UNIVERSITY OF CALIFORNIA,
IRVINE

Chronic Behavioral Changes and Associated Neuropathological Features In a Rodent Model of Repeated Mild Traumatic Brain Injury

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Eric Mitchell Gold

Dissertation Committee:
Professor Brian J Cummings, Chair
Professor Aileen J Anderson
Professor Richard Robertson

2017

DEDICATION

To all of my mentors and influencers over the years who have guided me along my path

To my high school anatomy teacher, Mrs. Dorothy Harris who told me to apply for a fellowship at a place called the National Institutes of Health

To my first PI, Dr Jaqueline Crawley, who so kindly introduced me to scientific research, and to my first post-doc, Dr Andrew Holmes, who taught me the nuances of animal behavior

To Dr Joseph Frank, who introduced me to an unknown field of MRI research, leading me to pursue studies of traumatic brain injury, stem cells, and ultimately a PhD

To Dr Baris Bingol and Cécile Chalouni who helped me peak behind the curtain of industry and introduce me to a whole new world

To Brian Cummings and Aileen Anderson, who taught me how to think critically, to defend yourself, to manage an audience, and to act like a leader

To my parental units, who have always let me find my own path and take my own route

If you're walking down the right path and you're willing to keep walking, eventually you'll make progress

-POTUS #44

TABLE OF CONTENTS

		Page
LIST OF FIGURES		iv
LIST OF TABLES		vi
ACKNOWLEDGMENTS		vii
CURRICULUM VITAE		ix
ABSTRACT OF THE DISSERTATION		xiv
CHAPTER 1	Introduction	1
CHAPTER 2	Functional Assessment of Long-Term Deficits in Rodent Models of Traumatic Brain Injury	13
CHAPTER 3	Repeated Mild Closed Head Injuries Induce Long-Term White Matter Pathology And Neuronal Loss That Are Correlated With Behavioral Deficits	90
CHAPTER 4	In depth rmCHI Model Characterization and Therapeutic Interventions	140
CHAPTER 5	Summary	173

LIST OF FIGURES

		Page
Figure 2.1	There are 5 main models of TBI in rodents	64
Figure 2.2	Nissl-stained rat brain sections of traumatic brain injuries induced by controlled cortical impact (CCI)	65
Figure 2.3	Comparison of the percentage of all rodent TBI papers by model which assessed functional outcomes short-term or long term post TBI.	66
Figure 3.1	Novel model of repeated mild closed head injury used in long-term behavioral testing of mice	128
Figure 3.2	rmCHI results in minimal motor behavior deficits 1DPI, returning to control levels by 4DPI and sustaining motor function chronically	130
Figure 3.3	rmCHI alters cognitive function at 1 and 2 months post-injury	131
Figure 3.4	Mice suffering rmCHI have significant white matter damage and over 30% atrophy of corpus callosum at 2 months post injury	132
Figure 3.5	rmCHI leads to neuronal loss in the cortex, negatively correlating with behavioral testing	134
Figure 3.6	Sustained deficits in behavioral tests persist up to 6 months post injury, as well as chronic white matter atrophy within the corpus callosum	136
Figure 3.7	rmCHI induces chronic astrogliosis at 2 and 6 months post injury	138
Figure 3.8	rmCHI induces chronic microglial inflammation at 2 and 6 months post injury in white matter tracts	139
Figure 4.1	Decreasing the speed parameter of rmCHI injuries does not induce behavioral changes, while decreasing the number of hits at full velocity still induces effects.	163
Figure 4.2	Electron microscopy analysis reveals decrease in G-ratio following rmCHI	165

Figure 4.3	rmCHI in Nod-*scid* Gamma mice does not induce changes in behavioral performance at both 1MPI or 6MPI, nor is there a decrease in corpus callosum volume	166
Figure 4.4	rmCHI in Nod-*scid* Gamma mice does not induce changes in Novel Object Recognition or Novel Place Recognition task at 2MPI	168
Figure 4.5	AVL-3288 does not improve behavior performance at 1mpi or 2mpi	169
Figure 4.6	Vivreon 2.0 does not improve behavior performance at 1mpi or 2mpi	171

LIST OF TABLES

		Page
Table 2.1	List of functional outcome tasks used in the TBI field.	67
Table 2.2	Models and functional outcome tasks with long-term deficits (2-, 3-, 6- and 12-months post injury).	68
Table 2.3	Human cellular therapy studies in rodent models of TBI.	69
Table 3.1	Summary of deficits observed on behavioral tasks assessed following repeated mild closed head injury	127

ACKNOWLEDGMENTS

I would like to thank Brian and Aileen for all their support and wisdom. The instant I came to Irvine, I knew this is the laboratory I wanted to join. An unparalleled tandem modeling how a lab is more a family than anything else. Your guidance over the past several years has shaped me to be the stubborn, meticulous, and questioning scientist that I am today. Your leadership within the laboratory, the building, the campus and the community are inspirational and have taught me a great deal about wearing the right hat at the appropriate time.

Brian, a special thank you to you for being the best mentor I could ask for. You've been extremely supportive in both my project progression and my professional development. Letting me take an internship in San Francisco was a pivotal moment in my life, opening up doors I didn't know previously existed. I know it was unconventional and I greatly appreciate your support.

To everyone in the Andermings Lab over the years, thank you! It would be nearly impossible to name everyone who's been impactful to my experience, but let's give it a shot: Andrea, Brittany, Casey, Cherie, Chris Ott, Chris Nishi, Cislo, Dan, Daniel Ho, Danny Hoa, Danquynh, Denisse, Diane, Emily, Francisca, FuFu, Gaby, Hal, Harvey, Hiro, Hongli, Joel, Jovanny, Kasuni, Katja, Kelly, Krystal, Mitra, Noriko, Oscarito, Phoebe, Rebecca, Ron, Sarah, Sean, Sheri, Sujin, Usha….to name a few. It's been a blast and I couldn't have survived without you all.

A special thank you to Chris and Rebecca for being the glue holding the it all together. Without you two, everything would fall apart, nobody would know how to do anything, and we'd be written up by IACUC, ULAR, EH&S, and every other regulatory body. You guys are amazing.

To my friends, old and new, near and far, thank you for not fully understanding what I'm doing, not questioning it, but embracing the adventure and being supportive over the past 6 years. You've kept my sanity intact, so keep up the good work.

To the Patterson CdM clan, thank you being so welcoming into the family and being so involved in Kate and my lives. It's been amazing to have family so close when mine is so far away, and I appreciate your open arms and support.

To my Parents and Sister, thank you for making me the person who I am today. Thank you for unwavering support of my dreams, my goals, and my decisions to move far away. You mean everything to me and I love you.

To Darwin and Penny, my true inspirations for waking up every day, thank you. Maybe not inspirations, but rather a persistant 5:58AM alarm clock needing to be fed and taken out. You're always ecstatic to see me and the feeling is mutual. Best dogs in the world.

To Kate, what can I say. You beat me to the finish line, I concede. Thank you for taking a New Balance wearing East Coaster and teaching me the finer ways of SoCal. It's been an amazing journey with you and I look forward to everything upcoming. You mean the world to me, I love you.

Financial support was provided by NIH Training Program in Stem Cell Translational Medicine for Neurological Disorders (T32 NS082174-01), NIH Training Program in the Neurobiology of Aging (T32 AG00096-30), & Dr Lorna Carlin.

CURRICULUM VITAE

EDUCATION

Ph.D. Candidate, Biomedical Sciences, University of California, Irvine *July 2011 – June 2017*
B.A. Psychology, Emory University *August 2003 – May 2007*

RESEARCH EXPERIENCE

PhD Candidate *July 2011 – Present*
University of California, Irvine, Department of Anatomy and Neurobiology
Mentor: Brian J. Cummings, Ph.D.

Intern *June 2016 – Dec 2016*
Genentech, Neuroscience Department & Department of Pathology
PI: Baris Bingol, Ph.D. & Cecile Chalouni, Ph.D.

Research Assistant *June 2009 – June 2011*
Center for Neuroscience and Regenerative Medicine, Uniformed Services University of the Health Sciences/NIH
PI: William D. Watson, M.D., Ph.D. & Joseph A. Frank, M.D.

Intramural Research Training Award (IRTA) Fellow *August 2007 – June 2009*
Laboratory of Diagnostic Radiology Research, Clinical Center, NIH
PI: Joseph A. Frank, M.D.

RESEARCH AWARDS

- NRSA Predoctoral Researcher Fellow, "Training Program in Stem Cell Translational Medicine for Neurological Disorders", T32 NS082174-01, 2014-2016
- NRSA Predoctoral Researcher Fellow, "Training in the Neurobiology of Aging", T32 AG00096-30, 2013-2014
- American Society for Neural Therapy and Repair, Travel Fellowship Award, 2015
- NeuroRenew, Inc. and MBF Bioscience, Inc. Practical Workshop in Confocal Microscopy and Stereology Scholarship, 2013
- HHMI-UCI Graduate Teaching Fellow, 2012
- NIH Post-Baccalaureate Intramural Research Training Award (IRTA), 2007-2009

ACTIVITIES

UCI Advanced Degree Business and Consulting Club, Co-President	2016-2017
UCI GPS-BIOMED Peer Career Mentor, Business-Related Careers	2016-2017
UCI School of Medicine Graduate Student Advisory Committee Member	2014-2017
UCI School of Medicine Advisory Committee, Career Options Subcommittee	2014-2017
UCI Anatomy and Neurobiology Department Graduate Student Representative	2013-2016
UCI Technology & Entrepreneurship Competition	2016
UCI Anatomy and Neurobiology Department Journal Club Chair	2013-2014
UCI Graduate InterConnect Program Peer Mentor	2013
UCI Interdepartmental Neuroscience Program Recruitment Chair	2012-2013
UCI Interdepartmental Neuroscience Program Recruitment	2011-2013

PUBLICATIONS

1. **Gold EM**, Tiefenthaler C, Hoa DP, Rawanaka K, Cummings BJ. (2017) Repeated mild closed head injuries induce long-term white matter pathology and neuronal loss that are correlated with behavioral deficits. *Journal of Neurotrauma*, submitted for review.
2. Sahyouni, R, Gutierrez P, **Gold EM**, Robertson RT, Cummings BJ. (2017) Effects of concussion on the blood-brain barrier in humans and rodents. *Journal of Concussion*, in press.
3. Beretta S, Cunningham KM, Haus DL, **Gold EM**, Perez H, López-Velázquez L, Cummings BJ. (2016) Effects of human ES-derived neural stem cell transplantation and kindling in a rat model of traumatic brain injury. *Cell Transplant*, epub ahead of print.
4. Haus DL, López-Velázquez L, **Gold EM**, Cunningham KM, Perez H, Anderson AJ, Cummings BJ. (2016) Transplantation of human neural stem cells restores cognition in an immunodeficient rodent model of traumatic brain injury. *Exp Neurol*, 281:1-16.
5. Turtzo LC, Budde MD, Dean DD, **Gold EM**, Lewis BK, Janes L, Lescher J, Yarnell A, Grunberg NE, Frank JA. (2015) Failure of intravenous or intracardiac delivery of mesenchymal stromal cells to improve outcomes after focal traumatic brain injury in the female rat. *PLOS ONE*, 10(5): e0126551.
6. Haus DL, Nguyen HX, **Gold EM**, Kamei N, Perez H, Moore HD, Anderson AJ, Cummings BJ. (2014) CD133-enriched Xeno-Free human embryonic-derived neural stem cells expand rapidly in culture and do not form teratomas in immunodeficient mice. *Stem Cell Research*, 13(2):214-226.
7. **Gold EM**, Su D, López-Velázquez L, Haus DL, Perez H, Lacuesta GA, Anderson AJ, Cummings BJ. (2013) Functional assessment of long-term deficits in rodent models of traumatic brain injury. *Regenerative Medicine*, 8(4): 483-516.
8. Turtzo LC, Budde MD, **Gold EM**, Lewis BK, Janes L, Yarnell A, Grunberg NE, Watson W, Frank JA. (2012) The evolution of traumatic brain injury in a rat focal contusion model. *NMR in Biomedicine*, 26(4): 468-479.
9. Ziadloo A, Burks SR, **Gold EM**, Lewis BK, Chaudhry A, Merino MJ, Frenkel V, Frank JA. (2012) Enhanced homing permeability and retention of bone marrow stromal cells (BMSC) by non-invasive pulsed focused ultrasound. *Stem Cells*, 30(6): 1216-1227.
10. Budde MD, **Gold EM**, Jordan EK, Frank JA. (2012) Differential microstructure and physiology of brain and bone metastases in a rat breast cancer model by diffusion and dynamic contrast enhanced MRI. *Clinical & Experimental Metastasis*, 29(1): 51-62.
11. Budde MD, Janes LA, **Gold EM**, Turtzo LC, Frank JA. (2011) The contribution of gliosis to diffusion tensor anisotropy and tractography following traumatic brain injury: validation in the rat using Fourier analysis of stained tissue sections. *Brain*, 134(Pt 8): 2248-2260.
12. Budde MD, **Gold EM**, Jordan EK, Smith-Brown M, Frank, JA. (2011) Phase contrast MRI is an early marker of micrometastatic breast cancer development in the rat brain. *NMR in Biomedicine*, 25(5): 726-736.

13. Song HT, Jordan EK, Lewis BK, **Gold EM**, Liu W, Frank, JA. (2011) Quantitative T_2* imaging of metastatic human breast cancer to brain in the nude rat at 3 T. *NMR in Biomedicine*, 24(3): 325-334.
14. Cole JT, Yarnell A, Kean WS, **Gold EM**, Lewis BK, Ren M, McMullen DC, Jacobowitz DM, Pollard HB, O'Neill JT, Grunberg NE, Dalgard CL, Frank JA, Watson WD. (2011) Craniotomy: true sham for traumatic brain injury, or a sham of a sham? *Journal of Neurotrauma*, 28(3): 359-369.
15. Lee JH, Smith M, Liu W, **Gold EM**, Lewis B, Song HT, Frank JA. (2009) Enhanced stem cell tracking via electrostatically assembled fluorescent SPION-peptide complexes. *Nanotechnology*, 20(35): 355102.
16. Holmes A, Li Q, Koenig EA, **Gold EM**, Stephenson D, Yank RJ, Dreiling J, Sullivan T, Crawley JN. (2005) Phenotypic assessment of galanin overexpressing and galanin R1 receptor knockout mice in the tail suspension test for depression-related behavior. *Psychopharmacology*, 178(2-3): 276-285.
17. Holmes A, Li Q, Murphy DL, **Gold EM**, Crawley, JN. (2003) Abnormal anxiety-related behavior in serotonin transporter null mutant mice: the influence of genetic background. *Genes, Brain and Behavior*, 2(6): 365-380.

ORAL PRESENTATIONS

1. **Gold EM**, Tiefenthaler CM, Hoa D, Anderson AJ, Cummings BJ. Long-term white matter pathology and cognitive deficits following a novel model of repetitive mild traumatic brain injury. Annual meeting of the *American Society for Neural Therapy and Repair*. Clearwater, FL. April 2015.

POSTERS

1. **Gold EM**, Tiefenthaler C, Hoa DP, Cummings BJ. (2015) Long-term white matter pathology and cognitive deficits following a novel model of repetitive mild traumatic brain injury. Annual *Military Health System Research Symposium.* Ft. Lauderdale, FL. August 2015.
2. Haus DL, **Gold EM**, Perez H, Nguyen HX, Anderson AJ, Cummings BJ. Transplantation of xeno-free, sorted human embryonic-derived neural stem cells into the intact and traumatically injured rodent brain. Annual meeting of the *International Society for Stem Cell Research*. Boston, MA. June 2013.
3. Turtzo LC, Janes L, **Gold EM**, Budde MD, Coppola T, Dean DD, Lescher J, Frank JA. The macrophage response after focal traumatic brain injury in the rat. Annual *National Neurotrauma Symposium*. Phoenix, AZ. July 2012.
4. Turtzo LC, Budde MD, **Gold EM**, Lewis B, Janes L, Watson W, Frank JA. The evolution of traumatic brain injury in a rat model: Implications for MRI cell tracking. Annual *National Neurotrauma Symposium*. Hollywood Beach, FL. July 2011.
5. Budde M, Janes L, **Gold EM**, Turtzo L, Frank JA. Microscopic determinates of anisotropy in the injured rodent brain using histological Fourier analysis. Annual meeting of the *International Society for Magnetic Resonance in Medicine*. Toronto, Canada. May 2011.

