin and the underlying fascia on the dorsal side of the head in order to expose the cranium. The skin was held to each side with hemostats to expose the surface of the skull which was cleaned with sterile saline and cotton tipped swabs to view the cranial sutures. A unilateral circular craniotomy (~5.0 mm) was performed with a micro drill and centered over the sensorimotor cortices 3 mm lateral to bregma in the left hemisphere. This area, the sensorimotor cortex, is located in the frontal cortex and has demonstrated that despite the direction of force for TBI, it is the most affected area in the brain for physical motor distress (Hoffman et al, 1994; Holbourn, 1943). Sham operated animals underwent the identical procedure except receipt of the CCI.

A contusion injury was produced with a commercially available impactor device from My NeuroLab (St. Louis, MO) fitted with a sterilized round stainless steel impactor (3 mm diameter) powered by an electromagnetically driven piston. The impactor was accelerated 3.0 m/second with a cortical contact time of 500 milliseconds and D/V -2.0 mm impact depth, which caused a mild to moderate trauma. The speed and contact time are considered of the medium velocity range (2.86 ± 0.192 m/sec) with a 3.0-4.5 mm diameter impactor according to Lighthall (1988). Any larger impact or increase in velocity would fatally injure the animal due to extensive damage causing hypertension and hemorrhage (Kessens, MacDonald, Brody, & Bayly, 2005; Lighthall, 1988). The low velocity range (1.9 ±0.075 m/sec) with a 2.0-3.0 mm diameter impact did not yield an injury to the cortical surface or dura mater and does not show physical motor deficits

post-operatively. The high velocity range (4.0 ± 0.01 m/sec) does yield a prolonged hypertensive period following injury with both 2.5 mm diameter and 3.5 mm diameter impacts; with the 3.5 mm impacts (n=3) resulting in fatal injuries to the animals twenty minutes post-operatively (Lighthall, 1988).

The area of the contusion showed an indentation and discoloration of the dura that was obvious following perfusion and removal of the brain. Following the contusion, any bleeding was controlled with sterile swabs. The incision was sutured with Ethicon® 4-0 nonabsorbable suture material and the wound was treated with the triple antibiotic ointment Vetropolycin® (Overland, KS). Animals were then transferred to a heated 37° Celsius post-operative recovery area and were monitored continuously until mobile. They were then transferred back to their home cages with their same cage mates and monitored daily for 14 days post-surgery. Buprenorphine, an analgesic, was administered at a dosage of 0.05 mg/kg intraperitoneally (IP) at 12 and 24 hours post-operative. Behavioral testing resumed on day 4 post-operatively a time reported to yield reliable data (Soblosky, et al., 1996).

2.4 Balance Beam Task

Since the brain area most often affected by TBI includes the sensorimotor cortex, (Holbourn, 1943), motor and sensory tests were used to assess function pre and post-operatively (Cenci, Whishaw, & Schallert, 2002; Kolb & Whishaw, 1983; Schallert & Woodlee, 2005). The balance beam assessed fine and gross motor function through the animals' ability to traverse the beam (Feeney, Gonzalez & Law, 1982; Goldstein, 1993; Goldstein & Davis, 1990; Kolb & Whishaw, 1983; Schallert et al, 2005). Soblosky and colleagues (1996) noted that prior to injury all of their animals could cross the beam with little difficulty while post-operatively on days one and two; none of the animals could

cross the beam. Soblosky and colleagues (1996) utilized a 4mm impact tip with a 5 mm craniotomy positioned 2.5 mm anterior and 2.5 mm posterior to bregma. On post-operative days three and four, only one and two rats out of 25, respectively, could successfully complete the task. In this study, a 3mm impact tip was utilized with a 4 mm craniotomoy positioned 3mm lateral to bregma. The impact that Soblosky and colleagues (1996) created was slightly larger and more midline than this study's. In this study, post-operative days four, seven and fourteen were scheduled to conduct behavioral testing.

Animals were shaped to traverse a 2 cm wide by 1 m long beam suspended 90 cm over soft padding for five trials a day for seven days prior to RSD and/or surgery. The length of the beam was marked lengthwise from 1-5 into equivalent sections. Baseline measures were taken four days prior to surgery and measures were taken post-operatively on day four, seven and fourteen. Scoring was rated from zero to five. Zero was defined as not traversing the beam and remaining at the starting point and five was defined as completion of the task and entering the goal box. Scores of one, two, three or four assigned for reaching equivalent sections along the beam. The latency to complete the task or fall off the beam was recorded in seconds. Prior to injury, all animals were able to successfully complete the task and post-operatively, animals that received a contusion injury had increased difficulty completing the task thus increasing their latency times as well as decreasing their completion rates. The result of these scores demonstrated the severity of injury on the motor function of the animal. Animals were motivated to cross the beam to escape the aversive stimuli of white noise and received positive reinforcement in the form of a Yogies (Spectrum Brands, Islandia, NY) yogurt fruit flavored rat treat. If the animal fell off the beam, the time and place of fall was recorded

and the trial immediately ceased. The rats were placed in the home cage for two minutes in between balance beam trials.

2.5 Bilateral Tactile Adhesive Removal Somatosensory Task

The BTARS task is a simple yet reliable measure of sensorimotor deficits as a result of various types of brain injury such as CCI and stroke (Cenci et al., 2002; Chen et al., 2001; Chen et al., 2007; Schallert & Woodlee, 2005; Wiley et al., 1996). Individual animals showed few or no performance variations during baseline measures which were collected four days prior to injury. Animals were shaped to remove 2 cm diameter round stickers (Avery #5466) from each of their distal radial forelimbs (right and left) in the hairless area while in a clear shoebox cage with lid. Each day consisted of two trials for each forelimb resulting in a total of 4 trials per day per animal. Animals were tested four days pre-operative and four, seven and fourteen days post-operative. Removal time was recorded in seconds and the trial was considered over when the animal either removed the sticker or two minutes elapsed. After the completion of each trial, the animal was removed from the testing cage, placed in his home cage for five minutes during the inter-trial interval, and the stickers were removed.

2.6 Histology

At the conclusion of behavioral testing, or fifteen days post-injury, animals were euthanized with a lethal dose of sodium pentobarbital (75 mg/kg, IP). Once animals were deeply anesthetized, they were intracardially perfused with 200 ml of 0.1M phosphate buffered saline (PBS, pH 7.4) followed by 200 ml of 4% paraformaldehyde solution (PAF, pH 7.4). Brain tissue was removed and post-fixed in 4% PAF for storage up to four hours. Tissue was then transferred to 30% sucrose for at least 48 hours or until the tissue sank to the bottom of the storage container. It was then embedded into gelatin and

refrigerated for one hour. The gelatin block was then trimmed to fit into the original container and fixed in a solution of 2.5% gluteraldehyde, 0.1M PBS and 10% sucrose until sectioning. The gelatin block was then rinsed in PBS for a minimum of one hour prior to sectioning on a sliding microtome equipped with a freezing stage. The cerebellum was removed and the cut area of the remaining tissue was frozen on the microtome. A wedge was cut into the right hemisphere side of the gelatin block to ensure correct orientation when floating and mounting tissue for all techniques. To determine the correct desired area of the brain to be kept for analysis, a comparison of the frozen tissue to the diagrams provided by Paxinos and Watson (2008) was performed until the correct area was visible. Brains were sectioned coronally at 50 micrometers (μm) and roughly half of the sections were approximately 1.5 mm anterior to bregma and the other half were about 1.5 mm posterior to bregma which was centered on the lesion area in the sensorimotor cortex. About 96 sections from each brain were kept in PBS with 5% Sodium Azide for preservation and for anatomical analysis. Every fourth section (25% of the total sections) was placed free floating in cryoprotectant and stored in a -80˚ Celsius freezer for later use.

2.7 Cresyl Violet

Every fourth section, or 25% of total brain tissue sections collected, was stained with Cresyl Violet cell body stain for neurons and nuclei in order to quantify lesion area. Saved sections were mounted from storage trays of PBS with 5% Sodium Azide onto gel subbed slides and put on the slide warmer. Mounted tissue slides were loaded into slide boats and all immersions took place while on a rotating shaker to ensure even distribution of solutions to the sections. The slides were placed into slide boats and dipped into jars containing increasing dilutions of EtOH and then into 1:1 mixture of Chloroform and

100% EtOH for 20 minutes for rehydration in preparation of staining. Tissue sections were then subjected to decreasing concentrations of EtOH and distilled water before being submersed in the Cresyl Violet stain for approximately seven minutes to ensure the stain had completely penetrated the tissue. After the staining step, slides were then rinsed in distilled water and increasing concentrations of EtOH. Two consecutive steps of Histoclear to clear any remaining stain were used prior to coverslipping with Permount. Digital images were obtained using a digital camera (RT Spot, Sterling Heights, MI) attached to a light microscope. Every third section of Cresyl Violet stained tissue per brain was photographed and cortical area by hemisphere was quantified using Image J software (NIH, Bethesda, MD). The Image J software allows the user to outline a specified area to be measured and calculated. The left hemisphere (injured side) and right hemispheres were outlined separately from the lateral edge of the corpus callosum to midline and up dorsally to connect the section. The injury was excluded in the outlined portion to allow for comparison of the left vs. right hemispheres. The difference between the left and right hemisphere provided was used to calculate lesion percent [100 - [(Ipsilateral measurement / Contralateral measurement) * 100]]. Once it was saved, the Image J software provided a calculation of the lesion percent for each section analyzed.

2.8 Fluoro-Jade B

Every fourth section or 25% of brain tissue sections was stained with Fluoro-Jade B (Millipore, Temecula, CA) cell stain for degenerating cells (Hoane, Gilbert, Holland, & Pierce, 2006). Fluoro-Jade B is a dye that specifically stains the soma and neurites of the degenerating neurons (Schmued & Hopkins, 2000). Sections were mounted from PBS with 5% Sodium Azide preservative on gel subbed slides and put on the slide warmer after sectioning. All tissue sections were loaded into slide boats and all immersions took

place on a rotating shaker to ensure even distribution of all solutions to the sections. Sections were dipped in 0.1M PBS, distilled water and then dried laying flat on a slide warmer for ten minutes. The slides were then dipped in 100% EtOH, a combination of 1% NaOH and 80% EtOH, 70%, 30% and distilled water again. The next step, $KMnO_4$, was utilized to help break the tissue down to allow for stain penetration and lasted for 30 minutes. The sections were rinsed in distilled water and then immersed in the Fluoro Jade-B stain covered with foil for 45 minutes. After staining, the sections were rinsed in distilled water three times and then placed on the slide warmer again for 10 to 15 minutes in the dark. The sections were immersed in two consecutive steps of Histoclear prior to being cover slipped with Permount. Digital images were obtained using a digital camera (RT Spot, Sterling Heights, MI) attached to a fluorescent light microscope. Two digital images were taken of each section of brain at bregma (center of the measured lesion) contralateral to the injury with the first image from the medial perspective and the second image from the lateral perspective and measure 1024 x 1024 pixels. Photomicrographs were analyzed through Metamorph Software (MDS Analytical Technologies) for the number of degenerating neurons on the contralateral side to the lesion. A number of filters were used to remove any stained objects that are outside the preset parameters and to count the number of objects that met the predetermined conditions in the photomicrograph. The predetermined conditions are the minimum and maximum number of pixels that group together with the Fluoro-Jade B stain in the medial and lateral sections that measure 1024 x 1024 pixels adjacent to midline at bregma. The Fluoro-Jade B positive cells fluoresced red under a green filter.