6. Budde M, **Gold EM**, Jordan EK, Frank JA. Breast cancer metastases in the rat spinal cord induce focal, but not distal, neurodegeneration measured with diffusion tensor imaging. Annual meeting of the *International Society for Magnetic Resonance in Medicine*. Toronto, Canada. May 2011.
7. Turtzo L, Budde M, **Gold EM**, Lewis B, Janes L, Watson W, Frank JA. The evolution of traumatic brain injury in a rat model: implications for cell tracking with MRI. Annual meeting of the *International Society for Magnetic Resonance in Medicine*. Toronto, Canada. May 2011.
8. Ziadloo A, Burks SR, Chaudhry A, **Gold EM**, Dean DD, Lewis BK, Jordan K, Frenkel V, Frank JA. Targeting mesenchymal stem cells (MSC) using pulsed focused ultrasound: implications for stem cell therapy. Annual meeting of the *International Society for Magnetic Resonance in Medicine*. Toronto, Canada. May 2011.
9. Cole JT, Yarnell A, Kean WS, **Gold EM**, Lewis B, Ren M, McMullen D, Jacobowitz D, Pollard HB, O'Neill JT, Grunberg N, Dalgard CL, Frank JA, Watson WD. Craniotomy: True sham for traumatic brain injury or a sham of a sham. Annual meeting of the *Society for Neuroscience*. San Diego, CA. November 2010.
10. Hancock H, **Gold EM**, Lewis B, Dean D, Smith M, Burks S, Frenkel V, Frank JA. Assessment of focused ultrasound induced inflammation in muscle by MRI and fluorescent microscopy. Annual symposium on *MR-guided Focused Ultrasound*. Chantilly, VA. October 2010.
11. Ziadloo A, Chaudhry A, **Gold EM**, Dean D, Jordan EK, Burks S, Frank JA, Frenkel V. Targeting mesenchymal stem cells using focused ultrasound exposures. Annual symposium on *MR-guided Focused Ultrasound.* Chantilly, VA. October 2010
12. Budde MD, Resnick M, **Gold EM**, Jordan EK, Frank, JA. Microscopic morphology of brain and bone metastases in a rat breast cancer model of diffusion MRI. Annual meeting of the *International Society for Magnetic Resonance in Medicine*. Stockholm, Sweden. May 2010.
13. Budde MD, **Gold EM,** Jordan EK, Smith-Brown M, Frank JA. Hypoxia detected with phase contrast MRI is an early event in micrometastatic breast cancer development in the rat brain. Annual meeting of the *International Society for Magnetic Resonance in Medicine*. Stockholm, Sweden. May 2010.
14. Chaudhry A, Pawelczyk E, **Gold EM**, Lewis B, Brown M, Balakumaran A, Frank JA. Pro-survival cocktail improves bone marrow stromal cells (BMSC) survival and homing to flank tumors as demonstrated by cellular MRI. Annual meeting of the *International Society for Magnetic Resonance in Medicine.* Stockholm, Sweden. May 2010.
15. Hancock H, **Gold EM,** Lewis B, Smith M, Frenkel V, Frank JA. Cellular MRI assessment of magnetic fluorescent bead labeled macrophage accumulation following high intensity focused ultrasound (HIFU) induced damage in a murine model. Annual meeting of the *International Society for Magnetic Resonance in Medicine.* Stockholm, Sweden. May 2010.

16. Chaudhry A, Pawelczyk E, **Gold EM**, Balakumaran A, Frank JA. Pro-survival cocktail improves human bone marrow stromal cell and human amniotic fluid cell survival under stress conditions. Annual meeting of the *International Society for Stem Cell Research*. Barcelona, Spain. July 2009.
17. Chaudhry A, Pawelczyk E, **Gold EM**, Balakumaran A, Frank JA. Pro-survival cocktail improves stem cell survival in stress conditions. Annual meeting of the *International Society for Cellular Therapy*. San Diego, California. May 2009.
18. **Gold EM**, Jordan EK, Frank JA. Intravenous administration of luciferin increases sensitivity in rat model of metastases. NIH Spring Research Festival. Bethesda, Maryland. May 2009.
19. Budde MD, Jordan EK, Smith M, **Gold EM**, Frank JA. BOLD changes in the microenvironment are an early marker of micrometastatic breast cancer in the rat brain. Annual meeting of the *International Society for Magnetic Resonance in Medicine*. Honolulu, Hawaii. April 2009.
20. Holmes A, **Gold EM**, Li Q, Crawley JN. Galanin modulation of antidepressant-related responses in mice. Annual meeting of the *Society for Neuroscience*. New Orleans, LA. November 2003.

ABSTRACT OF THE DISSERTATION

Chronic Behavioral Changes and Associated Neuropathological Features
In a Rodent Model of Repeated Mild Traumatic Brain Injury

By

Eric Mitchell Gold

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2017

Professor Brian J Cummings, Chair

Every year, there are 3.8 million traumatic brain injuries in the United States leading to an estimated 5.3 million Americans that are currently living with a disability due to these injuries. Of these traumatic brain injuries (TBI), 75% are considered to be *mild* in severity. Importantly, while a single mild TBI has transient symptoms and associated neuropathological features, multiple mild TBI can result in a wide range of debilitating chronic symptoms and neuropathological features. Laboratory rodent models of traumatic brain injury have primarily focused on acute outcomes following TBI, and have also not focused heavily on repeated mild closed head injuries. This dissertation first reviews the literature outlining rodent models of traumatic brain injury, and the lack of assessments at chronic timepoints following injury. We found that 68% of papers did not evaluate a functional outcome past 1-month post TBI, and 90% of papers reviewed did not make a functional assessment 2 or more months following injury. Of these papers that investigated a 2 month timepoint, 84% demonstrated a functional deficit in a behavioral measure, stressing the persistence of chronic deficits and the important of studying them. Next, we

focus on the development of a rodent model of repeated mild traumatic brain injury. We utilized components of classic rodent models of TBI to develop a model and protocol for repeated mild closed head injuries (rmCHI) that can be easily reproduced across users and laboratories, and mimics the clinical presentation. The parameters of our model of rmCHI produce behavioral changes and neuropathological features for multiple impacts, but not single impacts, at chronic timepoints of 1, 2, and 6 months post injury. Behavioral changes in measures of anxiety, depression, and learning are also closely associated with neuropathological features including white matter atrophy and cortical neuronal loss. rmCHI results in 33-35% corpus callosum loss, developing by 2 months and lasting out to 6 months post injury. Lastly, we dive deeper into the mechanisms underlying white matter loss, test more mild injury parameters to find injury thresholds, test rmCHI in an immunocompromised mouse strain, and explore potential novel therapeutics for rmCHI.

CHAPTER 1

Introduction

Traumatic brain injury is broadly defined as an injury resulting in an alteration of brain function, or other evidence of brain pathology, caused by an external force. In the United States, an estimated 5.3 million people are living with a traumatic brain injury (TBI) related disability (Langlois et al., 2005), resulting in direct and indirect costs estimated between 56 and 221 billion USD per year (Coronado et al., 2012). Every year, 1.7 million Americans go to a hospital to seek treatment for TBI (Faul et al., 2010). This statistic does not account for the millions of more head injuries that occur, but are left untreated. It is estimated that in addition to 1.7 million TBI treated in hospitals, 1.4 - 1.8 million concussions occur annually that were not seen at a hospital.

One of the largest problems facing the field of head injuries is defining and categorizing injury severities. An 'alteration in brain function' is defined as 1 of the following clinical signs (Menon et al., 2010): any period of loss of or a decreased loss of consciousness, any loss of memory for events immediately before or after injury, neurologic deficits (weakness, loss of balance, change in vision, sensory loss, aphasia, etc), or any alteration in mental state at the time of injury (confusion, disorientation). 'Caused by an external force' could include any of the following: the head being struck by an object, the head striking an object, the brain undergoing an acceleration/deceleration movement without direct eternal trauma to the head, a foreign body penetrating the brain, forces generated from events such as a blast or explosion. Clearly, this opens the door to a wide range of combinations of inciting events, acute symptoms, and chronic outcomes following TBI. It is therefore critical to have a wide range of robust, reproducible animal models that

are able to capture a subset of these injuries to investigate mechanisms underlying pathology and functional deficits associated with TBI.

Animal Modeling of Traumatic Brain Injury

The earliest reports of investigating traumatic brain injury in the laboratory come from Lidgren and Ridner in 1965 where they describe the fluid percussion model of TBI in rabbits (Lindgren and Rinder, 1965). It was then characterized in cats in 1976, rats in 1987 (Dixon et al., 1987), swine (Zink et al., 1993), and ultimately mice (Carbonell et al., 1998). The fluid percussion model involves trephining a hole into the animal's skill and cementing a plastic cap over the craniotomy. A weighted pendulum drops from a specified height, impacting a long plexiglass tube filled with saline, creating a fluid wave traveling through the tube connected to the plastic cap, ultimately causing a fluid wave to impact dura directly and filling into interstitial spaces. Varying severities of neuropathology and behavior can be achieved via more or less height from which the pendulum is dropped.

A second major model of TBI in animals is the controlled cortical impact (CCI) model (Lighthall, 1988; Smith et al., 1995). Similar to the fluid percussion model, a craniotomy is first performed in the rodent's skull exposing the intact dura and underlying brain. Commercially available devices to impact the brain directly have precise control of velocity, impact depth, and dwell time that can produce a wide range of pathologies and functional deficits.

The weight drop impact acceleration model is another major model of TBI that has been exhaustively characterized (Foda and Marmarou, 1994; Marmarou et al., 1994). A bit more crude than the more controlled fluid percussion and controlled cortical impact

models, it does however have another significant important characteristic these models do not have: rotational, acceleration/deceleration forces. In the weight drop model, rodents are most commonly placed onto a foam pad below a guide tube. A weight is dropped down the guide tube to impact the rodent's head below. Not only are there acceleration/deceleration forces involved, but this model does not involve excising the skin, making a craniotomy, or exposing the brain.

While these are the three primary historic models of traumatic brain injury, we set forth in this dissertation to improve on these models, and focus on being able to produce multiple head injuries over multiple days, mimicking multiple mild TBI.

Repeated Mild Traumatic Brain Injury

Recent evidence, both clinically and in the laboratory, has pointed to the significance and unique disease of repeated mild traumatic brain injury. First named 'punch drunk syndrome' (Martland, 1928), next 'dementia pugilistica' (Millspaugh, 1937), then 'chronic progressive traumatic encephalopathy of boxers' (Critchley, 1957), and ultimately 'chronic traumatic encephalophathy' (McKee et al., 2009), repeated head injuries result in a unique set of neuropathologies and behavioral changes. Laboratories over the past several years have begun to investigate the effects of multiple head injuries using novel experimental devices to mimic different components of repeated mild TBI (rmTBI).

One of the first reports to investigate repeated head injuries simply used a rubberized tip on a CCI impactor impacting the skull directly after excising the skin (Laurer et al., 2001). Mice were given 2 hits, 24 hrs apart, and were assessed on a wide range of behavioral measures out to 2 months post injury. The injury parameters used showed no

cognitive dysfunction after either a single or 2 hits, although there were deficits in motor function as assessed by rotorod, rotating pole test, and neuroscore, out to 2 months. They observed no Nissl abnormalities, and no tau or amyloid beta deposition in either animal group.

John Trojanowski's group then went on to investigate repeated TBI in mice in Alzheimer's models, utilizing wildtype, Tg2576, and Tg T44 mice (Uryu et al., 2002; Yoshiyama et al., 2005). First, using a rubberized tip, they impacted the skull directly once or twice (24 hrs apart). There were no behavioral deficits observed for either group in Morris water maze testing at 16WPI. However they did show that two hits accelerated amyloid beta accumulation and oxidative stress. They also saw that 2 hits induced GFAP+ positive astrocytes around the impact site, as well as in the white matter. In their follow-up study, they looked at much more chronic timepoints of 3, 6, and 9MPI. No wildtype or Tg injured mouse showed any increased tau pathology due to injury. This study gave 16 total hits, 1 every 20 minutes, weekly for 4 weeks. This paper lacked strong quantitative or qualitative data.

Tracy McIntosh's group also began modeling rmTBI in mice, in their first study where they impacted mice twice, varying the time between impacts at 3, 5, or 7 days apart (Longhi et al., 2005). They used a CCI device, with a siliconized tip, impacting the skull directly. Unfortunately however, this was an extremely short study, where the animals were sacrificed just 3 days after their final injury. Slight cognitive deficits were observed for mice impacted 3 and 5 days apart, but not at 7 days apart in a measure of Morris water maze testing. They also observed increased and exacerbated axonal damage for the 3 and 5 days separated impacts.

Rats have also been used to investigate repeated mild traumatic brain injury. David Hovda's group created a model in which a rat is slightly affixed within a stereotaxic holder, but they impacted the skin directly with a CCI piston, as opposed to excising the skin and impacting the skull (Prins et al., 2010). A very quick study, they impacted either once or twice, 24 hrs apart, and sacrificed just a day or two later. They observed increased axonal injury, astrocytic reactivity, and a memory impairment at 1DPI in the novel object recognition task for rats hit twice. In a followup study, they investigated the effects of increasing the interhit interval (Prins et al., 2013), testing 1 hit vs 2 hits either 24 hrs or 120 hrs (5 days) apart. However no histology was presented, and novel object testing was completed at 1-3 days post injury. They argue that the duration of metabolic depression reflects the vulnerability to a second injury and could be used as a biomarker in establishing the window of vulnerability guidelines.

More exhaustive model development, characterization and experimentation began to take off after 2010. David Brody's group began testing repeated TBI (rTBI) using a rubberized tip on mice affixed into a stereotaxic chamber (Shitaka et al., 2011). They impacted either once or twice (24hr apart) and tested a wide range of histological and behavioral outcome measures. Morris water maze deficits were observed in the first week after injury, but diminished by 7 WPI. rTBI caused extensive argyrophilic abnormalities, axonal injury and reactive microgliosis. At 7WPI, microglia were still present, as assessed by stereology and particularly in the corpus callosum, as well as abnormal silver staining in rTBI mice.

Fiona Crawford's group began studying rTBI with their first study of either single or 5 hits (one injury every 48 hrs) using a metal tip directly to the skin, and assessing rotarod

and Barnes maze out to 2 weeks. An interhit interval of 48 hrs demonstrated greater cognitive impairment, microglial activation, reactive astrocytosis, and axonal pathology. There was no hippocampal cell loss, although there were deficits in rotator and Barnes maze performance. It should be noted that all histological analyses were assessed at 1DPI. However, in a followup study, they investigated more chronic timepoints of 6, 12, and 18MPI (Mouzon et al., 2014). Mice were hit 5 times, and assessed on Water maze and Barnes maze. rmTBI mice had learning deficits compared to single TBI mice at 12 and 18 months in these tests. Their model also quantified white matter atrophy of corpus callosum, by measuring the thickness. They saw a 12% reduction of single TBI compared to sham at 6 months and a 10% reduction at 12 months. The rmTBI group had a 21% decrease at 6 months compared to sham, and a further 5% more reduction compared to 6 months. However, no stereological assessment was investigated.

A very unique and exhaustively characterized model was developed by Cheryl Wellington's group. The closed-head impact model of engineered rotational acceleration (CHIMERA) model was developed with a homemade apparatus that allows for extensive acceleration-deceleration forces (Namjoshi et al., 2014). They looked at 1 or 2 impacts, and only took the animals out to 2WPI. They observed short-term deficits in rotarod at 1, 2, and 7DPI, neurological severity score deficits at 1, 2, and 7DPI, as well as passive avoidance and Barnes maze deficits. They also observed widespread microglial activation especially in white matter tracts including corpus callosum, optic tract, superior colliculus, qualitatively assessed.

The studies mentioned thus far have utilized various controlled cortical impact devices, either commercially available or homemade pneumatic devices. Many of these

groups also placed the mice into stereotaxic chambers, impacting the skull directly after excising the skin. In order to minimize skull fracture, many of these groups modified the impactor tip to either be siliconized or rubberized to diffuse the energy and avoid fractures. In addition, the CHIMERA model is the only that allows for rotational forces. We believe that using a controlled cortical impact device is advantageous in that you have precise control over impact parameters such as velocity, depth, and dwell time. Moreover, it is enticing to use a commercially available device that can be easily taught across laboratories minimizing variability. While these models used a CCI device component, many groups have created modified weight drop acceleration injuries to rodents without excising the skin.

Michael Whalen's group has developed a model where rodents are placed on a kimwipe, as opposed to a foam block, underneath a weight drop apparatus. When a weighted rod is dropped from above, it strikes the rodents head, pushing the rodent through the kimwipe, to flip and land on a foam pad beneath. In their first study, they ran two experiments (Meehan et al., 2012). First they looked at 1, 3, 5, or 10 hits, 24 hours apart. The next experiment they tests 5 hits, either daily, weekly, or monthly as interhit intervals. They found that after 3, 5, or 10 daily hits, performance on Morris water maze was impaired at 1DPI, but was not reported for any other time points. Then looking at changing the interhit interval, they found that daily and weekly impacts, but not monthly injuries, led to learning deficits 1 month after their last injury. In a limited sample size, 5 daily hits had significantly worse performance in MWM compared to sham. 5 weekly hits trended to significance but the sample size was too low. Using the same injury model, they then tested a larger cohort at timepoints of 3DPI, 2MPI, 6MPI, and 12MPI. Injury groups

included 5 daily hits, 7 daily hits over 9 days, 1 hit every week for 5 weeks, 1 hit every other week for 10 weeks, 1 hit every month for 5 months, or just a single hit (Mannix et al., 2013). Overall, daily or weekly hits, but not biweekly or monthly hits had deficits in water maze performance. Interestingly, MRI was performed and no differences were observed in any measure when looking at white matter tracts.

Overall, there many models have been to better understand the effects of repeated multiple mild traumatic brain injuries. We believe that in order to have a meaningful model, clinical parallels must exist. We believe it important to have a closed head injury model that includes acceleration/deceleration forces. The ideal model must have robust, measurable behavioral deficits similar to those observed in patients suffering from multiple traumatic brain injuries. Moreover, the model itself should be easy reproducible across laboratories.

Summary

First, this dissertation reviews the literature of rodent models of traumatic brain injury with an emphasis on studies investigating behavioral tasks associated with injuries. Importantly, we focus on papers that assess functional deficits at chronic timepoints of at least 1 month post injury, with an emphasis on longer timepoints for chronic therapeutic value. Next, I present a novel model of repeated closed head injury designed to mimic the clinical pathology and cognitive outcomes patients develop after suffering repeated mild traumatic brain injuries. We hypothesized that repeated, but not single injuries of a particular injury severity will result in cognitive and pathological changes at chronic timepoints. This model results in white matter atrophy, cortical neuronal loss, behavioral

changes in measures of anxiety and learning performance. This study is also the first to report a distinct correlation between pathology and behavioral outcomes. Next, the model is further characterized to find different levels of injury severity and investigate mechanisms underlying the developed white matter neuropathology. Lastly, a set of experiments tested potential therapeutics acting on injury pathways associate with rmTBI, as well as testing the model in an immunocompromised mouse strain.

References

Carbonell, W.S., Maris, D.O., McCall, T., Grady, M.S., 1998. Adaptation of the fluid percussion injury model to the mouse. J Neurotrauma 15, 217-229.

Coronado, V.G., McGuire, L.C., Sarmiento, K., Bell, J., Lionbarger, M.R., Jones, C.D., Geller, A.I., Khoury, N., Xu, L., 2012. Trends in Traumatic Brain Injury in the U.S. and the public health response: 1995-2009. J Safety Res 43, 299-307.

Critchley, M., 1957. Medical aspects of boxing, particularly from a neurological standpoint. Br Med J 1, 357-362.

Dixon, C.E., Lyeth, B.G., Povlishock, J.T., Findling, R.L., Hamm, R.J., Marmarou, A., Young, H.F., Hayes, R.L., 1987. A fluid percussion model of experimental brain injury in the rat. J Neurosurg 67, 110-119.

Faul, M., Xu, L., Wald, M.M., Coronado, V.G., 2010. Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations and Deaths 2002-2006, Available: http://www.cdc.gov/traumaticbraininjury/pdf/blue_book.pdf.

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

Langlois, J.A., Rutland-Brown, W., Thomas, K.E., 2005. The incidence of traumatic brain injury among children in the United States: differences by race. J Head Trauma Rehabil 20, 229-238.

Laurer, H.L., Bareyre, F.M., Lee, V.M., Trojanowski, J.Q., Longhi, L., Hoover, R., Saatman, K.E., Raghupathi, R., Hoshino, S., Grady, M.S., McIntosh, T.K., 2001. Mild head injury increasing the brain's vulnerability to a second concussive impact. J Neurosurg 95, 859-870.

Lighthall, J.W., 1988. Controlled cortical impact: a new experimental brain injury model. J Neurotrauma 5, 1-15.

Lindgren, S., Rinder, L., 1965. Experimental studies in head injury. I. Some factors influencing results of model experiments. Biophysik 2, 320-329.

Longhi, L., Saatman, K.E., Fujimoto, S., Raghupathi, R., Meaney, D.F., Davis, J., McMillan, B.S.A., Conte, V., Laurer, H.L., Stein, S., Stocchetti, N., McIntosh, T.K., 2005. Temporal window of vulnerability to repetitive experimental concussive brain injury. Neurosurgery 56, 364-374; discussion 364-374.

Mannix, R., Meehan, W.P., Mandeville, J., Grant, P.E., Gray, T., Berglass, J., Zhang, J., Bryant, J., Rezaie, S., Chung, J.Y., Peters, N.V., Lee, C., Tien, L.W., Kaplan, D.L., Feany, M., Whalen, M., 2013. Clinical correlates in an experimental model of repetitive mild brain injury. Annals of Neurology 74, 65-75.

Marmarou, A., Foda, M.A., 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.

Martland, H.S., 1928. Punch drunk. Journal of the American Medical Association 91, 1103-1107.

McKee, A.C., Cantu, R.C., Nowinski, C.J., Hedley-Whyte, E.T., Gavett, B.E., Budson, A.E., Santini, V.E., Lee, H.S., Kubilus, C.A., Stern, R.A., 2009. Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. J Neuropathol Exp Neurol 68, 709-735.

Meehan, W.P., Zhang, J., Mannix, R., Whalen, M.J., 2012. Increasing recovery time between injuries improves cognitive outcome after repetitive mild concussive brain injuries in mice. Neurosurgery 71, 885-891.

Menon, D.K., Schwab, K., Wright, D.W., Maas, A.I., Demographics, Clinical Assessment Working Group of the, I., Interagency Initiative toward Common Data Elements for Research on Traumatic Brain, I., Psychological, H., 2010. Position statement: definition of traumatic brain injury. Archives of physical medicine and rehabilitation 91, 1637-1640.

Millspaugh, J.A., 1937. Dementia pugilistica (punch drunk). US Naval Med Bull 35, 297-303.

Mouzon, B.C., Bachmeier, C., Ferro, A., Ojo, J.O., Crynen, G., Acker, C.M., Davies, P., Mullan, M., Stewart, W., Crawford, F., 2014. Chronic neuropathological and neurobehavioral changes in a repetitive mild traumatic brain injury model. Ann Neurol 75, 241-254.

Namjoshi, D.R., Cheng, W.H., McInnes, K.A., Martens, K.M., Carr, M., Wilkinson, A., Fan, J., Robert, J., Hayat, A., Cripton, P.A., Wellington, C.L., 2014. Merging pathology with biomechanics using CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration): a novel, surgery-free model of traumatic brain injury. Mol Neurodegener 9, 55.

Prins, M.L., Alexander, D., Giza, C.C., Hovda, D.A., 2013. Repeated mild traumatic brain injury: mechanisms of cerebral vulnerability. J Neurotrauma 30, 30-38.

Prins, M.L., Hales, A., Reger, M., Giza, C.C., Hovda, D.A., 2010. Repeat traumatic brain injury in the juvenile rat is associated with increased axonal injury and cognitive impairments. Dev Neurosci 32, 510-518.

Shitaka, Y., Tran, H.T., Bennett, R.E., Sanchez, L., Levy, M.A., Dikranian, K., Brody, D.L., 2011. Repetitive closed-skull traumatic brain injury in mice causes persistent multifocal axonal injury and microglial reactivity. J Neuropathol Exp Neurol 70, 551-567.

Smith, D.H., Soares, H.D., Pierce, J.S., Perlman, K.G., Saatman, K.E., Meaney, D.F., Dixon, C.E., McIntosh, T.K., 1995. A model of parasagittal controlled cortical impact in the mouse: cognitive and histopathologic effects. J Neurotrauma 12, 169-178.

Uryu, K., Laurer, H., McIntosh, T., Praticò, D., Martinez, D., Leight, S., Lee, V.M.Y., Trojanowski, J.Q., 2002. Repetitive Mild Brain Trauma Accelerates Aβ Deposition, Lipid Peroxidation, and Cognitive Impairment in a Transgenic Mouse Model of Alzheimer Amyloidosis. The Journal of Neuroscience 22, 446-454.

Yoshiyama, Y., Uryu, K., Higuchi, M., Longhi, L., Hoover, R., Fujimoto, S., McIntosh, T., Lee, V.M., Trojanowski, J.Q., 2005. Enhanced neurofibrillary tangle formation, cerebral atrophy, and cognitive deficits induced by repetitive mild brain injury in a transgenic tauopathy mouse model. J Neurotrauma 22, 1134-1141.

Zink, B.J., Walsh, R.F., Feustel, P.J., 1993. Effects of ethanol in traumatic brain injury. J Neurotrauma 10, 275-286.

CHAPTER 2

Functional Assessment of Long-Term Deficits in Rodent Models of Traumatic Brain Injury

Abstract

Traumatic Brain Injury (TBI) ranks as the leading cause of mortality and disability in the young population worldwide. The annual incidence of TBI (new cases each year) in the general population is estimated at 1.7 million Americans per year, with an estimated financial burden in excess of $75 billion a year in the US alone. Despite the prevalence and cost of TBI to individuals and society, no treatments have passed clinical trial to clinical implementation. The rapid expansion of stem cell research and technology offers an alternative to traditional pharmacological approaches targeting acute neuroprotection. However, pre-clinical testing of these approaches depends on the selection and characterization of appropriate animal models. In this article we consider the underlying pathophysiology for the focal and diffuse TBI subtypes, discuss the existing pre-clinical TBI models and functional outcome tasks used for assessment of injury and recovery, identify criteria particular to pre-clinical animal models of TBI in which stem cell therapies can be tested for safety and efficacy, and review these criteria in the context of the existing TBI literature. We suggest that 2-months post-TBI is the minimum period needed to evaluate human cell transplant efficacy and safety. Comprehensive review of the published TBI literature revealed that only 32% of rodent TBI papers evaluated functional outcomes 1-month or greater post-TBI, and only 10% evaluated functional outcomes 2-months or greater post-TBI. Not all published papers that evaluated functional deficits at a minimum of 2-months post-TBI reported deficits; hence, only 8.6% of overall TBI papers captured in

this review demonstrated functional deficits at two months or more post-injury. A 2-month survival and assessment period would allow sufficient time for differentiation and integration of hNSCs with the host. Critically, while trophic effects might be observed at earlier time-points, it will also be important to demonstrate the sustainability of such an effect, supporting the importance of an extended period of *in vivo* observation. Furthermore, regulatory bodies will likely require at least 6 months survival post-transplantation for assessment of toxicology/safety, particularly in the context of assessing cell abnormalities.

Background

1.1 Definition of Traumatic Brain Injury (TBI). Broadly defined, traumatic brain injuries encompass any injury resulting in "altered brain function" or brain pathology as the result of an external force. Under this definition, altered brain function includes any period of any one of the following: a decrease in or loss of consciousness; pre- or post- event memory loss (amnesia); neurological deficits such as confusion, slowed thinking, or aphasia; or vision or sensory changes, loss of balance, muscle weakness, or paralysis (Management of Concussion/mTBI Working Group, 2009; Menon et al., 2010). An individual need not lose consciousness to sustain a brain injury. A mild traumatic brain injury, or concussion, can still damage the brain at the cellular level and increase the risk of neurodegenerative disorders such as Alzheimer's disease in the future (Moretti et al., 2012). Even persons experiencing a minor concussion that does not result in hospitalization can have persistent symptoms of headaches, anxiety, and/or fatigue coupled with cognitive deficits in memory, concentration, and/or attention; this constellation of symptoms is referred to as post-concussion syndrome (PCS) (Hall et al., 2005b). Not all individuals who sustain trauma to

the head will experience a traumatic brain injury, but anyone with a history of head trauma resulting in altered brain function can be said to have sustained a TBI.

Repetitive head injuries, even minor ones, can also lead to serious repercussions to the recipient – including permanent brain damage or death. Repetitive brain injury was initially termed "punch drunk" syndrome (Martland, 1928), then "traumatic encephalopathy" (Parker, 1934) or "dementia pugilistica" (Millspaugh, 1937), and finally "chronic progressive traumatic encephalopathy of boxers" (Critchley, 1957). This terminology reflects the early characterization of repetitive brain injury in professional boxers. More recently, the resulting neuropsychological problems and underlying brain pathology seen in some professional athletes following repeated brain trauma has been termed chronic traumatic encephalopathy or CTE. The first National Football League player diagnosed with CTE was 50 years old and had sustained multiple concussive injuries over a 17 year professional career (Omalu et al., 2005). Since then, numerous reports of CTE in athletes have been linked to a variety of professional contact sports (McKee et al., 2009).

1.2 Incidence, prevalence, and cost estimates of TBI. TBI is often referred to as "a silent epidemic", a status borne out by the fact that it ranks as the leading cause of mortality and disability in the young population worldwide (Langlois et al., 2005). Every 18.5 seconds, someone in the United States suffers a TBI (Scudellari, 2010). The annual incidence of TBI (new cases each year) in the general population was estimated at 1.7 million Americans per year (based on 2002-2006 data) (Faul et al., 2010). This is higher than the incidence of Alzheimer's disease, Parkinson's disease, and multiple sclerosis combined, and greater than the incidence of individuals diagnosed with brain, breast, colon, lung, or prostate

cancer combined (Scudellari, 2010). Critically, these figures do not include military personnel. TBI-related injuries are the signature injury of military personnel who served in either the Iraq and/or Afghanistan wars. It is estimated that nearly 60% of all casualties among soldiers admitted to Walter Reed National Military Medical Center suffer from TBI (Combat Casualty Care Research Program, 2013). The Department of Defense Medical Surveillance System estimated that the number of personnel who experienced some level of TBI between 2000-2012 was 266,810 (Department of Defense, 2013).

During the same time period (2002-2006), 1.1 to 1.4 million civilians per year were treated in US hospital emergency rooms for TBI, resulting in an average of 52,000 deaths per year (Coronado et al., 2011). Mortality in severe TBI cases remains around 30% (Narayan et al., 2002). Greater than 40% of US citizens with TBI have residual disability one year post-injury (Corrigan et al., 2010). It is difficult to estimate the prevalence of TBI world-wide due to sparse data, differing definitions and changing patterns over time (Roozenbeek et al., 2013). The changing pattern is due to a variety of factors, including demographic/population shifts, the institution of modern safety measures, changes in reporting criteria, etc… which make determining the worldwide incidence and prevalence of TBI difficult. Prevalence estimates for long-term TBI-related disability in the US range from 3.2 million (Corrigan et al., 2010) to 5.3 million (Thurman et al., 1999) people; thus nearly one out of every fifty to seventy-five American civilians are currently living with disabilities from TBI. Direct medical costs coupled with rehabilitation and lost productivity result in a financial burden estimated to be in excess of $75 billion a year in the US (in 2010 dollars) (Coronado et al., 2012; Finkelstein et al., 2006). This number would be much higher when projections are made for lost productivity of the care-givers. In a study of

post-TBI employment rates among adults with TBI who received inpatient care and rehabilitation at the University of Missouri TBI Model Systems center, only 36% of those who were employed at the time of their injury were employed two years post-injury. The percentage on some form of public assistance rose from 18% pre-injury to 38% at two years post-injury (data were not broken down by injury severity) (Shigaki et al., 2009).

1.3 Underlying pathophysiology of TBI. Primary TBI reflects the early damage that is the direct result of the physical displacement of brain structures, including contusion, vascular injury, and axon shearing. Secondary TBI reflects damage that is an indirect result of the initial trauma; secondary damage therefore encompasses a variety of cellular processes that contribute to the progressive loss of cells and damage to underlying pathways over hours, days and weeks (McIntosh et al., 1996). TBI can also be classified as focal or diffuse in type, with the latter accounting for up to 70% of TBI in humans . The forces producing focal and diffuse TBI are notably different. Clinically, focal TBI results from a direct impact to the skull, e.g. following a fall, which causes compression of the brain region underlying the impact (*coup*) and rebound impact to the brain region directly opposite (*contrecoup*) (Andriessen et al., 2010; Morganti-Kossmann et al., 2010). As a result, the location of the impact is directly related to the neuroanatomical locations of damage and resulting neurological deficits. Pathophysiologically, the principal characteristics of focal TBI are laceration, contusion, and hematoma.