2.9 Glial Fibrillary Acidic Protein (GFAP)

25% of brain sections were labeled with mouse anti-glial fibrillary acidic protein

(GFAP conjugated to Cy-3, 1:2000, Sigma-Aldrich, Saint Louis, MO) using a free floating technique. The GFAP labels astrocytes and Bergmann glial cells. Tissue sections were washed three times for ten minutes each in 0.1M PBS and then blocked for one hour in 5% normal goat serum in 0.1M PBS with 0.1% Triton-X at room temperature in a dark location since it is extremely light sensitive. The GFAP antibody was allowed to incubate overnight at 4˚ Celsius covered with foil to ensure darkness. The next day, sections were washed in 0.1 M PBS at room temperature three times for twenty minutes each. At the completion of this washing, sections were mounted onto slides and cover slipped with Vectashield with DAPI (Vector, Burlingame, CA). Images were obtained using a digital camera (Axiocam, Zeiss, Jena, Germany) attached to a confocal microscope. Images were taken on the contralateral side to the lesion approximately 225 microns from midline and 225 microns from the dorsal edge of the tissue for each section. The contralateral side was analyzed since the corpus collosum is near the injury site and transfers cellular injury information to the contralateral side of the injury (Hellige, 1993). All the sections were mounted onto slides with the same orientation; therefore the measures could be taken the same way in all sections even if an injury was not present. A 225 x 225 micron box was placed electronically at the midline portion of the section approximately 225 microns from the dorsal edge of the section and moved laterally 225 microns from midline. This area was photomicrographed and analyzed through Metamorph Software (MDS Analytical Technologies, Sunnyvale, CA) for the number of astrocytes in the contralateral side of the lesion in a 225 x 225 micron size area. Metamorph uses a number of filters to remove any stained objects that are outside the preset parameters and to count the number of objects that meet the predetermined

conditions in the photomicrograph. The predetermined conditions were the minimum of 80 and maximum of 250 pixels that group together with the GFAP stain in the selected area contralateral to the injury. The GFAP positive cells fluoresced red under a green filter.

CHAPTER III

RESULTS

3.1 Statistical Analysis

The primary data analysis consisted of several two-way mixed model ANOVA's. The between-groups factor was group (Sham, NH CCI, SI CCI and RSD CCI) and the within groups factor was day of testing (Baseline, Day 4, Day 7 and Day 14). The data from CCI animals showing no visible lesion were removed, from any animal not completing behavioral tasks during baseline and from outliers according to the Grubb's test for outliers ($p > 0.05$). For all statistical analyses, $p < 0.05$ was considered significant for two-tailed tests.

3.2 Cresyl Violet Lesion Area Data Analysis

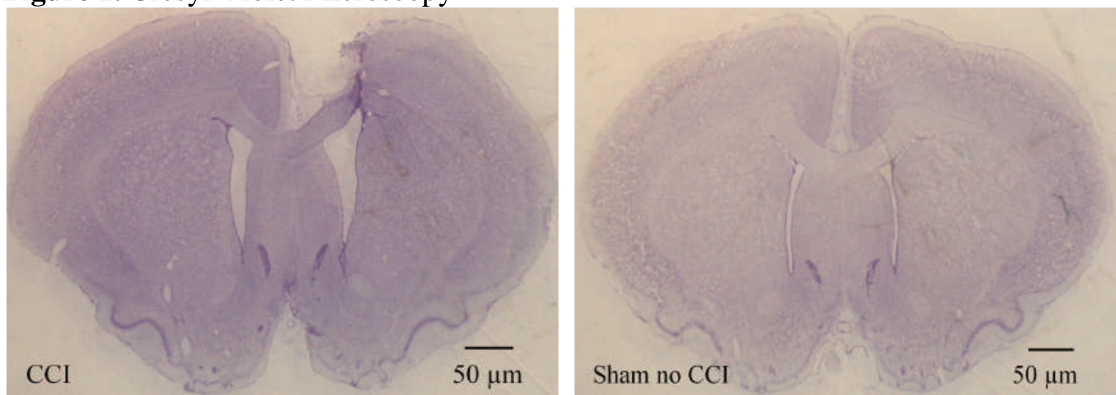
It was expected that all groups that received an injury would exhibit approximately the same lesion area since the same size impact (3 mm in diameter) was utilized for each surgery. The Sham group, which did not receive a CCI injury, showed minimal deficits from the craniotomy. The Cresyl Violet data were analyzed by evaluating the differences between the contralateral to the ipsilateral area [100 - [(Ipsilateral measurement / Contralateral measurement) * 100]] to arrive at the lesion percent for the section. The mean lesion percents for each group as well as the standard errors are presented in Table I.

Table I. Cresyl Violet Lesion Percent Means and Standard Errors

Group (n=27)	Mean	SE
RSD (n=7)	13.86	3.15
SI (n=6)	11.71	3.40
NH CCI (n=6)	8.93	4.40
SHAM (n=8)	-0.16	1.73

The Sham group had an average lesion size (M = -0.16, SE = 1.73) whereas the CCI groups combined (RSD, SI and NHCCI) exhibited significantly larger lesions (M = 11.62, SE = 2.04), t (22.808) = 4.396, p = 0.001. A sample of Cresyl Violet stained tissue shows a section from the injured and sham groups in Figure 1.

Figure 1. Cresyl Violet Microscopy



3.3 GFAP and Fluoro Jade-B Cell Count Data Analysis

One quarter of all collected tissue sections were labeled with an anti-glial fibrillary acidic protein (GFAP) antibody which labels astrocytes and Bergmann glial cells. These cells were counted on the contralateral side to the injury. The Fluoro Jade-B and GFAP labeled cell count data were evaluated by calculating the average number of stained or labeled cells in each group (Sham, NH CCI, SI CCI and RSD CCI) and compared them to the other groups. Data were log transformed due to non-normality. As expected all groups that received a CCI injury exhibited inflammation. The inflammation was measured by counting the GFAP label above a threshold of 80 pixels grouped together via Metamorph Software (MDS Analytical Technologies). A one-way ANOVA

revealed that GFAP cell counts were significantly different between groups F (3, 21) = 4.322, p< 0.05. GFAP original cell count data means and standard errors are presented in Table II.