In contrast, diffuse TBI results from rapid acceleration-deceleration of the head, e.g. following a high speed motor vehicle accident, which causes widely distributed white matter, vascular, and hypoxic-ischemic damage (Andriessen et al., 2010; Morganti-

Kossmann et al., 2010). Macroscopically, the diffuse TBI brain presents few abnormalities; the true extent of axonal damage is only detected using markers or modalities sensitive to abnormalities in axons and white matter structures (Buki and Povlishock, 2006; Graham et al., 1995; Newcombe et al., 2011). In situ, this includes staining for neurofilament (NF) or amyloid precursor protein (APP). In vivo, this includes analysis by diffusion tensor magnetic resonance imaging (DT-MRI). Clinical studies suggest that diffuse TBI results in progressive, chronic atrophy of white matter that may continue for years post-injury (Sidaros et al., 2009). This is thought to be associated with detachment of distal axons from the cell body, resulting in the formation of retraction bulbs, a histological hallmark of diffuse injury. In experimental animal models, the underlying processes are termed traumatic axonal injury (TAI), and include changes in axolemma permeability, activation of proteases, cytoskeletal degradation and impairment of axonal transport (Buki and Povlishock, 2006). Axonal damage in diffuse TBI is thought to result from shearing and compressive forces that exceed the maximum elasticity potential of axons. As a result, the direction of shear and strain forces in diffuse TBI plays a critical role in the severity of damage. Critically, however, MRI studies suggest that 50% or more of patients with moderate to severe TBI exhibit a mixed etiology, in which both focal lesions and diffuse axonal injury are observed (Skandsen et al., 2010). These complex and mixed clinical observations suggest that, despite the contribution of distinct pathophysiological mechanisms to focal neuronafl loss versus diffuse axonal pathology, the distinction between focal and diffuse TBI is an artificial one (Andriessen et al., 2010). This blurred distinction highlights the importance of animal model selection, the need to replicate positive findings in more than one model, and the potential need for combinatorial

therapeutic approaches.

1.4 Predictive validity of pre-clinical animal models for clinical translation. It is clear that TBI in humans causes a wide range of damage and deficits. Basic and pre-clinical animal research attempts to model aspects of TBI pathology using different injury paradigms and measuring different types of motor and cognitive outcomes. Common to most injury paradigms are two of the main pathological hallmarks of TBI: neuronal cell death and white matter damage. Basic and pre-clinical TBI research has focused heavily on strategies to attenuate the early expansion of damage. However, although even a modest improvement in outcome for patients would have significant quality of life benefits and financial benefits, no therapy has yet to be found to improve outcomes in TBI patients. To date, there have been 45 phase II or III clinical trials in the US for a drug or procedure (e.g. hypothermia or hyperbaric oxygen) to treat TBI; yet there are still no FDA approved therapies for TBI (ClinicalTrials.gov). This failure rate has highlighted the necessity of re-evaluating both clinical translation strategies and pre-clinical models.

In part, these failures have been suggested to result from heterogeneity in the clinical face of TBI; as a result, the reliance of clinical trials incorporating broad inclusion criteria and the use of pre-clinical data from highly homogeneous animal models may be a significant contributing factor in trial failure (Maas and Menon, 2012). Experience from the stroke field suggests a relevant and cautionary tale, where a high rate of failure in translation from bench to bedside led to the formulation of criteria for the conduct and evaluation of pre-clinical research supporting clinical trial initiation (the STAIR criteria), as well as standards to ensure that the design of clinical trials reflects, as accurately as possible, the pre-clinical

data and models available (Fisher et al., 2009; Stroke Therapy Academic Industry, 1999). More recently, parallel criteria have been established by NIH. In August 2011, NINDS released formal guidelines for the conduct of pre-clinical and clinical research, called the RIGOR criteria [NOT-NS-11-023]. Both sets of criteria are designed to improve pre-clinical study design and enhance transparency of study reporting. For example, the RIGOR guidelines re-emphasize the basics of good experimental design, including: rational selection of models, route/timing of interventions, and study endpoints; adequacy of controls, sample size, and statistical methods; appropriate blinding, randomization, and complete data reporting; data reproducibility/replication and verification of biological activity; consideration of alternative interpretations/hypotheses; discussion of effect sizes; and disclosure of conflicts of interest.

While, if consistently applied, STAIR and RIGOR criteria may improve the success of clinical translation over time, it may also be the case that a mis-match between pre-clinical models and the human condition can contribute to translation failure. This latter issue may be particularly critical for the advent of stem cell translational approaches for neurological disease and injury, which is our focus in the following sections.

1.5 Criteria for predictive models of TBI and assessment of stem cell therapies. The rapid progression of stem cell research in the last fifteen years has opened a new aspect of regenerative medicine research and clinical translation for neurological disease & injury. Stem cell or other cellular therapy trials have either been completed or are still underway for the treatment of patients with Batten's disease, spinal cord injury, Pelizaeus-Merzbacher Disease (a fatal neurodegenerative disorder of myelin), amylotrophic lateral

sclerosis (ALS), Huntington's disease, Parkinson's disease, and ischemic stroke (De Feo et al., 2012). Stem cell approaches to regenerative medicine for CNS disorders are thought to proffer several broad mechanisms of action: **(A) immune-modulation** of the host environment, **(B) trophic factor** secretion in the local microenvironment, and/or **(C)** underlying **functional integration** with the injured host following terminal differentiation of neural stem cells (NSCs) into neurons, astrocytes and/or oligodendrocytes. Critically, these mechanisms need not be mutually exclusive. In our experience, human NSCs have the potential to simultaneously target neuronal replacement and white matter repair, to replenish lost cells following injury and/or to restore myelination of demyelinated axons (Cummings et al., 2005; Hooshmand et al., 2009).

While stem cell therapy strategies for TBI may offer the possibility for a new and mechanistically combinatorial approach, testing of donor cell populations in animal models will add to the complexity of the criteria necessary for pre-clinical animal models with good predictive validity for clinical translation. In particular, at least two conditions would need to be met for any relevant pre-clinical model to enable evaluation of the success or failure of a therapeutic stem cell approach. First, a model in which ***sufficient engraftment*** of donor human cells can be achieved to reliably test safety and efficacy across a xenotransplantation barrier will be necessary; this requirement will likely require immunodeficient animal models, or dramatically improved methods of achieving adequate immunosuppression (Anderson et al., 2011). Second, a model in which ***sufficient time duration*** from transplant to assessment allows for the potential functional impact is necessary, so that either improvement (efficacy) or determent (safety) can be measured. In the case of TBI, the persistence of sustained deficits that can be reliably measured are an

especially critical variable in this context. Human donor cells, in particular, may require a significant period of time for proliferation, migration, differentiation and integration; the time required for these processes can be anticipated to be an essential variable in determining the effect (or not) of donor cells on Mechanism C (functional integration). Critically, based on our experience with hNSC and spinal cord injury (Cummings et al., 2005; Hooshmand et al., 2009; Piltti et al., 2013; Salazar et al., 2010), we would predict that sustained deficits post-TBI of at least 1 month, and more likely at least 2-months post-injury, are needed to allow for detection of cell transplant mediated functional effects if such effects are via integration and not via trophic mediated mechanisms. Even in the case of either Mechanism A (immune-modulation) or B (trophic factors), functional improvements at greater than 1 month post-injury would be desirable. As many TBI studies have focused on short term outcomes, it is unclear which of the existing TBI models may meet these criteria to support safety and efficacy studies, particularly in the context of long-term deficits.

Accordingly, the goal of this review was to ascertain if there is an optimal combination of injury model and functional assessments that yields prolonged functional deficits, i.e. ≥ 1 month post-TBI, to enable detection of safety and efficacy in the context of stem cell transplantation strategies. Towards this end, we summarize current animal models of TBI and the cognitive and motor tasks that have been used in post-injury assessments in these models. With these categorizations in hand, we conducted a survey of the literature from the beginning of PubMed indexing to March 31, 2013, in order to objectively evaluate the range of functional assessments that exhibit prolonged deficits (≥ 1 month) in various TBI models. We conducted ten PubMed searches with the following

search terms: “controlled cortical impact AND water maze”, “controlled cortical impact AND cognition”, “fluid percussion AND water maze”, “fluid percussion AND cognition”, “blast AND water maze”, “blast AND cognition”, “weight drop AND water maze”, “weight drop AND cognition”, “closed head injury AND water maze”, and “closed head injury AND cognition”. All papers matching these search terms were merged into one list.

Initially, 817 papers were found with these search terms, but results were then filtered for studies in rats or mice only (371 remained), which were not reviews (362 remained), and which included an uninjured/sham control group in comparison to an injured group on one or more functional assessments (45 papers were excluded). This filtering indicates ~12% of rodent TBI papers did not include sham to injured comparisons and resulted in a total of 314 unique papers for review.

Injury Models

There are a wide range of injury paradigms used to model TBI in rodents. Broadly, these are typically divided into focal and diffuse injury models, although considerable pathological and functional overlap between the focal and diffuse models exists. Below, we briefly describe the main features of each model (see **Figure 2.1,** from (Xiong et al., 2013)).

2.1 Fluid Percussion Injury (FPI) models. Fluid percussion injury (FPI) is the most commonly used model for studying traumatic brain injury. First described in 1965, Lidgren and Rindner developed the fluid percussion model in rabbits (Lindgren and Rinder, 1965). In 1976, FPI was characterized by Sullivan *et al.* in cats (Sullivan et al., 1976), and finally by Dixon *et al.* in rats in 1987 (Dixon et al., 1987). Fluid percussion has also been adapted for

swine (Stern et al., 2000; Zink et al., 1993) and mice (Carbonell et al., 1998). FPI involves trephining a hole in the skull and cementing a plastic cap over the craniotomy. The FPI device has a saline filled plexiglass cylindrical tube attached to the plastic cap via a Leur-Loc connection. A weighted pendulum swings down from a desired height and impacts the opposite end of the tube, starting a fluid wave which ends on the intact dura of the animal. The fluid wave impacts dura while also filling into interstitial spaces, causing both focal and diffuse brain injury. The craniotomy can be placed directly on top of the sagittal suture for a Medial Fluid Percussion Injury (MFPI) (Dixon et al., 1987), or can be placed lateral to midline for a Lateral Fluid Percussion Injury (LFPI) (McIntosh et al., 1989). Standard LFPI in rodents causes neuronal damage to the hilar region of the dentate gyrus (Lowenstein et al., 1992), the ipsilateral cortex, hippocampus, and thalamus (Hicks et al., 1996). Small changes in the lateral, rostral, or caudal location of the craniotomy for LFPI can have different effects on behavior and pathology (Floyd et al., 2002; Vink et al., 2001). LFPI also results in increased extracellular glutamate and aspartate, widespread reactive astrocytosis, cavity formation, BBB disruption, ipsilateral cortical and subcortical hemorrhage, and cerebral edema (Cortez et al., 1989; Faden et al., 1989; McIntosh et al., 1989; Soares et al., 1992).

2.2 Controlled Cortical Impact (CCI) model. The CCI model of TBI employs a pneumatic impact device to drive a rigid impactor to deliver mechanical energy onto the exposed and intact dura mater. This mechanical energy causes a deformation of the brain. The advantage of this model is the reproducibility and precise control of the mechanical parameters: dwell time, velocity and depth of impact (Morales et al., 2005; Xiong et al.,

2013). The first CCI was described in ferrets (Lighthall, 1988) and was later adapted for use in rats (Dixon et al., 1991) and mice (Hannay et al., 1999; Smith et al., 1995). CCI has also been described in swine and monkeys (Xiong et al., 2013). The neuropathology induced by CCI includes contusion, subdural hematoma, subarachnoid hemorrhage, edema, hypoperfusion, neurodegeneration, and cavitation (Dixon et al., 1991; Hall et al., 2005a; Kochanek et al., 1995). While some diffuse damage also occurs, CCI is considered to induce focal brain injury. This kind of damage occurs proximal to the mechanical impact and induces brain injury of both cortical and subcortical structures and ventricular enlargement (Morales et al., 2005) (**See Figure 2.2**).

2.3 Weight Drop Impact-Acceleration model (WDIA)/Closed Head Injury (CHI). The weight drop impact-acceleration (WDIA) model was developed by Marmarou in 1994 (Foda and Marmarou, 1994; Marmarou et al., 1994). WDIA is a popular model for TBI, involving very inexpensive equipment and minimal invasiveness. A rodent is strapped onto a foam block after having a metal disk affixed its skull. A weight is dropped from a desired height to directly strike the disc. This results in a diffuse injury with widespread axonal damage, whilst still keeping the cranium intact (Foda and Marmarou, 1994). Subarrachnoid hemorrhage, rapid and transient BBB disruption, and brain edema are observed following WDIA in rodents (Barzo et al., 1996; Foda and Marmarou, 1994). For more precise control and manipulation of diffuse injuries to a rodent's head without trephination, many investigators are developing experimental closed head injury (CHI) models utilizing CCI devices to directly strike a disk attached to the skull, or the head itself without any protection. These injuries produce very similar pathologies to WDIA but with increased

reproducibility.

2.4 Blast Injury model. The blast model of TBI has become an increasingly popular model to study in the laboratory due to its ability to mimic blast waves associated with explosions. Blast or explosions are the most common cause of TBI to soldiers in Iraq (Hoge et al., 2008), leading to long term cognitive and emotional deficits. The blast injury model uses a long metal tube that is closed on one end. An air pressure wave or an explosion is used to deliver over- or under-pressure waves beginning at the closed end of the tube. Rodents are placed at the open end of the tube to receive the shockwave, affecting the whole body and the head (Cernak et al., 2001). Blast injuries result in neuronal damage in the temporal cortex, cingulate gyrus, piriform cortex, dentate gyrus, and the CA1 region of the hippocampus (Saljo et al., 2000).

2.5 Penetrating Ballistic-Like Brain Injury model (PBBI). PBBI is considered a type of focal brain injury (Xiong et al., 2013), even though there may also be diffuse damage as well. Firearm related injuries are on the rise nationwide, with bullet wound injuries to the head being a common cause of PBBI/TBI related injuries. Williams *et al.* (Williams et al., 2006a; Williams et al., 2006b) describe PBBI as a high-energy transfer wound which causes direct damage to the brain via formation of a temporal intracranial cavity. The injury is produced by a specially designed probe inserted into the brain at the desired location, leaving a permanent injury tract, followed by a fast inflation of an attached balloon to mimic the temporary cavity provoked by a penetrating bullet (Williams et al., 2005). The first penetrating brain injury was described in rhesus monkey (Allen et al., 1982), and

subsequently in cats (Carey et al., 1989) and rats (Williams et al., 2005). PBBI results in intracerebral hemorrhage, brain edema, and degeneration of neurons and fiber tracts remote from the core lesion (Shear et al., 2011a; Williams et al., 2006a; Williams et al., 2007). This model isrelatively rare (only 4 papers were found matching the search criteria); hence the PBBI model was not analyzed further.

2.6 Instant Rotational model. A instant rotational model of traumatic injury was first developed by Gennarelli *et al.* in 1982 (Gennarelli et al., 1982). Primates were originally studied in this model which involves quickly accelerating the animal's head in a given direction. In 1994, this model was also utilized to study TBI in swine (Ross et al., 1994). The instant rotational model is limited to use in larger animals, as the inertial force required to cause damage is inversely related to brain size and is impractical for use in rodents. Hence, this model was not included in the set of papers in this review.

2.7 Summary of TBI models. While there are many different methods to model TBI in rodents, and each method has variations in its application, the most common models are the fluid percussion injury models (FPI), the controlled cortical impact (CCI), and the weight drop impact -acceleration/closed head injury models (WDIA/CHI). Overall, we found 140 FPI papers (either lateral or medial) which met our inclusion criteria, 130 CCI papers, 24 WDIA papers, 15 CHI papers and 7 blast papers during our review of the literature from inception of PubMed to the present (March 31, 2013) (**See Figure 2.3A**).

Assessments of functional outcome in rodents after TBI

There are a host of functional outcome measures suitable for use in rodents. Functional recovery and post-TBI deficits can be assessed in a subject's global neurological performance, in a range of gross and fine motor tasks, on sensorimotor tasks, tests of pure sensory response, cognition (including long- and short-term memory, spacial memory, etc...) and emotional responses. We describe each of these domains in order followed by an analysis of the number of papers reporting deficits in various domains at a minimum of 1-month post injury, as defined by 28 days post-injury (dpi).

3.1 Global Assessments of general impairment

Following TBI, a global assessment of the animals' neurological status is often conducted by using a set of generalized neurological screening tasks. Such global assessments are used to verify injury severity, as inclusion/exclusion tests for assignment to treatment groups, or as final endpoint measures. Currently, there are several neurological test/scoring protocols employed by different research groups in the brain trauma field.

3.1.1 Neurological Severity Score (NSS). One of the first global scoring systems was developed by Shapira *et al.* in 1988 (Shapira et al., 1988) to measure the clinical relevance of a closed head injury rat model by determining a neurological severity score (NSS) based on an animal's response on a series of simple tests post-TBI. Rodents were evaluated for their ability to exit a 50cm circle, for righting reflex, seeking behavior, hemiplegia or hemiparesis, with higher scores indicating more deficits. Increased NSS scores were correlated with damaged tissue pathology (Shapira et al., 1988). The NSS was expanded and refined by Shohami *et al.* in 1995 to include more reflex tasks (Shohami et al., 1995).

Further adaptations to the NSS include a 14 point scale consisting of motor (muscle status, abnormal movement), sensory (visual, tactile and proprioceptive), and reflex tasks (Lu et al., 2007; Lu et al., 2001), which became the basis for the modified NSS (mNSS).

3.1.2 Modified Neurological Severity Score (mNSS). The Modified NSS is a global assessment of function gauged by a composite of motor, sensory, balance, and reflex tasks (Chen et al., 2001b; Lu et al., 2007). The mNSS is based on the NSS (as adapted from (Shapira et al., 1988) by (Shohami et al., 1995)) as well as incorporating additional tasks that target motor function (Borlongan et al., 1995; Longa et al., 1989; Schallert et al., 1997), sensory function (Hillary et al., 2011), balance (Germano et al., 1994), and reflex function (Chen et al., 1997; Germano et al., 1994; Schallert et al., 1997) on a scale of 1 to 14 points in mice (Li et al., 2000). The mNSS is frequently used to evaluate long term neurological function after unilateral traumatic brain injury in rodents (Meng et al., 2011; Ning et al., 2011; Xiong et al., 2010; Xiong et al., 2011b; Xiong et al., 2012; Zhang et al., 2009). While the current mNSS was originally designed using mice (Li et al., 2000), there is a trend of including balance beam tasks as part of the mNSS when evaluating rats (Chen et al., 2001b)(Chen et al., 2001c; Xiong et al., 2010; Xiong et al., 2011b; Xiong et al., 2012). However, the "mouse" mNSS has been used for global assessments in rats as well (Chen et al., 2001a). The mNSS as modified for rats consists of an 18 point deficit score, where a composite score of ≤6 designates a mild TBI, 7 to 12 designates moderate TBI, and ≥13 designates severe TBI. In a unilateral model of TBI, a high score in the motor portion of the mNSS indicates asymmetric behavior due to contralateral limb paralysis related to damage to the cerebral cortex; a high score in the sensory tasks of the mNSS is indicative of damage

to the cerebral cortex and cortiocospinal pathways (which overlaps with motor deficits); a high score in balance portion indicates damage to the pons and mesencephalon; a high score in the reflex portion of the mNSS indicates damage to the medulla, pons, and upper cervical cord (Lu et al., 2007; Tupper and Wallace, 1980).

3.1.3 Composite Neuroscore. The composite neuroscore originates from a scale designed by McIntosh *et al.* (McIntosh et al., 1987; McIntosh et al., 1989). The composite neuroscore initially consisted of five tests: (i) forelimb flexion upon suspension by the tail, (ii) decreased resistance to lateral pulsion, (iii) circling behavior upon spontaneous ambulation, (iv) the angle board task, which tests the animal's ability to stand on an inclined angle board and (v) the grip test, which requires the animal to use its 4 paws and tail to remain on a narrow, 2cm wide wooden beam (McIntosh et al., 1987). The five tasks are combined for a possible score of 20 points (McIntosh et al., 1987; McIntosh et al., 1989). A composite neuroscore of 20 would indicate a normal animal; 15 indicates slight motor impairment; 10 indicates moderate motor impairment; 5 indicates a severely impaired animal; and 0 indicates an afunctional animal. This set of criteria was then modified and expanded to seven tasks with a maximum score of 28 (Saatman et al., 1997). The seven task composite neuroscore is employed by various groups to detect long term neurological deficits (Hayward et al., 2010; Hoover et al., 2004; Lenzlinger et al., 2005), although variations of the task composition and/or scoring approaches have also been used (Dixon et al., 1987; Mattiasson et al., 2000; Okiyama et al., 1992; Sinson et al., 1995; Smith et al., 1993).

Overall, 14% (45 of 314) of the articles in this review contained a global neurological

assessment as part of their functional analysis. Of these, 23 assessed a global neurological assessment using LFPI, 14 using CCI, 4 using WDIA, and 4 using CHI. Studies which looked for deficits at 1-month or more include 10 using LFPI, 6 using CCI, and 4 using CHI. Deficits in a global neurological assessment in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 9 papers using LFPI (Hayward et al., 2010; Hoover et al., 2004; Keck et al., 2007; Marklund et al., 2007; Pierce et al., 1998; Rau et al., 2012; Riess et al., 2001; Schutz et al., 2006; Wahl et al., 2000) and 6 papers using CCI (Longhi et al., 2011; Longhi et al., 2004; Meng et al., 2011; Shelton et al., 2008; Xiong et al., 2011b; Xiong et al., 2012). TBI papers that detected sustained neurological deficits ≥ 1-month post injury via global neurological assessment of function account for less than 5% of the total number of articles reviewed (15/315).

The global assessments used in the papers using CCI were relatively uniform in protocol and application among researchers using the mNSS. A common element of the CCI papers reviewed which found deficits at 1-month post injury is that 6 of 7 articles were conducted using common parameters: 300-400g, male Wistar rats (6-8 per per group), a moderate CCI injury (2.5mm deep, 4 m/s) over the left cortex, mNSS analysis, and a maximum assessed time-point of 35 days post injury (Bradwell et al., 2012; Longhi et al., 2011; Meng et al., 2011; Ning et al., 2011; Xiong et al., 2010; Xiong et al., 2011b). However, global assessment protocols used in papers using FPI more often used a composite neuroscore which had more variation in individual components and scoring (Hayward et al., 2010; Hoover et al., 2004; Lenzlinger et al., 2005; Rau et al., 2012; Wahl et al., 2000). One concern regarding the validity of any global assessment is the reproducibility and subjectivity of human scoring, as opposed to more objective measurements. In early TBI

articles reviewed, reproducibility was often supported through confirmation of inter-rater reliability (McIntosh et al., 1987; McIntosh et al., 1989).

3.2 Motor Assessments

Motor behavior tasks can be divided into many categories but within the papers reviewed two main categories emerge. The first category is gross motor behavior, which includes walking, running, and torso movements; these movements typically use large muscle groups. Tasks that are included in gross locomotor behavior are balance beam, beam walking, foot fault, grip test, inclined plane, open field, righting reflex, rotarod, rotating pole, and the swim speed component of the Morris water maze. The second category of motor behavior tasks is fine motor behaviors, which use smaller muscles such as muscles in the paws. From the papers reviewed beam walking, bilateral tactile adhesive task, and the cylinder task were used as tasks assessing fine motor control. Of course, it is difficult to separate out tasks which are purely motor or purely sensory. Tasks which are sensorimotor in nature are included in this motor section whereas pure sensory tasks are in a separate section.

***3.2.1 Balance Beam*.** Balance beam is a test for motor and vestibular function. Most papers describe this as a motor function task, however, some emphasize the task's sensory assessment. Balance beam involves an elevated narrow beam, which the animal needs to balance on; the latency for the animal to fall off the beam is recorded (typically with a maximum latency of 60 seconds). A round, or narrower, beam can be used to increase the difficulty of the task. Neuroanatomy that is reported to be involved in balance beam is the

motor cortex *(Singleton et al., 2010)* and possibly cerebellum *(Colombel et al., 2002; Lekic et al., 2011)*. Of all papers reviewed, 43 assessed balance beam using CCI, 9 using LFPI, 7 using MFPI, 3 using CHI, and 2 using WDIA. Studies looking at deficits at 1-month post injury or later include 1 using CCI and 1 using LFPI. Deficits in balance beam performance in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 1 paper using CCI *(Cheng et al., 2012)* and 1 using LFPI *(Keck et al., 2007)*.

3.2.2 Beam Walking. Similar to balance beam, beam walking is a fine motor coordination task for rodents that uses a negative reinforcement paradigm. Animals must escape from ambient light and/or high decibel white noise by crossing an elevated narrow beam and entering a dark goal box on the other end of the beam. The time that it takes an animal to cross the beam and the number of foot slips it makes while crossing are recorded. Beams are generally placed 3m above the ground so that the animal is fearful from the height. Foot slips and latency to reach the goal are measured during this task. Scoring ranges from 1 to 7, with 1 being unable to traverse the beam and unable to place the affected limb on the horizontal surface, and 7 being the animal was able to traverse the beam normally with no more than two foot slips. Regions of the brain that may affect this performance in this task are the motor cortex, sensory cortex, thalamus, brain stem, and cerebellum (Hallam et al., 2004; Lee et al., 2004). Of all papers reviewed, 36 assessed beam walking using CCI, 25 using LFPI, 8 using MFPI, 2 using WDIA, 1 using CHI, and 1 using Blast. Studies looking at deficits at 1-month post injury or later include 9 using LFPI, 7 using CCI, and 1 using WDIA. Deficits in beam walking performance in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 6 papers using CCI (Fox and Faden, 1998;

Fox et al., 1998a, b; Fox et al., 1999; Longhi et al., 2011; Zhao et al., 2012), 4 using LFPI (Bao et al., 2012; Hallam et al., 2004; Lee et al., 2004; Lyeth et al., 2001), 1 using repeated LFPI (Shultz et al., 2013), and 1 using WDIA (Hallam et al., 2004).

3.2.3 Bilateral Tactile Adhesive Removal Task. The bilateral tactile adhesive removal task involves placing small rectangular patches of adhesive bracelets onto a rodent. These bracelets are wrapped above the front paws and the time for removal from both forelimbs is recorded. This task measures sensorimotor deficits in the caudal forelimb region, rostral forelimb region, and anteromedial cortex of the brain *(Barth et al., 1990)*. Of all papers reviewed, 4 assessed the bilateral tactile adhesive removal test using CCI, 3 using LFPI, and 1 using WDIA. Studies looking at deficits at 1-month post injury or later include 3 using LFPI and 2 using CCI. Deficits in the bilateral tactile adhesive removal task in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 1 paper using LFPI *(Riess et al., 2001)* and 1 using CCI *(Hoane et al., 2004)*.

3.2.4 Cylinder Test. The cylinder test is used to assess asymmetries in forelimb function. An animal is placed in a cylinder and the number of times it places its contralateral, ipsilateral, or both paws on the wall of the cylinder during rearing are counted. Injured animals will often not use their contralateral limbs to balance themselves while rearing along the walls of the cylinder (Hanell et al., 2010; Woodlee et al., 2005). Structures suggested to be involved in the cylinder test are midbrain median raphe and dorsal raphe nuclei (Carballosa Gonzalez et al., 2013). Of all papers reviewed, 2 assessed the cylinder test using CCI and 1 using LFPI. Studies looking for deficits at 1-month or later include 1

using CCI (Hanell et al., 2010) and 1 using LFPI (Carballosa Gonzalez et al., 2013), both of which observed deficits in TBI injured rodents compared to uninjured controls.

3.2.5 Foot Fault. Foot fault or locomotor placement tasks utilize a wire grid to examine the number of times individual paws slip through the grid during a preset number of paw placements. Increases in foot faults might be due to tissue loss in the cortex, striatum, and corticostriatal connections (Xiong et al., 2008a). Of all papers reviewed, 12 assessed the foot fault task using CCI, 5 using LFPI, 2 using CHI, and 1 using WDIA. Studies looking at deficits at 1-month post injury or later include 8 using CCI and 1 using LFPI. Deficits in foot fault performance in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 7 papers using CCI (Hoane et al., 2004; Meng et al., 2011; Xiong et al., 2008b; Xiong et al., 2010; Xiong et al., 2011a; Xiong et al., 2011b; Zhang et al., 2009) and 1 using LFPI (Rau et al., 2012).

3.2.6 Forelimb Flexion. Forelimb flexion is an assessment of neurological function examining flexion or adduction of an animal's forelimbs after lifting the animal by its tail. The amount of flexion an animal exhibits is graded on a scale of 1 to 4, with complete adduction as 1 and the normal lack of adduction as 4. Of all papers reviewed, 4 assessed forelimb flexion using CCI and 3 using LFPI. Studies looking at deficits at 1-month post injury or later include 3 using CCI. No deficits in forelimb flexion in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in any paper.

3.2.7 Inclined Plane. The inclined plane test is a sensorimotor task where rodents climb an

inclined plane at varying degrees of difficulty. Animals are placed perpendicular to the downward slope of an inclined plane. The inclination when animals lose their footing is recorded. There are several additional parameters that can be measured, including first fall angle, threshold angle, total falls for inclined plane, and best, mean, and median latencies to finish the task. Anatomy that may be involved in the inclined plane task are cortical and sub-cortical regions, as well as white matter tracts, especially those found in the brainstem and cerebellum *(Hallam et al., 2004)*. Of all papers reviewed, 3 assessed the inclined plane test using LFPI, 3 using WDIA, and 2 using CCI. Studies looking at deficits at 1-month post injury or later include 1 using LFPI and 1 using WDIA. Deficits in the inclined plane task in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 1 paper using LFPI *(Hallam et al., 2004)*.

3.2.8 Limb Placing Function. Limb placing function is an assessment to detect tactile and proprioceptive deficits after injury. Animals are held in the experimenter's hand, taking care not to stimulate the face. Forward and sideways placing is tested by lightly touching the edge of a table with the lateral or dorsal edge of a paw, which stimulates the animal to place the paw down and push against the table edge. The limb of animal that is contralateral or ipsilateral to the injury is also gently pulled away from the body and the placement after release is recorded. Animals are given a score from zero, no limb placement, to two, immediate and complete placing for each limb. This motor task is used to detect damage in the frontal and parietal cortex area of the brain *(De Ryck et al., 1992)*. Only one LFPI injury paper used limb placing as a measure of motor deficit, but prior to 1-month post injury.

***3.2.9 Morris Water Maze, Swim Speed*.** Swim speed during the Morris Water Maze is one of many measurements that can be obtained from the water maze task; while swim speed is a motor based score, it is discussed with the other MWM tasks, which are more cognitive (see below).

***3.2.10 Open Field*.** Open field assessments are conducted in an open field, typically a square or round open topped box. Animals are allowed to freely explore the arena for five minutes and movements are recorded either by a visual tracking system or hand scored. Possible outcome measures recorded are time spent in the center of the area, total distance traveled, speed, and number of rearings *(Hoffman et al., 1994; Washington et al., 2012; Zhao et al., 2012)*. These behaviors can be affected by damage to the prefrontal cortex *(Hoffman et al., 1994)*. Of all papers reviewed, 9 assessed the open field performance using CCI, 4 using WDIA, 3 using LFPI, 2 using CHI, and 1 using Blast. Studies looking at deficits at 1-month post injury or later include 3 using LFPI and 2 using WDIA. Deficits in open field behavior in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 2 papers using WDIA *(O'Connor et al., 2003; Vink et al., 2003)*.

***3.2.11 Rotarod*.** Rotarod is a considered a sensitive measure for integrated motor abilities by requiring animals to maintain balance on a rotating rod *(Hamm et al., 1994)*. The amount of time spent balanced on the rotarod before falling off or gripping the rod itself and spinning around once is measured. Of all papers reviewed, 14 assessed rotarod using CCI, 6 using LFPI, 5 using CHI, 3 using MFPI, 2 using WDIA and 1 using Blast. Studies looking at

deficits at 1-month post injury or later include 6 using CCI, 2 using LFPI, 2 using WDIA and 2 using CHI. Deficits in rotarod performance in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 2 papers using CCI *(Brody et al., 2007; Shear et al., 2011b)*, 2 papers using WDIA *(O'Connor et al., 2003; Vink et al., 2003)*, 1 using LFPI *(Riess et al., 2001)* and 1 using repeated CHI *(Laurer et al., 2001)*.

3.2.12 Rotating Pole. The rotating pole task primarily tests motor coordination. The rotating pole is a wooden pole with platforms on each end. The animal is expected to cross from one platform to the other while the pole is rotating *(Philips et al., 2001)*. The pole rotations generally change directions three to 10 times per minute. Scores, from 0 to 6, are given based on an animal's ability to cross without falling, while also taking into account the number of foot slips. Of all papers reviewed, 4 assessed the rotating pole test using LFPI and 2 using CHI. Studies looking at deficits at 1-month post injury or later include 2 using LFPI and 2 using CHI. Deficits in the rotating pole task in TBI injured rodents compared to uninjured controls at 1-month post injury were reported in 2 papers using LFPI *(Hoover et al., 2004; Keck et al., 2007)* and 1 using repeated CHI *(Laurer et al., 2001)*.

3.2.13 Wire Grip Test. The wire grip test is performed by suspending a wire between two poles and allowing the animal to hang on the wire. Functional performance is assessed by grading the degree of attachment and movement of the animal. Typical scoring of this task is: 0 - fell from wire within 30 seconds; 1 - unilateral grasp of either upper or lower extremities; 2 - midline grasp of both upper and lower extremities but not tail; 3 - midline grasp of all extremities plus tail; 4 - movement along the wire after achieving a score of 3; 5 - climbing to and down one of the poles within 60 seconds **(Mannix et al., 2011)**. Of all

papers reviewed, 12 assessed the wire grip test using CCI and 2 using WDIA. None of these studies looked at deficits at 1-month post injury or later.

3.3 Sensory Assessments

3.3.1 Acoustic Startle Response. Acoustic startle response, ASR, and pre-pulse inhibition, PPI, are generally tested together. In standard ASR, a loud noise is presented, causing a flinching response from the rodent. While this test assesses auditory function, motor and emotional components are also involved in the flinching reflex. PPI is a more sensitive measurement of auditory function. When presenting a pre-pulse of lower decibels, it inhibits the response to a louder pulse (Washington et al., 2012). One paper using CCI and one paper using Blast utilized the acoustic startle task, but neither assessed startle response after 1-month post injury.