Table II. GFAP Cell Count Means (units of 80 pixels) and Standard Errors

Group (N=25)	Mean	SE
RSD (n=6)	1732.34	347.65
SI (n=5)	1564.54	748.97
NHCCI (n=6)	677.22	227.57
SHAM (n=8)	535.72	202.27

It was expected that the RSD group would exhibit the highest cell counts due to the combined sleep deprivation and brain injury. The RSD group cell counts were significantly higher than Sham (p = 0.036) but not significantly different from the remaining groups (SI and NHCCI). There were no other significant differences found between the groups. Figure 2 shows the area of the brain contralateral to the injury site where the GFAP microscopy image was taken and analyzed. Figure 3 is a representative example of the GFAP cell count stain for each group.

Figure 2. Contralateral brain section where GFAP microscopy images were taken.

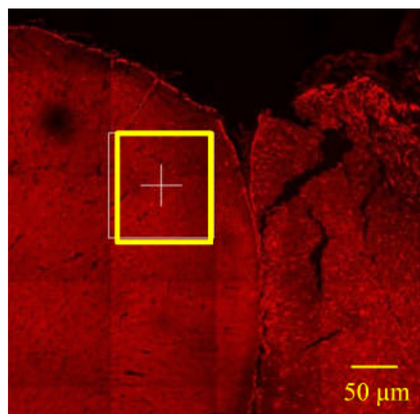
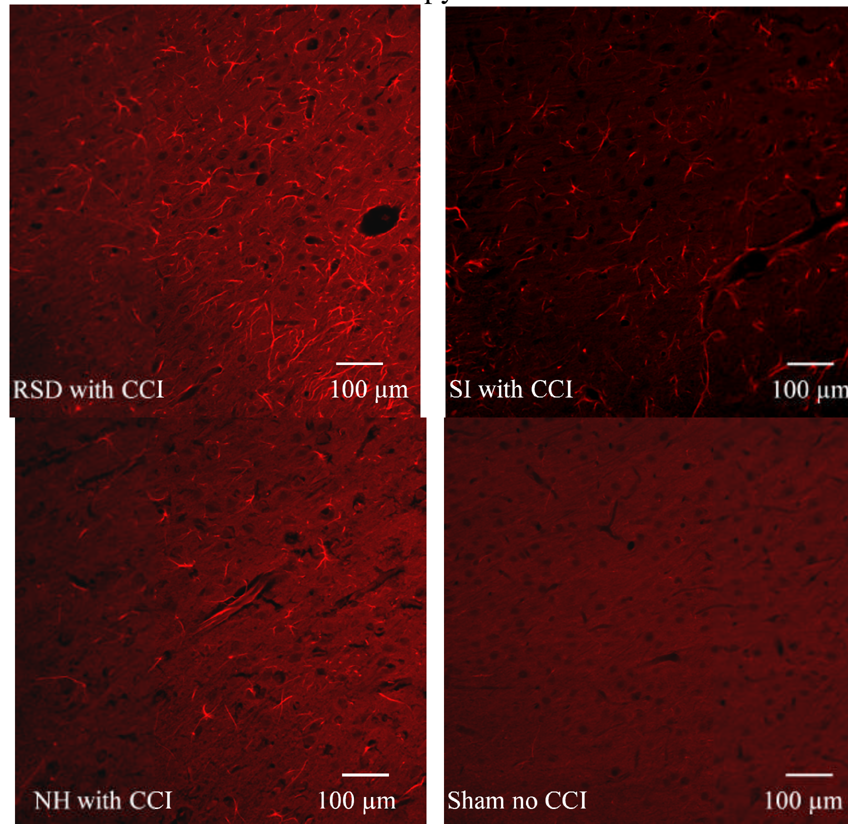


Figure 3. GFAP Cell Count Stain Microscopy



For Fluoro Jade-B, it was expected that all groups that received an injury would exhibit neuronal death near the injury site as well as on the contralateral side since the injury was near the corpus callosum. The corpus callosum is known to transfer cellular injury information to both sides of the brain (Hellige, 1993). The contralateral side was analyzed because the injury was so large that it was easier to examine the uninjured side for information as well as to see how much cellular injury had been transferred to this side as opposed to the obvious cellular injury on the injured side of the brain. The data were log transformed and the analysis was not statistically significant, $F(3, 24) = 1.787$, $p > 0.05$. All means and standard errors for original FJB cell count data are presented in Table III.

Table III. FJB Cell Count Means (units of 15 pixels) and Standard Errors (Original Values)

Group (N=25)	Mean	SE
RSD (n=6)	244.83	214.54
SI (n=5)	90.24	78.90
NHCCI (n=6)	193.69	135.70
SHAM (n=8)	7.57	4.67

3.4 Balance Beam Data Analysis

Time to traverse the balance beam and number of beam sections successfully completed were recorded. However, many animals fell off the beam before reaching the end of the beam therefore speed per section was used for analysis. Speed per section was calculated by dividing the number of sections completed by the time it took to complete the task for each trial and then averaged over the five trials for each animal on baseline, day four, seven and fourteen. The percent deviation [(1-day 4, 7 or 14 / baseline) * 100] from baseline was used to determine the rate of recovery (see Table IV).

Table IV. Percent deviation from baseline balance beam speed means and standard errors

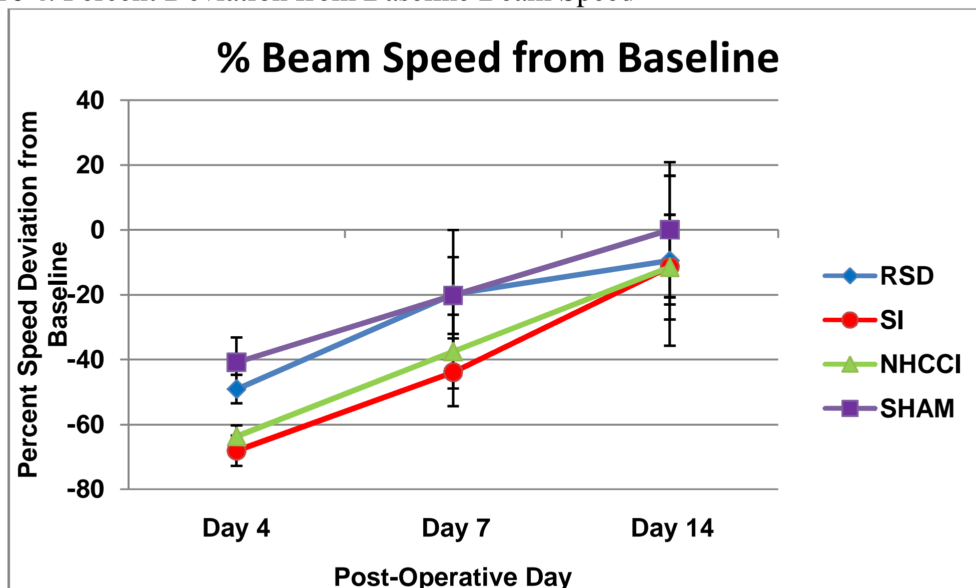
	% Dev Post-Op Day 4		% Dev Post-Op Day 7		% Dev Post-Op Day 14	
Group (N=27)	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
RSD (n=7)	-49.08	4.37	-19.83	19.80	-9.48	26.19
SI (n=6)	-68.13	4.68	-43.89	10.43	-11.38	11.62
NH CCI (n=6)	-63.71	3.42	-37.51	11.36	-11.45	16.13
Sham (n=8)	-40.88	7.74	-20.25	11.83	0.05	20.81

Mean balance beam speeds differed significantly between time points ($F(2, 46) = 19.142$, $p < 0.001$). Tukey post hoc tests revealed that balance beam speeds from baseline were significantly slower on all post-operative days (four, seven and fourteen; ($p < 0.001$). Post-operative day four performance was significantly slower from post-operative days seven and fourteen ($p < 0.001$) with post-operative day seven also significantly slower from post-operative day fourteen ($p < 0.05$). This main effect

indicates an improvement in performance from post-operative days four to fourteen. A one-way ANOVA revealed that post-operative day four was the only day to demonstrate significant differences between groups ($F(3, 26) = 4.905$, $p < 0.05$). More specifically, Sham was found to be significantly faster than SI and NH CCI ($p < 0.05$) but not significantly faster than RSD ($p = 0.719$) for post-operative day four only.

Post-operatively, the animals demonstrated slower speeds when compared to baseline during the task. The animals' speeds increased across trials with a near return to baseline by post-operative day fourteen. It was expected that the Sham group would perform significantly better than the rest of the conditions, but this group only performed significantly better than the SI and NHCCI conditions. It was also expected that the RSD group would have significantly reduced performance when compared to the remaining conditions (SI, NHCCI and Sham). However, it was only slightly and non-significantly different. Figure 4 shows the behavioral pattern of the four groups throughout the balance beam speeds task with the percent deviation from baseline.

Figure 4. Percent Deviation from Baseline Beam Speed



3.5 Bilateral Tactile Adhesive Removal Somatosensory Task Data Analysis

Latency to remove adhesive stickers for right and left forelimbs and a combined total was recorded (all four trials summed). The percent deviation from baseline measure was analyzed across post-operative days four, seven and fourteen. The lesion was only located in the left hemisphere (this means that sensory function in the right forelimb will be affected so the rat will have decrease sensory and motor function in the right forelimb which may manifest in decreased removal time from right forelimb), therefore it was expected that the animals should have experienced more difficulty in removing the adhesive sticker post-operatively from the right forelimb due to sensory impairments and from the right forelimb due to motor impairments. All animals were expected to show improved performance rates over the course of the experiment for both forelimbs as they learned to compensate for their impairments (Neumann, et al., 2009; Soblosky, et al., 1997). Aggregate total removal time for left and right percent deviation [(1-day 4, 7 or 14 / baseline) * 100] from baseline means and standard errors are presented in Table V.

Table V. BTARS Total Latency to Remove Percent Deviation from Baseline Means and Standard Errors

	% Dev Post-Op Day 4		**% Dev Post-Op Day 7**		**% Dev Post-Op Day 14**	
Group (N=22)	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
RSD (n=5)	-115.5	89.7	-133.4	75.7	-57.2	72.3
SI (n=6)	-23.0	20.7	11.2	25.8	18.0	22.9
NHCCI (n=4)	31.0	27.1	42.9	29.1	65.3	6.8
Sham (n=7)	33.6	15.1	39.0	21.5	36.1	15.4

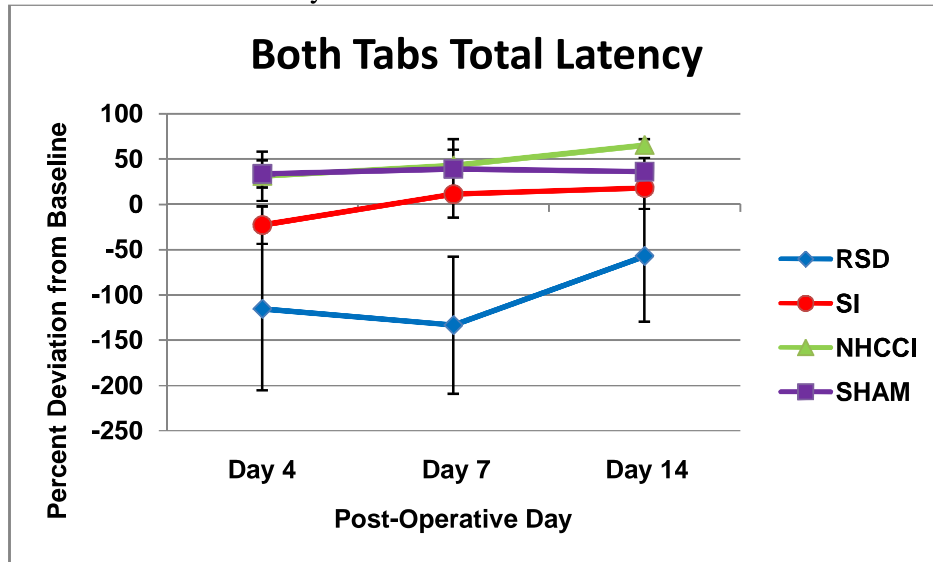
The percent deviation from baseline total latency to remove the tabs differed significantly between time points ($F(2, 36) = 5.107$, $p < 0.05$). Specifically, post-operative day four was significantly different from post-operative day fourteen ($p <$

0.001) while post-operative day seven when compared to post-operative day fourteen approached significance ($p = 0.052$). No other differences between time points were significant.