3.3.2 Gap Cross Test. Rats can be trained to jump across gaps in the dark and considerably larger gaps in the light for a food reward *(Jenkinson and Glickstein, 2000)*. In the dark they use their vibrissae at greater distances. Animals are typically trained to jump 2 cm gaps initially, then incrementally increasing the distance. Barrel field lesions and trimming of whiskers has been shown to decrease performance of gap crossing *(Jenkinson and Glickstein, 2000)*. Out of all the TBI papers reviewed, none used the gap cross test as a behavioral task.

3.3.3 Whisker Nuisance Task. Whisker nuisance task is a sensory task (Jenkinson and Glickstein, 2000). During this sensory task, whiskers are stimulated using a wooden

applicator for periods of five minutes for three different periods of time. Recordings of a subjects' responses are analyzed for posture, grooming, evading, whisker position, and general response to the applicator. Abnormal responses are scored by the experimenter. Of all the TBI papers reviewed, none use the whisker nuisance task.

3.4 Cognitive Assessments

One of the most common and debilitating features of TBI is alterations in cognition, including confusion, memory impairments, and deficits in executive function. There are a variety of tests in rodents that assess cognitive function. Deficits using these tasks could suggest that there is damage in hippocampus and other brain structures involved in learning and memory.

3.4.1 Barnes Maze. The Barnes Maze is a cognitive test of spatial learning and memory (Barnes, 1979). Rodents are placed on a circular board raised several feet from the ground. Holes lining the entire perimeter of the board allow the rodents to look through and explore what is below. One hole on the board contains a dark, escape compartment below. Rodents are trained over several days to learn the location of this compartment. Latency to find the compartment is used as a measurement of spatial learning. By moving the location of the box during training, the task can assess working memory. Of all papers reviewed, 4 assessed Barnes maze performance using CCI, 3 using LFPI, and 2 using WDIA. Two studies looking at deficits at 1-month post injury using WDIA. Deficits in Barnes maze performance in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 2 papers using WDIA (O'Connor et al., 2003; Vink et al., 2003).

3.4.2 Fear Conditioning. Fear conditioning is used as a measure of associative learning of a presented cue (often a tone or light) with a subsequently presented stimuli, most often a foot shock (Cahill, 2000). Training includes a single day of tone-shock pairings. Testing occurs the following day to the context or to the cue in a novel context. Animals having learned the association will freeze upon being placed into the context or to the cue. The hippocampus is involved, mostly in contextual fear conditioning, while the amygdala is involved in cued fear conditioning. Of all papers reviewed, 3 assessed contextual fear conditioning using using LFPI, 2 using CCI, and 2 using CHI. Studies looking at deficits at 1-month post injury or later include 1 using LFPI and 1 using CCI. Neither of these studies found deficits after 1 month. Of all papers reviewed 2 papers assessed cued fear conditioning using CCI and 1 using LFPI. Studies looking at deficits at 1-month post injury or later include 1 using CCI and 1 using LFPI. Neither of these studies found deficits after 1-month.

3.4.3 Morris Water Maze. In 1984, Morris developed the Morris Water Maze (MWM) task (Morris, 1984). The MWM is primarily a cognitive assessment of spatial learning and memory. Rodents are placed into a large tank filled with water to find a platform hidden below the surface of the water. They use distal cues in the testing room to find the platform (Morris, 1984; Vorhees and Williams, 2006). The animals are tested individually and placed into different starting positions of the tank (N, S, W, E, NW, SW, SE or NE). Spatial learning is evaluated with repeated trials to locate the hidden platform. Spatial learning refers to the process through which animals encode information about their environment to facilitate

navigation through space and recall the location of motivationally relevant stimuli. The animal must learn to use distal cues to navigate and find a hidden platform (Vorhees and Williams, 2006). Reference memory is evaluated subsequently in which the platform is removed and the rodent must search for where the platform used to be (Vorhees and Williams, 2006). Reference memory represents knowledge for aspects of a task that remains constant between trials. It is a long term memory (LTM), which can last for days, weeks, months, and years (Nadel and Hardt, 2011). This memory is evaluated at the end of learning, commonly by use of a probe trial 24 hours after the last acquisition day. Reversal learning of MWM is a modification of the original testing protocol in which the platform is relocated to another quadrant (commonly in the opposite quadrant) and administering another four testing trials per day for an additional five days, after already learning the initial location of the platform. This tests whether or not animals can extinguish their initial learning of the platform's position and acquire the new platform position (Vorhees and Williams, 2006). Another variation of MWM is to test for working memory, or short-term memory (STM). The term STM implies a repository, a place for the temporary storage of facts (Nadel and Hardt, 2011). To test this memory, the platform is relocated every day during several days of testing. Specific deficits in MWM testing have been found in animals with damage in hippocampus, striatum, basal forebrain, cerebellum and neocortex (prefrontal cortex, insular cortex, entorhinal, and perirhinal cortex) (D'Hooge and De Deyn, 2001).

While there are many variations to the MWM protocol and specialized subtests. The five MWM components most commonly used in the TBI field include: learning latency, probe trial, working memory, reversal, and swim speed. We discuss each separately below.

3.4.3.1 Morris Water Maze, Learning Latency (spatial learning). The most common outcome measure when using the MWM is the time it takes the subject to locate the hidden platform. Learning latency is often referred to as escape latency. Of all papers reviewed, 107 assessed learning latency using CCI, 97 using LFPI, 18 using MFPI, 19 using WDIA, 17 using CHI, and 3 using Blast. Studies looking at deficits at 1-month post injury or later include 29 using LFPI, 28 using CCI, 7 using CHI, 6 using WDIA and 2 using MFPI. Deficits to find the platform in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 22 papers using LFPI (Bao et al., 2012; Baranova et al., 2006; Browne et al., 2006; Browne et al., 2004; Carballosa Gonzalez et al., 2013; Giza et al., 2005; Hayward et al., 2010; Hoover et al., 2004; Keck et al., 2007; Lenzlinger et al., 2005; Marklund et al., 2007; Phillips et al., 1997; Pierce et al., 1998; Rau et al., 2012; Reid and Hamm, 2008; Riess et al., 2001; Sanders et al., 1999; Schmidt et al., 1999; Schutz et al., 2006; Sinson et al., 1997; Sun et al., 2007; Thompson et al., 2006), 21 papers from CCI (Byrnes et al., 2012; Chauhan and Gatto, 2010, 2011; Cheng et al., 2012; Dixon et al., 1994; Dixon et al., 1999; Fox and Faden, 1998; Hamm et al., 1992; Han et al., 2009; Hanell et al., 2010; Longhi et al., 2011; Longhi et al., 2008a; Longhi et al., 2008b; Longhi et al., 2004; Marklund et al., 2009; Meng et al., 2011; Shear et al., 2011b; Tomasevic et al., 2012; Xiong et al., 2011c; Xiong et al., 2012; Zhang et al., 2012), 4 using WDIA (Adelson et al., 2000; Maughan et al., 2000; Zohar et al., 2006; Zohar et al., 2003), 3 using CHI (Huh and Raghupathi, 2007; Maruichi et al., 2009; Raghupathi and Huh, 2007), 2 using repeated LFPI (Shultz et al., 2012; Shultz et al., 2013), 1 paper MFPI (Hamm et al., 1995), 1 using repeated WDIA (Meehan et al., 2012), and 1 using repeated CHI (Uryu et al., 2002).

3.4.3.2 Morris Water Maze, Probe (reference memory). In the probe test, the platform is removed and the rodent is allowed to search for the platform. Most commonly, time spent in the target quadrant, or in the platform zone, is measured. Of all papers reviewed, 43 assess the probe test using LFPI, 33 using CCI, 9 using WDIA, 7 using CHI, 2 using MFPI, and 1 using Blast. Studies looking at deficits at 1-month post injury or later include 13 using LFPI, 11 using CCI, 4 using WDIA, 4 using CHI, and 2 using MFPI. Deficits in probe trial performance in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 8 using LFPI (Giza et al., 2005; Griesbach et al., 2004; Hayward et al., 2010; Immonen et al., 2009; Marklund et al., 2007; Rau et al., 2012; Schmidt et al., 1999; Thompson et al., 2006), 6 papers using CCI (Chauhan and Gatto, 2010, 2011; Dixon et al., 1999; Han et al., 2009; Marklund et al., 2009; Xiong et al., 2010), 3 using WDIA (Adelson et al., 2000; Maughan et al., 2000; Zohar et al., 2003), 2 papers using MFPI (Yamaki et al., 1997; Yamaki et al., 1998), and 2 using CHI (Huh and Raghupathi, 2007; Raghupathi and Huh, 2007).

3.4.3.3 Morris Water Maze, Working Memory (short term memory). Of all papers reviewed, 5 assessed the working memory task of MWM using CCI, 2 using LFPI, and 1 using CHI. Only 1 study looked at deficits at 1-month post injury or later, which did see deficits using a LFPI (Carballosa Gonzalez et al., 2013).

3.4.3.4 Morris Water Maze, Reversal Learning. Of all papers reviewed, 7 assessed reversal learning test using LFPI and 1 using CCI. Studies looking at deficits at 1-month post injury or later include 6 using LFPI. Deficits in reversal learning in TBI injured rodents

compared to uninjured controls at 1-month post injury were observed in 2 papers using repeated LFPI (Shultz et al., 2012; Shultz et al., 2013), and 1 paper using LFPI (Thompson et al., 2006).

3.4.3.5 Morris Water Maze, Swim Speed (motor). The Morris water maze swim speed is one of many measurements that can be obtained from the water maze task. Swim speed is the distance covered in the water maze over how long it took the animal to find the hidden platform. This is an important measure for motor function (not cognition) because if there is no difference in swim distance but a difference in time to reach platform, it could suggest that there is a motor deficit. Of all papers reviewed, 43 assessed MWM swim speed using LFPI, 31 using CCI, 10 using WDIA, 8 using CHI, 2 using MFPI, and 2 using Blast. Studies looking at deficits at 1-month post injury or later include 14 using LFPI, 7 using CCI, 4 using CHI, 3 using WDIA, and 1 using MFPI. Deficits in MWM swim speed in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 2 papers using LFPI *(Giza et al., 2005; Hayward et al., 2010)* and 1 using repeated LFPI *(Shultz et al., 2012)*.

3.4.3.6 Morris Water Maze, Other. There are several other variants or sub-tasks which can be assessed during the MWM, including computation of a "Memory Score", measurement of the total distance traveled, using a visible platform rather than a submerged one, calculating the percentage to time animals spend in the "correct" quadrant, or counting the number of platform crossings. However, these alternative measures are not commonly used or as uniform as the five MWM tests reviewed above.

3.4.4 Novel Object Recognition. Novel object recognition (NOR) evaluates non-spatial

hippocampal mediated memory. Animals are allowed to explore two identical objects in a chamber for a predetermined amount of time and then, after an inter-trial interval, are placed back into the chamber with one familiar object and one novel object. The time spent with the novel and familiar object are recorded. Control animals will spend more time exploring novel object. Brain structures implicated in this task are the dentate gyrus, CA1, and CA3 regions of hippocampus (Zhao et al., 2012) and perirhinal cortex (Ennaceur et al., 1996). Of all papers reviewed, 3 assessed NOR performance using CCI and 1 using WDIA. Studies looking at deficits at 1-month post injury or later include 1 using CCI and 1 using WDIA. Deficits in NOR performance in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 1 paper using WDIA (Edut et al., 2011).

3.4.5 Novel Place Recognition. Novel place recognition (NPR) evaluates spatial hippocampal mediated memory. Animals are allowed to explore two identical objects in a chamber for a predetermined amount of time and then, after an inter-trial interval, are placed back into the chamber with two identical objects but one has been moved to a new location. Control animals will spend more time exploring the object in the new location. In this review no papers used this task as a measure for TBI deficits.

3.4.6 Passive Avoidance. The passive avoidance task is used to assess simple non-spatial learning. Animals are placed into a lit chamber and a door opens to an adjoining dark chamber. Once the animal crosses to the dark chamber, the door closes and the animal receives a mild foot shock. The animals are tested the following day and the latency to cross over to the dark chamber is recorded. Structures thought to be involved in this behavior

are the nucleus basalis, amygdala, nucleus accumbens, and to an extent, the frontal cortex (Hamm et al., 1993). Of all papers reviewed, 3 assessed passive avoidance performance using WDIA, 1 using CCI, 1 using MFPI, and 1 using Blast. Studies looking at deficits at 1-month post injury or later include 3 using WDIA and 1 using CCI. Deficits in passive avoidance performance in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 3 papers using WDIA (Baratz et al., 2010; Milman et al., 2005; Milman et al., 2008) and 1 using CCI (Zhao et al., 2012).

3.4.7 Radial Arm Maze. Radial arm maze is a measure of spatial learning and memory. Food restricted rodents navigate an 8- or 12-armed maze using spatial cues on the walls of the testing room, exploring each arm for food reinforcements. After several days to weeks of training, assessments can be made to see how well the rodent systematically goes down each arm looking for reinforcements. Performance is assessed by counting number of errors, or times a rodent goes down an arm they have already gone down. Brain structures that may be involved in this task are the hippocampus and the nucleus accumbens (Floresco et al., 1997). Of all papers reviewed, 3 assessed radial arm maze performance using LFPI and 2 using WDIA. Studies looking at deficits at 1-month post injury or later include 2 using LFPI and 1 using WDIA. Deficits in radial arm maze performance in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 1 paper using LFPI (Hallam et al., 2004) and 1 using WDIA (Hallam et al., 2004).

3.4.8 Y-Maze. The Y-maze task is a hippocampal based task that measures working memory (Malleret et al., 1999; Tanaka et al., 2009). The apparatus of the Y-maze is

composed of three arms of equal length with a central entry zone. This task can either be assessed as a single trial (Chauhan and Gatto, 2010; Malleret et al., 1999; Tanaka et al., 2009) or a two trial (Baratz et al., 2010) task. In the single trial task, an animal is allowed to explore for five minutes and the time spent in each arm, number of arm entries, and the order of arm entries are recorded, where a normal animal should explore all arms equally due to spontaneous exploration. In the two trial Y-maze one arm is blocked during the first trial and then all arms are open during the second trial. The time spent and entries into each arm are recorded. Of all papers reviewed, 4 assessed Y-maze performance using CCI, 2 using WDIA, and 1 using CHI. Studies looking at deficits at 1-month post injury or later include 2 using CCI, 2 using WDIA, and 1 using CHI. Deficits in Y-maze performance in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 1 paper using CCI (Chauhan and Gatto, 2010), 1 paper using WDIA (Lloyd et al., 2008), and 1 using CHI (Baratz et al., 2010).

3.5 Emotional Assessments

***3.5.1 Active Avoidance*.** The active avoidance task is a fear-motivated associative avoidance test that uses an electrical shock as negative reinforcement. In this task the animal has to learn to move from the dark chamber to the light chamber when a cue is presented. The number of crossings into the light chamber after the cue presentation, freezing, and the latency for the animal to cross to the other non-shocking chamber are recorded. Structures thought to be involved in this behavior are the prefrontal cortex and the amygdala (Moscarello and Ledoux, 2013). Of all papers reviewed, 2 assessed active avoidance performance using Blast and 1 using WDIA. No papers assessed active avoidance

at 1-month post injury or later.

3.5.2 Elevated Plus Maze. The elevated plus maze is a task that assesses anxiety. The maze itself is "+ shaped", with two open arms and two closed arms. Rodents have a preference for dark places, therefore behavior differences are assessed by the time spent in the closed arms of the maze relative to the open arms. Animals are placed in the center of the maze facing an open arm and tracked using motion detection software. Number of entries, time spent in each arm (open vs closed), and distance traveled are typically measured. Of all papers reviewed, 5 assessed elevated plus maze performance using LFPI, 4 using CCI, and 2 using WDIA models. Studies looking at deficits at 1-month post injury or later include 5 using LFPI and 2 using WDIA. Differences in anxiety between TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 2 papers using repeated LFPI (Shultz et al., 2012; Shultz et al., 2013), 1 paper using LFPI (Bao et al., 2012) and 1 using WDIA (Baratz et al., 2010).

3.5.3 Forced Swim Task. Depression-like behavior can be assessed using the forced swim task (Washington et al., 2012). This task typically uses a large beaker filled with water for the animals to swim in. There is no escape and trials last <10 min. Depression-like behavior is observed when the animal floats and is immobile. Of all papers reviewed, 3 assessed forced swim task performance using LFPI, 2 using WDIA, and 1 using CCI. Studies looking at deficits at 1-month post injury or later include 3 using LFPI and 2 using WDIA. Deficits in forced swim task performance in TBI injured rodents compared to uninjured controls at 1-month post injury were observed in 2 papers using WDIA (Milman et al., 2005; Milman et

al., 2008) and 1 paper using LFPI (Shultz et al., 2012).