The main effect of group was also statistically significant $F(3, 18) = 3.902$, $p < 0.05$, indicating that groups differed total latency on removing the tactile stimuli. Tukey post hoc tests revealed that RSD was significantly slower than NHCCI and Sham ($p < 0.05$) with the group SI comparison approaching significance ($p = 0.07$). This suggests that the RSD group was most impacted by the sleep deprivation while the remaining groups showed more recovery progress following surgery.

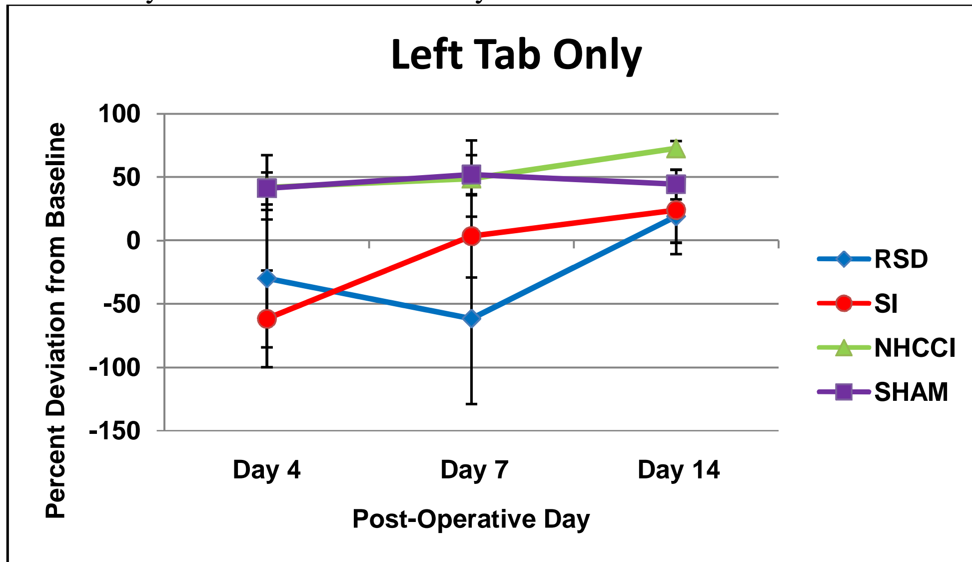
Post-operatively, for total latency to remove, from day four through day seven, all groups except RSD showed improvement in removal latency times (a positive behavioral result) while the RSD group increased their overall latency times which was a negative behavioral result. However, from day seven through day fourteen, the RSD group as well SI and NHCCI decreased their latency times while Sham showed a slightly worse performance pattern. It was expected that the Sham group would perform significantly better than the rest of the conditions. However, they only performed slightly and non-significantly better for total latency to remove. It was also expected that the RSD group would demonstrate lower performance when compared to the SI, NHCCI and Sham groups and this too was found. Figure 5 shows the behavioral pattern of the four groups throughout the BTARS total latency to remove task with a percent deviation from baseline as the performance standard.

Figure 5. BTARS Total Latency to Remove Percent Deviation from Baseline



Latencies for left and right tab removal were analyzed separately using a repeated measures ANOVA with percent deviation from baseline. Post-operative days four, seven, and fourteen for left tab latency to remove was significant ($F (2, 36) = 3.362$, $p < 0.05$). Pairwise comparisons revealed that post-operative day four performance was significantly slower than post-operative day fourteen ($p = 0.008$) while post-operative day seven approached significance compared to post-operative day fourteen ($p = 0.06$) for left tab removal only. The RSD group also showed a trend toward significance ($p = 0.059$) when compared to the Sham group. No other groups exhibited significant differences for the left tab removal latency. Figure 6 shows the latency to remove the left tab only percent deviation from baseline.

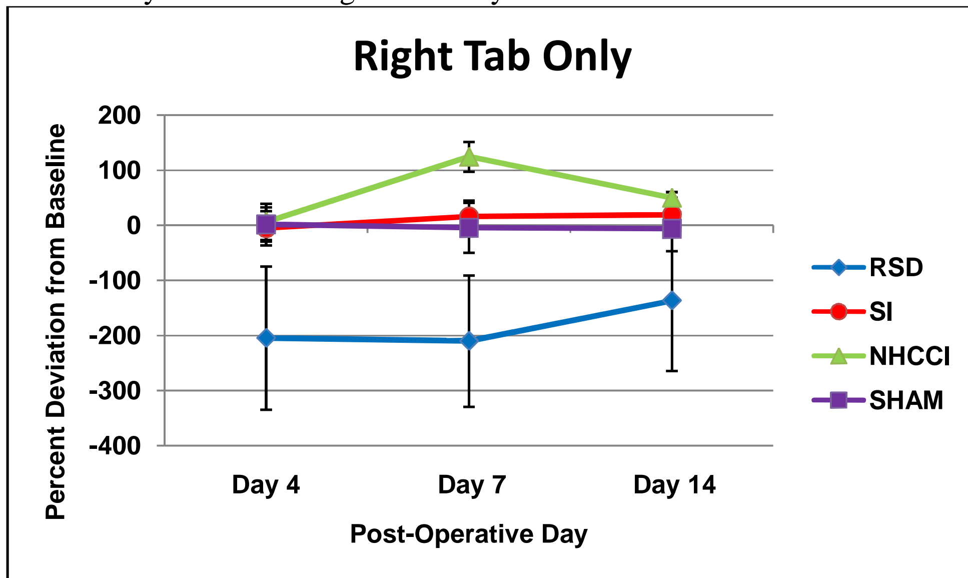
Figure 6. Latency to Remove Left Tab Only Percent Deviation from Baseline



Right tab only latency removal percent deviation from baseline across time points (post-operative day four, seven and fourteen) was also found to be significant ($F (2, 36) = 3.886$, $p < 0.05$). Post-operative day four performance was found to be significantly slower than post-operative days seven and fourteen ($p = 0.048$ and $p = 0.007$, respectively); however, post-operative day seven was not significantly different than post-operative day fourteen for right tab removal only. Pairwise comparisons revealed that RSD performance was significantly slower than SI and NHCCI but only showed a trend toward significance with respect to Sham ($p = 0.052$). For right tab only latency to remove, the RSD group seemed to have slower recovery of function possibly as a result of the significant effect of the sleep deprivation since the Sham group did not experience the sleep deprivation but did undergo a craniotomy procedure under anesthesia for surgery.

No other groups were found to be significantly different for right tab latency to remove only. The latency to remove right tab only percent deviation from baseline behavioral pattern is shown in Figure 7.

Figure 7. Latency to Remove Right Tab Only Percent Deviation from Baseline



Post-operatively, animals in RSD and SI demonstrated slower latency times when compared to baseline on day four for right tab only removal. By post-operative day seven, the SI and NHCCI groups were faster with right tab only removal latencies compared to baseline than both RSD and Sham. By post-operative day fourteen, groups SI, NHCCI and Sham were nearly back to pre-operative right tab only removal latencies while the RSD group was continuing to show improved progress. It was expected that the Sham group would perform significantly better than the other groups; however, the Sham group performed only slightly and non-significantly, better than the remaining conditions. The tab latency to remove means and standard errors, left vs. right, percent deviation from baseline are presented in Table VI.

Table VI. BTARS Latency to Remove Means and Standard Errors Left vs. Right

	% Dev Post-Op Day 4		**% Dev Post-Op Day 7**		**% Dev Post-Op Day 14**	
Group (N=22)	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
RSD Left (n=5)	-29.9	54.2	-61.6	67.2	19.1	29.7
RSD Right (n=5)	-204.5	129.9	-209.9	119.2	-136.5	127.7
SI Left (n=6)	-61.7	38.1	3.4	32.4	23.9	25.6
SI Right (n=6)	-5.2	30.9	16.0	29.2	19.3	31.2
NHCCI Left (n=4)	42.0	25.3	48.9	30.0	72.6	5.9
NHCCI Right (n=4)	6.5	32.8	124.4	27.1	50.0	10.5
Sham Left (n=7)	41.1	12.6	52.0	15.4	44.2	11.7
Sham Right (n=7)	1.7	31.0	-4.4	45.2	-6.3	40.4

CHAPTER IV

DISCUSSION

4.1 Current Research

It was expected that the Cresyl Violet stained tissue would demonstrate that the CCI groups would exhibit a similar lesion size and the Sham group to exhibit minimal effects from the craniotomy. Although there was a slight variability in each of the injured groups, the lesion size for the injured groups was significantly larger than the Sham group.

It was expected that there would be neuron loss at the point of injury and that the sleep deprived group would exhibit the highest count of degenerating neurons as indicated with the Fluoro Jade-B stain. It was unexpected that the normal cellular debris removing process would remove these degenerating cells by the time the behavioral work (fourteen days post-operative) was completed and the histological process started. Stained degenerating neurons were mostly localized in the corpus callosum tracts in both hemispheres (injured and uninjured) since inflammation and injury information is transferred through this structure. The lesion size was so large that it was easier to examine the uninjured (contralateral side) for cellular information.

Moldovan and colleagues noted that at seven days post injury, degenerating neurons were nearly absent so they switched staining type to CC3 to try to detect any remaining cells (Moldovan et al., 2010). Fluoro Jade-B staining for degenerating neurons was informative to work at 6 and 24 hours post operatively, but not at eight and thirty days post injury where cell labeling indicated either necrotic cells or where labeling was completely absent (Hoane et al., 2006; Sutton, et al., 1993). At fifteen days post-operative, Fluoro Jade-B seemed to only stain the remaining necrotic cells in the fiber tracts of the corpus callosum and a small surrounding area of the injury site.

With respect to the GFAP cell count analysis, it was expected that the sleep deprived group would exhibit the highest number of labeled cells, with the SI and NHCCI to have significantly lower cell count levels. However, the latter groups had only slightly lower non-significant cell counts. To account for the sleep deprivation factor only, a sleep deprivation *only* group should have been utilized to control for the CCI combined with sleep deprivation. The Sham group did have significantly lower cell counts than the RSD group, but not from the SI and NHCCI groups.

The present study demonstrated that 24 hours of sleep deprivation prior to TBI had an effect on sensorimotor function and recovery and specific underlying cellular mechanisms of trauma. This was evident with both of the behavioral tasks. All the animals, regardless of group (injury and treatment) improved in the beam speeds over post-injury time points. The Sham group did slightly better than the other groups, apparently because this group did not experience the brain injury, but did undergo the craniotomy procedure. All groups did worse immediately after surgery and showed continuous improvement through day fourteen with RSD performing slightly better than

the other conditions only on day 4 and 7 and only compared to other CCI groups. This pattern was unexpected since previous research has demonstrated that that sleep deprivation, even for 24 hours, posed a negative effect on the motor recovery. This was clearly not the case in this study. It was also unclear whether the sensorimotor improvement in each of the behavioral tasks was due to the recovery process, practice effects from the task or simply learning to compensate for any sensorimotor impairment that was experienced.