3.5.4 Tail Suspension Test. Depression-like behavior is assessed using the tail suspension test (Steru et al., 1985). Rodents are hung from their tails in an isolation chamber for a given time period. Depression-like behavior is seen when the animals cease to right themselves. Immobility can be measured using tracking software or by scoring immobility by hand. More depressive behavior is observed when an animal is more immobile than the control group (Washington et al., 2012). One paper using CCI used the tail suspension test (Washington et al., 2012), but not after 1-month post injury.

Analysis

4.1 Numerical Summary. In total, 314 unique papers were reviewed, of which 101 (32%) conducted a functional test at or after 1-month post-injury, where 43 used CCI, 41 used fluid percussion, 12 used weight drop, and 6 used CHI (one used both LFPI and WDIA) (**Figure 2.3A**). Of these 101 papers assessing function at ≥ 1 month post-TBI, 88 papers (87%) demonstrated deficits in one or more a functional outcomes (35 CCI, 35 FPI, 12 WDIA, and 6 CHI [one used both LFPI and WDIA]) (**Figure 2.3B**). Since we have suggested that even a 1-month assessment may not be sufficient time to allow for integration of human NSCs with the host, we also evaluated the number of papers which assessed function at 2-, 3-, 6-, and 12-months post-TBI. Combining papers using any injury model and any functional assessment demonstrating sustained deficits, we found 27 papers (9%), 17 papers (5%), 8 papers (2.5%) and 4 papers (1%) at these respectively longer time-points post-TBI exhibited deficits on one or more tasks (**See Figure 2.3B**).

The most commonly used functional assessment was learning latency on the Morris Water Maze (261 papers), a measure of cognitive function. The next most common functional assessments were MWM swim speed (96 papers), MWM, Probe test (95 papers), Beam Walking (73 papers), Balance Beam (64 papers), Global Neurological Assessment (45 papers), and Rotarod (31 papers) (**See Table 2.1**).

Less than 33% of papers reviewed performed functional assessments at 1 month post-injury or later (101 of 314). The most common assessments used at 1-month or more after injury were MWM, Learning Latency (72 papers), MWM, Probe Test (34 papers), MWM, Swim Speed (29 papers), Global Neurological Assessment (20 papers), Beam Walking (17 papers), Rotarod (12 papers), Foot Fault (9 papers), Elevated Plus Maze (7 papers), and MWM, Reversal (6 papers).

The ten most common functional tasks used in two or more studies where deficits were observed at 1-month or greater post injury (demonstrating replication of a sustained deficit), ranked by number of papers demonstrating a deficit include (where percentage indicates how many showed deficits): MWM, learning latency (76%), MWM, probe (62%), Global Neurological Assessment (75%), Beam Walking (71%), Foot Fault (89%), Rotarod (50%), MWM, % in correct quadrant (100%), Elevated Plus Maze (57%), Passive Avoidance (100%), and Water Maze, swim speed (10%) (**Table 2.2**).

4.2 Survival and proliferation of human stem cells in rodent models of TBI. In section 1.5, we proposed two criteria necessary to fully evaluate therapeutic success of human stem cell transplantation strategies in preclinical animal studies of TBI. First and foremost, for the safety/efficacy of transplanted cells to be appropriately evaluated, survival and/or

proliferation potential of transplanted cells must be maximized for the duration of the study (Anderson et al., 2011). We define "survival" in relation to the initial dose and without respect to whether nearly all of the initial dose died off and only a small subset of the initial cells proliferated extensively, or whether all cells survived the transplant and only double once – as very few studies actually investigate the dynamics of such proliferation. In this context, if 50,000 cells were transplanted initially and upon sacrifice, 100,000 human cells were quantified stereologically, this would be reported as 200% cell survival.

Second, to evaluate therapeutic potential, there needs to be sufficient time for engrafted cell integration to effect functional outcome. Long-term cell survival is particularly difficult to achieve in pre-clinical *in vivo* studies, and human NSCs require more time to differentiate than rodent NSCs. Furthermore, both xenogenic and allogeneic transplantation require robust attenuation of immunorejection mechanisms in order to achieve good cell engraftment, either in the form of pharmacological immunosuppression of T-cells, or constitutive suppression of T-cells by virtue of immunodeficient rodents. In this regard, it is not surprising that the highest reported human cell survival in a rodent TBI model is only 21% of the initial transplantation dose (Nichols et al., 2013), as this study used immunosufficient animals and no immunosuppression. The majority of human cell transplant studies into TBI models report cell survival under 10% or do not report on cell survival quantitatively at all. Fully 26 of 32 papers (81%) transplanting a human cell population in a rodent TBI model do not report on cell survival (**Table 2.3**). Similar low xenografted cell survival rates in the CNS (averaging 17% of the initial dose) have been reported in a recent review by Anderson *et al.* across multiple different traumatic injury

types in which human cells were transplanted into immunocompetent animal models receiving immunosuppressive drug treatment (Anderson et al., 2011).

Pharmacological, but combinatorial, immunosuppression may be an alternative approach to the use of immunodeficient animals. In some cases, for example, in immunocompetent C57BL/6 mice transplanted with hNSCs following spinal cord injury, FK506 immunosuppression alone resulted in no survival of transplanted hNSCs, while a combination of FK506 and anti-CD4 was required to achieve significant human cell survival (45% of initial dose) at 10-weeks post transplantation (Sontag, Anderson, & Cummings, *unpublished data*). Despite the lower level of overall engraftment in comparison with immunodeficient animals in this study, animals receiving combinatorial immunosuppression exhibited sufficient cell survival to enable demonstration of functional locomotor recovery following SCI. However, assessment of safety in the context of a cell population with proliferative potential is sub-optimal under these conditions, as we have discussed previously (Anderson et al., 2011), and should be carefully evaluated. Conversely, when immunodeficient animal models are used in traumatic CNS studies, human cell survival is dramatically increased (averaging 263%), enabling a more robust evaluation of safety and efficacy (Anderson et al., 2011).

4.3 Sufficient duration to allow for evaluation of functional outcome. As noted above, the second criteria for evaluating therapeutic success is sufficient time for transplanted cells to proliferate, migrate, and either integrate with the host TBI brain or produce trophic support. Thus, study length of human stem cell transplantation experiments in TBI models must also be considered. Functional integration of transplanted stem cells as neurons has

been demonstrated via electrophysiological measurement of neuronal firing between transplanted and host cells (although not in a TBI model) (Weick et al., 2011). Integration of hNSC or oligodendrocyte precursors via electron-microscopic evidence of remyelination of host axons by transplanted cells in spinal cord models (Cummings et al., 2005; Wang et al., 2013) has also been shown. In contrast to functional integration with the host, promotion of recovery due to neurotrophic support of spared host tissue is also a possibility. Transplanted stem cells have been shown to secrete both pro-survival and immune-modulatory factors *in vivo* (Andres et al., 2011; Horie et al., 2011; Yasuhara et al., 2006), which could lead to an increase in the amount of spared tissue or an increase in the health of the spared tissue, thus enabling greater neural network efficiency.

Depending on the type of human stem cell transplanted and the transplant location, the time-frame for integration and terminal differentiation may range from several months in the case of human fetal-derived neural stem cell transplanted spinal cord (Hooshmand et al., 2009), to 6 months in the case of human embryonic-derived neural stem cell transplanted brain studies (Nasonkin et al., 2009). Accordingly, in order to adequately assess the potential of a given donor cell population to yield repair, long-term studies (2-months or more) are required. Despite the desirability of long-term studies, very few (16%) of the human stem cell transplantation studies in rodent TBI models reviewed here (5 of 32 papers flagged in red in **Table 2.3**) have *exceeded* 6 weeks duration post-transplant.

Of the 32 publications using a human stem cell population in a rodent model of TBI, 27 looked 6 weeks or less post-transplant. Of these, 10 did not perform functional assessments. Of the 17 studies that did perform some functional assessment, two reported

no difference between controls and transplanted animals while 15 reported improvements on MWM (n=10), mNSS (n=8), rotarod (n=4), beam walk (n=1), and/or limb function (n=1) (the total does not equal 15 as some groups used more than one task). Only 2 of the 15 with positive functional outcomes also reported on human cell survival; Mahmood *et al.* (Mahmood et al., 2003) with 0.5% of surviving hMSCs at 4-weeks post-transplant and Hong *et al* (Hong et al., 2011) with 6.8% surviving hUCSCs at 3-weeks post-transplant. Accordingly, the lack of surviving human cells and the short duration of the majority of TBI experiments make interpretation of mechanism and capacity to exert sustained functional effects difficult. In particular, the short time course of survival and functional assessment suggest that while the positive effects seen could be due to immune-modulation and/or trophic mechanisms, cellular integration is unlikely as a mechanism (as discussed above).

Of the 5 studies that conducted evaluations for 6 weeks or greater, the results have been mixed in terms of human cell survival and/or functional recovery. Two studies reported partial data on long-term cell survival, but did not examine functional outcomes after such long-term engraftment. Wennersten *et al.* (Wennersten et al., 2006) noted human cell survival at both 6 weeks and 6 months post-transplant of human fetal NSCs, but reported only 0.2% human cell survival at 6 weeks (determined by counting human nuclei in 4 sequential sections) in cyclosporin treated Sprague-Dawley rats. Quantification of human cells at 6 months was not performed. Approximately 5% of human cells co-localized with neuronal or astrocytic markers; no oligodendrocytes were detected. The authors noted that the hippocampus appeared more conducive to neuronal differentiation than the cortex. No functional tasks were evaluated at any time-point in Wennersten's study. Nichols *et al.* (Nichols et al., 2013) transplanted retinoic acid (RA) primed CD133+ABCG2+CXCR4+

human MSCs into the lateral ventricle of male Sprague-Dawley rats 24 hours after FPI; no immunosuppression was used. The transplanted cells were reported to integrate within the host and differentiate into cells expressing both immature and mature neural lineage markers. The cells, however, were pre-labeled with a fluorescein dye (CFSE) prior to transplantation and not detected with human-specific markers post-mortem, making positive identification difficult and co-localization with neural lineage markers uncertain. Further, the methods section indicated that 500,000 cells were transplanted while figure 9A shows 100,000 cells transplanted with ~21,000 surviving in TBI rats (21%). Finally, function on the MWM was only examined 11-15 days post transplant, not long-term, where significance relative to uninjured controls was observed for "primed" hMSCs but not unprimed hMSCs (Nichols et al., 2013).

Additionally, three studies reported on both engraftment/survival and long-term functional assessments. Zhang *et al.* (Zhang et al., 2005) transplanted human NT2N neurons into a FPI model of TBI in cyclosporine treated Sprague-Dawley rats and reported human cell survival at 12 weeks post-transplant and the presence of human synaptophysin positive structures. However no human cell quantification was performed and there were no functional differences in cognition or motor performance 12 weeks post-transplant between NT2N or human fibroblast transplanted and vehicle injected controls. Tasks assessed included a composite neuroscore, beam balance, rotating pole, and adhesive tape removal. Skardelly *et al.* (Skardelly et al., 2011) transplanted human fetal derived neural progenitor cells (hfNPCs) via local or systemic injection into Sprague-Dawley rats 24 hours after a severe CCI (2.5mm depth, 4m/s impact). Cyclosporine and prednisolone were used for immunosuppression. Functional motor improvements (by blinded observer) on both

rotarod and mNSS were observed, but only for systemically administered cells. PKH-26 pre-labeled human cells were detected 12-weeks post transplant, but no human cell quantification was performed. Similarly, Mahmood *et al.* reported motor improvement (mNSS) at 12 weeks post-administration of human marrow stromal cells into Wistar rats. Two, four or eight million cells were administered systemically via tail vein 24 hours post-CCI induced TBI (2.5mm depth, 4m/s impact). All three doses of hMSCs resulted in significant improvements on mNSS, which was assessed blind to treatment. Non-stereological human cell quantification was performed in three sections per animal and showed that human cells survived 12 weeks post injection; none were NeuN or GFAP positive.

While two 12-week duration studies report functional improvements after human stem cell treatment, the absence of stereological quantification of surviving human cells makes it difficult to associate functional gains with human cells when the extent of human cell survival and integration after transplantation are unclear. Given the time, expense, and use of animals, we must be rigorous when designing human stem cell transplantation studies to enable sufficient cell survival and allow adequate time for terminal cell differentiation, and we must also be rigorous in our quantification and analysis so that the potential for misinterpretation, whether beneficial or detrimental, is minimized. Taken together, these studies highlight the potential for both long-term survival of transplanted human cell populations as well as the ability for transplanted cells to possibly integrate within the host and lead to functional improvements post-TBI. However, these studies also suggest that to improve the interpretability of future CNS injury/human cell transplantation studies, researchers must 1) accurately report engraftment (number of

transplanted animals with any surviving cells) and quantify cell survival (absolute number of cells per animal) so that comparisons of different treatments can be made, and 2) use immunodeficient animal strains, or combinatorial immunosuppression protocols whenever possible to maximize cell survival and ensure that safety/efficacy can be adequately evaluated.

Executive Summary - Recommendations

Despite the fact that distinct pre-clinical models of focal and diffuse TBI exist, imaging data in the majority of clinical TBI cases shows evidence characteristic of a mixed etiology. Further, the pathophysiological features of focal TBI (prominent neuronal loss), and diffuse TBI (prominent axonal pathology), may both benefit from neuroprotective and/or cell replacement strategies. As a result, and as many researchers have suggested, it may be likely that 1) combinatorial therapeutic approaches will ultimately be necessary for success in the clinical setting, and 2) the selection of outcome measures in pre-clinical experiments must be sufficiently characterized and match clinical features of TBI that are planned for assessment in a clinical trial.

Because human stem cells take longer to mature and differentiate than rodent stem cells, we recommend a minimum of 2-months *in vivo* incubation to allow for differentiation and integration of the human cells with the host. Of course, allowing the survival of human NSCs for greater than 2-months post-transplant would clearly be ideal, but there are many factors that limit such studies: financial resources, personnel, animal lifespan, and housing are all limited. Nonetheless, it seems clear that this minimal timeframe will be necessary for adequate assessment of efficacy and mechanisms of action. As stated in section 1.5,

there are three primary mechanisms that could underlie the efficacy of a human stem cell transplant: (A) immune-modulation, (B) trophic factors, and/or (C) functional integration. If, in fact, Mechanism C (differentiation and integration), and not Mechanism B (trophic factor secretion), underlies the activity of a given population of transplanted cells, then 2-months would be the minimum time needed to demonstrate *in vivo* efficacy. This 2-month period is based on extensive preclinical data in rodent models of SCI (where there are more human stem cell studies to date than the TBI field). Studies which show rapid improvements post transplant (≤4 weeks) with human cells have been shown to correlate with trophic factor generation (Sharp et al., 2010); in contrast, studies with integration as an underlying mechanism have not shown evidence of improvements until 6-8 weeks post-transplantation (Cummings et al., 2005; Hooshmand et al., 2009; Salazar et al., 2010). Critically, while trophic effects might be observed at earlier time-points, it will also be important to demonstrate the sustainability of such an effect, supporting the importance of an extended period of in vivo observation. Furthermore, regulatory bodies will likely require at least 6 months survival post-transplantation for assessment of toxicology/safety, particularly in the context of assessing cell abnormalities.

Related to these considerations and a particular issue in the context of cell-based therapeutic candidates, a variety of data in animal models has suggested that direct pharmacological modulation of the immune response after TBI can exert neuroprotective effects; for example, administration of clinical immunosuppressants such as cyclosporin A (Albensi et al., 2000; Sullivan et al., 2000). Conversely, it is increasingly clear that traumatic CNS injury, including both TBI and SCI, can result in peripheral immunodepression in both animal models and in the clinical setting (Esmaeili et al., 2012; Lu et al., 2009; Zhao et al.,

2011), suggesting the potential for increased susceptibility to post-injury infectious complications and that the inflammatory environment post-TBI may be a double edge sword (Morganti-Kossmann et al., 2002). It will be particularly important to consider immunosuppressive regimens and the timing of cell transplantation paradigms in this complex context.

In summary, we recommend two principal criteria for testing the safety and efficacy of human donor cell populations in pre-clinical TBI models. First, a model in which sufficient engraftment of donor human cells can be achieved to reliably test safety and efficacy across a xenotransplantation barrier will be necessary; this requirement will likely require immunodeficient animal models, or dramatically improved methods of achieving adequate immunosuppression. Second, a model in which the potential functional impact, either in terms of improvement (efficacy) or determent (safety) can be reliably measured for an extended period of time post-transplantation (≥ 2-months).

In this context, several key points are apparent from the TBI literature.