In all behavioral tasks, it was expected that the RSD group would perform significantly worse than the other groups due to the combined effects of brain injury and sleep deprivation. However, for total latency to remove, this group was significantly slower from NHCCI and Sham while demonstrating a trend toward significance in comparison to the SI group ($p = 0.07$). It was also expected that the Sham group would have significantly better performance compared to RSD, SI and NHCCI on total tab removal task but they performed only slightly and non-significantly better. Perhaps the effects of the craniotomy and surgical procedure were more traumatic than previously planned for this group. Each group (SI, NHCCI and Sham) improved their total latency removal times while the RSD seemed to have much slower latency times prior to improving their performance.

Since only the left hemisphere was injured in this study, the BTARS task was expected to demonstrate significant differences between left and right adhesive removal. The left tab removal task should have evidenced little to no difference across groups since this is correlated with the uninjured hemisphere. However, the SI, NHCCI, and Sham Groups displayed latency to remove improvements from post-operative day four to

post-operative day seven with the RSD group actually demonstrating a longer latency (slower) to remove the left tab, only in this time period. Each group demonstrated a different behavioral pattern from the other with RSD, SI and NHCCI demonstrating improvements from post-operative day seven to post-operative day fourteen. The Sham Group did not show improvement across these time points.

As for the right tab removal, which was contralateral to the injured left hemisphere, the animals' performance post injury did not differ across the time points. The sleep deprived group did have a significantly different performance pattern than the other three groups. It is clear from Figure 5 that the latency to remove the adhesive stimuli was increasingly slower for the sleep deprived group vs. the other three conditions. The sleep deprived group had difficulty sensing the sticker or lacked the motor control to remove the sticker. In the future, noting the latency to first contact may help to clarify this issue.

4.2 Limitations

An acknowledged limitation to the current study was the length of time for the sleep deprivation. The length of sleep deprivation in the literature ranges from six hours to seven days and the brevity of deprivation in this study may be responsible for the mixed results obtained. Instead, once 24 hour deprivation baseline has been collected, a longer sleep deprivation period could be investigated for more illumination of both acute and chronic effects. A 72 hour sleep deprivation time frame to solidify the effects of sleep deprivation prior to inducing any type of brain injury should be considered for future studies. Corticosterone levels should have been taken from each group to determine and quantify the level of stress that each animal was enduring while in each of the respective conditions. This would determine if the sleep deprived and socially

isolated animals experienced more stress in the novel environment of the inverted flower pot housing than the other animals experienced in the normal housing environment.

A limitation to the surgery was the craniotomy drilling which was completed by hand with a micro drill. There was a learning curve involved for the technique and the investigator's skill substantially improved by the close of the study. This learning curve likely created a direct effect on the Cresyl Violet lesion analysis evidenced with the high variability of the lesions in all the animals. The CCI contusion device is equipped with autoclavable metal tips that cause tissue deformation at the point of contact. The tip used in this study measured 3 mm in diameter and it is possible that this tip may have caused an injury that was too large. This may have masked other cellular processes occurring in the injury location. Possibly using a smaller tip would control for some of the masked cellular processes in the injury site. It is also possible that the sleep deprivation effects would be more prominent when using a smaller tip since the larger tip size may have caused variability in the underlying sleep deprivation effect.

Another limitation is in the balance beam task. The beam was suspended 90 centimeters over soft padding. When an animal's foot slipped, it lost its balance and fell onto the soft padding. This was quite aversive and may have hindered the rate at which the animal crossed the beam, slowing their overall latency for this task. A ledged beam which would catch the animals' foot slip may offer the stability that the animal needs to increase its speed when completing the balance beam task. The ledged beam has been shown to reduce compensation for the injury (Schallert & Woodlee, 2005).

An additional limitation noted in this study was with regard to the BTARS task. The animals were trained several days prior to taking baseline readings. The animals may

have had the opportunity to over-learn the task through repeated practice. It is unclear if they mastered the task; that is if their performance was due to progress post surgery or through practice effects from pre-operative training. In the future, pre-operative exposure (training) for removal of the adhesive tab would be limited to obtaining baseline data and post-operative performance to attempt to eliminate practice effects on this task. Another limitation with regard to this task was that it was unclear whether the task primarily tested for a somatosensory or motor deficit since it was not recorded when the animal first contacted the adhesive stimuli. Future studies should include this information as well as latency to remove.

Further investigation into the types of stains and techniques used for labeling degenerating neurons with a combination of the time frames allowed for behavioral work should be completed prior to implementation. However, there were few studies in the literature with regard to this particular stain/technique at the fifteen day or more time point for histological analysis. Therefore, it was not known that the degenerative processes would work so quickly and obscure labeling of degenerating neurons post behavior analysis. This stain should only be utilized at more acute time points.

4.3 Future Research

The combination of sleep deprivation and TBI is not restricted to military scenarios. In our fast paced world, much of society seems to be hard pressed to pack as much as they can into every waking moment while sleep is sacrificed. This lifestyle depletes motor and cognitive skills that everyone needs to function on a daily basis. However, little is known about the underlying cellular mechanisms that take place if an injury occurs during a sleep deprived state. Future research into these underlying mechanisms is needed to pinpoint what is happening, why it is happening, and what

neuroprotective factor(s) can be included to obtain the best recovery or therapy plan. This would apply to falls, and car accidents as well as military casualties.

Future research should also focus on the TBI diagnostic procedure itself. Many TBI afflicted patients are also dealing with Post Traumatic Stress Disorder, anxiety and depression. It is very difficult to treat this combination of factors or know which may be the primary contributor to the condition. If there were more accurate diagnostic procedures for TBI, physicians, nurses and the healthcare community itself may have more success treating a multi-faceted injury instead of focusing on one aspect of their condition at a time. This would create an integrated treatment and recovery plan with potentially better outcomes. However, to do this, the basic mechanics and underlying cellular mechanisms would need to be more clearly understood.

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