Most TBI preclinical research is short-term

- Most research in the TBI field has focused on short-term outcomes. The majority (68%) of papers reviewed did not evaluate functional outcomes past 1-month post TBI (213 of 314 papers).
- 90% of TBI papers reviewed did not make a functional assessment 2 or more months following injury (282 of 314); whereas 32 papers have looked long term (≥ 2-months).
- Most (84%) TBI papers that looked long-term (27 of 32) demonstrated functional deficits at two months post-injury, the minimum time during which one could

reasonably expect cell integration to exert an effect as a recovery of function mechanism.

Studies of Longer Duration are Needed

- Sustained and significant functional deficits of at least 2-months duration post-TBI are necessary before safety and efficacy can be demonstrated using any transplanted human stem cell population. Deficits can be cognitive, emotional, and/or motor in origin.
- Two months post-transplantation is also the minimum amount of time needed to allow for proliferation, migration, differentiation and/or integration of human neural stem cells into the injured host.

No immunodeficient animal models of TBI exist

- No pre-clinical TBI models from the review period employed immunodeficient animal models nor characterized TBI in an immunodeficient pre-clinical model that would enable maximal theoretical cell engraftment for assessment of safety and efficacy (Anderson et al., 2011).
- TBI cell-therapy studies do not routinely use combinatorial immunosuppression.

Human cell quantification is rare - efficacy even rarer

- Only 19% of TBI transplantation papers from the review period where human cell transplants were administered (6 of 32 papers) quantified human cell engraftment/survival. 81% did not report on cell survival.
- Human cell survival, when reported, was low, ranging from 0.03% of the initial dose to a maximum of 21% (average 4.7%).

- Only 6% of TBI transplantation papers using a human cell population reported functional efficacy at greater than 2-months post-transplantation (2 of 32 papers).

Future Perspective:

In the future, it is likely that better animal models of TBI will be developed, including models of repeated TBI, used of humanized immune system animals, and/or the characterization of immunodeficient animals models to better allow for assessment of human donor cell populations. Preclinical testing will also expand to more long-term studies and studies which utilize multiple outcome measures that better overlap functional outcome assessment in the human population. We also predict that advancements in understanding and testing mechanisms of action will reveal that different cell populations exert effects via distinct but overlapping actions on the injured host. Finally, we expect that increased application of RIGOR and SAINT standards will strengthen the translatability of preclinical animal testing to support clinical trials in TBI.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, patents received or pending, or royalties. AJA is PI on a DoD funded TBI grant, BJC is PI on a CIRM funded TBI grant (see support section below). No writing assistance was utilized in the production of this manuscript.

Support and Acknowledgments.

The authors would like to thank Aparna Sudarshan for assistance reviewing a portion of papers. This work was supported by a CIRM ETA II grant (TR2-01767) to BJC, a DoD DMRDP grant (DM103001) to AJA, a CIRM Research Training Program II grant (TG2-01152) to DLH, and CIRM Stem Cell Research Biotechnology Training Program at CSULB grant to Lisa Klig (TB1-01182), which supported GAL.

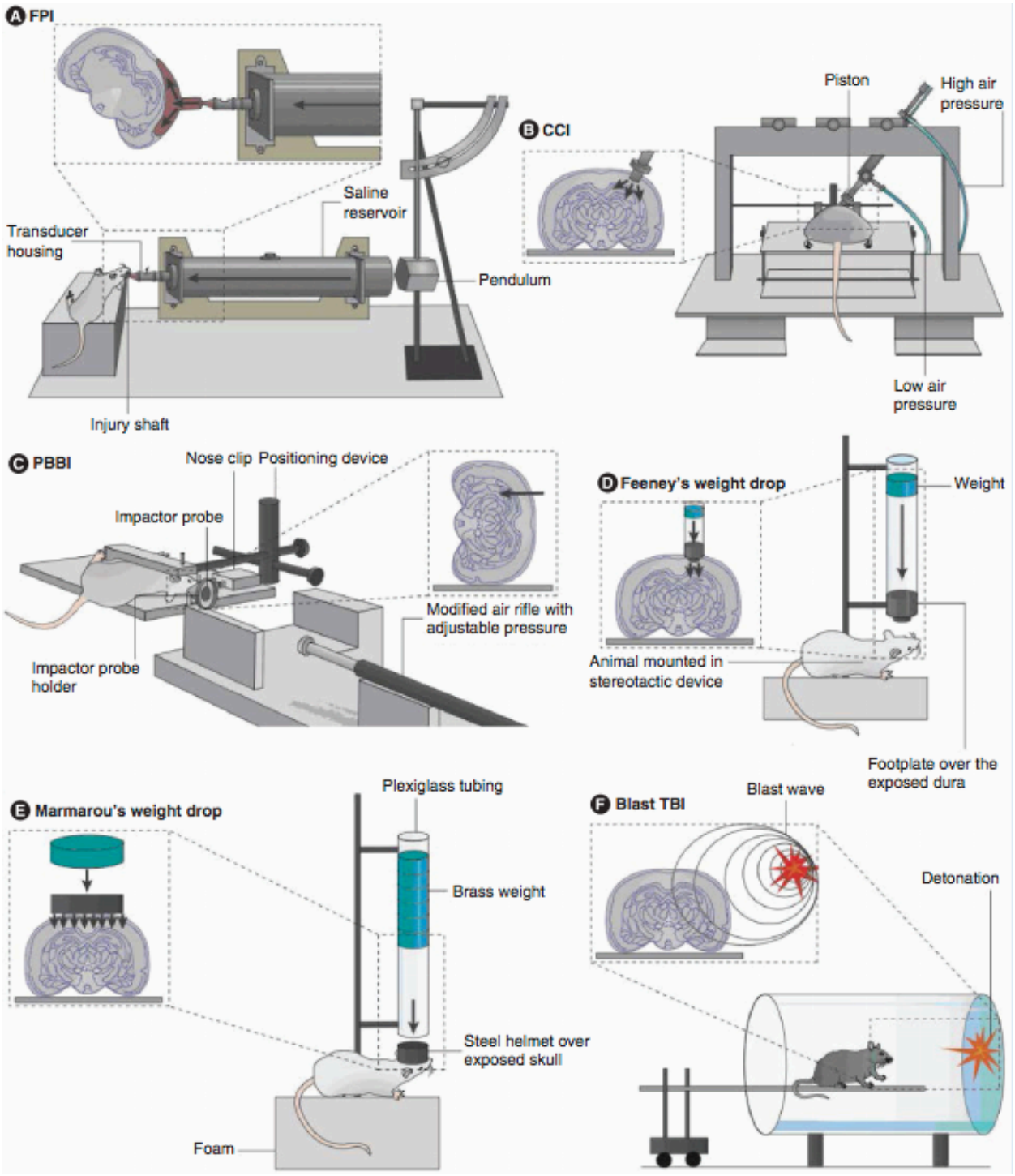

Figure 2.1. There are 5 main models of TBI in rodents. (**A**) Fluid Percussion Injury (FPI) (**B**) Controlled Cortical Impact (CCI) (**C**) Penetrating Ballistic-Like Brain Injury (PBBI), (**D&E**) Weight Drop Injuries (closed head or open), and (**F**) Blast injury. Illustration taken with permission from Xiong *et al.* 2013.

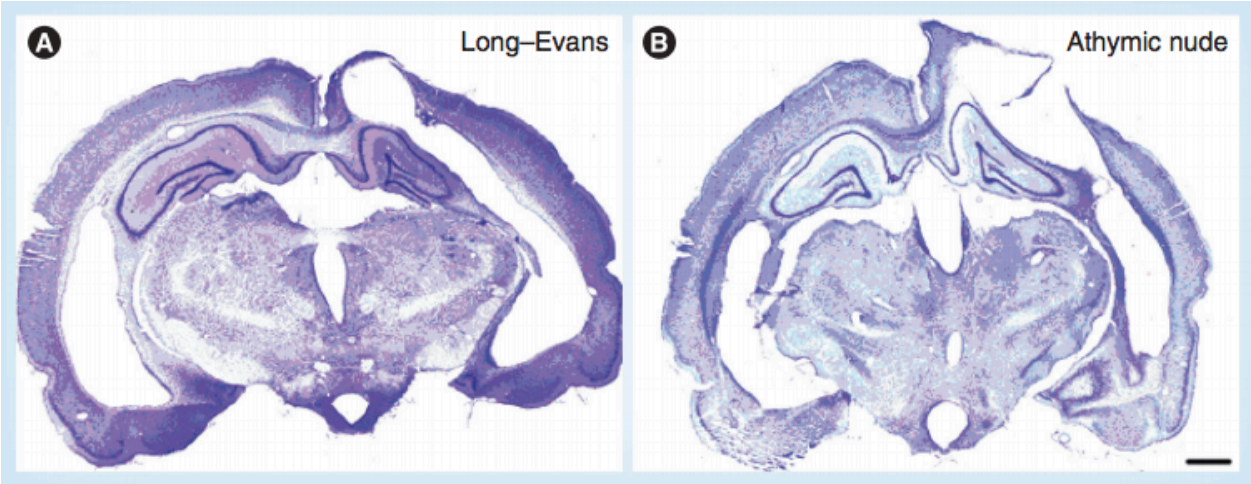

Figure 2.2. Nissl-stained rat brain sections of traumatic brain injuries induced by controlled cortical impact (CCI). (**A**) Long-Evans and (**B**) Athymic Nude rats display similar pathologies of cortical tissue loss and hippocampal damage at 22 weeks post-injury following a 2.00 mm deep, 3.5 m/s impact using a TBI-0310 traumatic brain injury device (Precision Systems & Instrumentation). Scale bar = 1 mm.

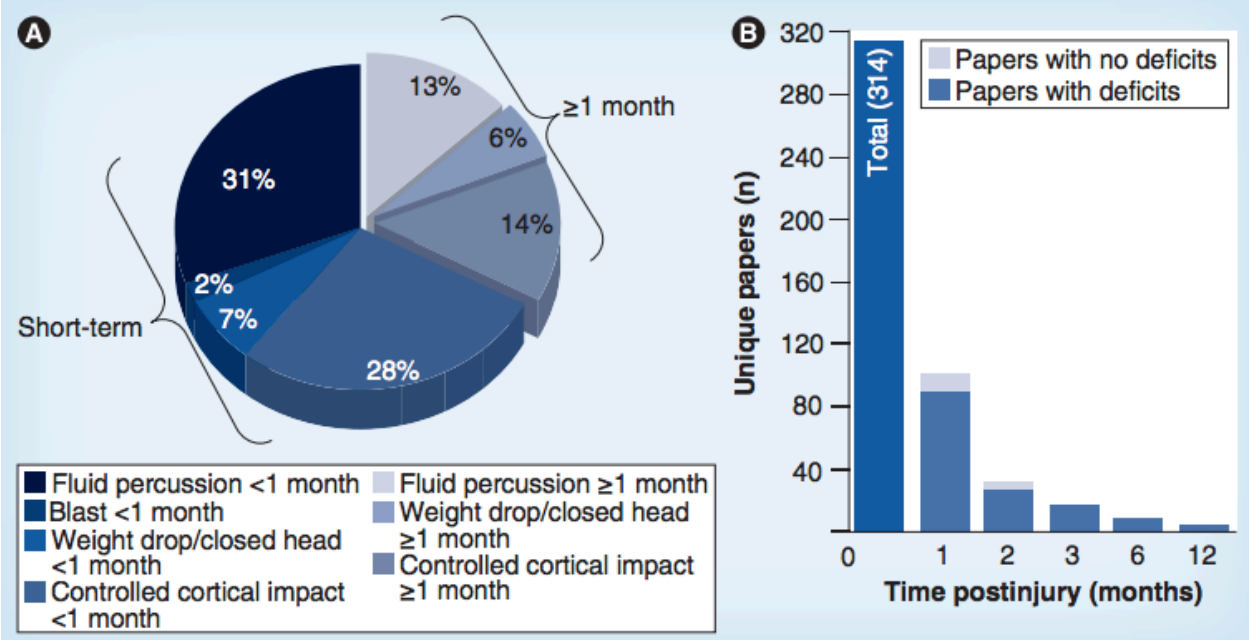

Figure 2.3. Comparison of the percentage of all rodent TBI papers by model which assessed functional outcomes short-term or long term post TBI. (A) 314 total papers were reviewed that used either FPI, WDIA/CHI, CCI or Blast models. Only 33% of the reviewed papers assessed functional outcomes at 1 month or more, the majority of these (41) were in FPI or (43) CCI models. **(B)** An analysis of papers reporting long-term deficits in functional outcomes (red bars) versus papers reporting no deficits (blue bars) at 1-, 2-, 3-, 6-, and 12-months post TBI. Few papers assess long-term deficits in TBI models. The green bar represents all papers.

Functional Assessment	Total Papers Assessing Task	Papers Assessing Task at ≥ 1 month	Papers with Deficits in Task at ≥ 1 month	% of Papers with Deficits in Task at ≥ 1 month
Water Maze, Learning Latency (LL)	261	72	55	76%
Water Maze, Probe (P)	95	34	21	62%
Global Neurological Assessment	45	20	15	75%
Beam Walking	73	17	12	71%
Foot Fault	20	9	8	89%
Rotarod	31	12	6	50%
Water Maze, % in Correct Quadrant	7	6	6	100%
Elevated Plus Maze	11	7	4	57%
Passive Avoidance	6	4	4	100%
Water Maze, Swim Speed (SS)	96	29	3	10%
Water Maze, Reversal (R)	8	6	3	50%
Y Maze	7	5	3	60%
Forced Swim Test	6	5	3	60%
Rotating Pole	6	4	3	75%
Water Maze, Visible Platform	12	3	3	100%
Open Field	19	5	2	40%
Bilateral Tactile Adhesive Removal Test	8	5	2	40%
Water Maze, Distance (D)	31	3	2	67%
Radial-Arm Maze	5	3	2	67%
Balance Beam	64	2	2	100%
Barnes Maze	9	2	2	100%
Vibrissae-Forelimb Placing Test	4	2	2	100%
Cylinder	3	2	2	100%
Inclined Plane Test	8	2	1	50%
Novel Object Recognition	4	2	1	50%
Water Maze, Memory Score (MS)	30	1	1	100%
Water Maze, Working Memory (WM)	8	1	1	100%
Water T-Maze	2	1	1	100%
Dry Maze Test	1	1	1	100%
Forelimb Flexion	7	3	0	0%
Fear Conditioning, Contextual	7	2	0	0%
Fear Conditioning, Cued	3	2	0	0%
Social Interaction	2	2	0	0%
Forepaw Contraflexion	1	1	0	0%
Hot Plate Test	1	1	0	0%
Reflexive Forelimb and Hindlimb Placing	1	1	0	0%
Staircase Test	1	1	0	0%

Table 2.1. List of functional outcome tasks used in the TBI field. Functional assessments reported to have deficits ≥ 1 month post-TBI. Of 314 papers reviewed, 32% of papers with an uninjured/sham group compared to an injury group performed functional assessments ≥ 1 month post-TBI. Functional tasks in black represent tasks that reveal a deficit and have been reported in at least 2 or more papers (replicated); tasks in blue have been reported to reveal deficits at ≥ 1 month in only one publication; tasks in grey have not been shown to reveal deficits at ≥ 1 month.

		Functional Deficits Detected Over Time			
		2 Months	3 Months	6 Months	12 Months
Fluid Percussion Injury	Beam Walking	23414334			
	Elevated Plus Maze	21933013 23414334			
	Forced Swim Test	21933013			
	Global Neurological Assessment		16424733	20839948	
	Water Maze, Distance	9262173			
	Water Maze, Learning Latency	9272642 10547100 16424733 21933013 23414334	16764859	15334605 20839948	9740398
	Water Maze, Probe	16720946		19101638 20839948	
	Water Maze, Reversal	16720946 21933013 23414334			
	Water Maze, Swim Speed	21933013		20839948	
Weight Drop Impact Acceleration/ Closed Head Injury	Forced Swim Test		16156715 17669633		
	Passive Avoidance	17669633			
	Rotarod	11702878			
	Rotating Pole Test	11702878			
	Water Maze, Learning Latency		10776912 16356639 11784789 12732240		22743360
	Water Maze, Probe		10776912 12732240		
	Water Maze, Visible Platform		10776912		
Controlled Cortical Impact	Balance Beam			22774771	
	Global Neurological Assessment				18164073
	Water Maze, Learning Latency	19927173	22373400	22774771	10098956
	Water Maze, Probe	19927173 20833152 21335666			10098956
	Y Maze	20833152			

Number denotes PMID of supporting publication

Table 2.2. Models and functional outcome tasks with long-term deficits (2-, 3-, 6- and 12-months post injury). Of 314 papers reviewed, 27 papers found functional deficits at ≥ 2 months post-TBI. PMIDs are shown in the latest time-point post-injury where deficits were observed for the given task.

First Author	Year	PMID	Injury Model	Human Cell Population	Terminal Time-point Post Injection	Quantified Cell Survival (%)	Behavioral Assessment
Nichols JE	2013	23290300	FPI	hMSC	12 Weeks	21	Improved MWM
Hong SQ	2011	21877237	WDIA	Human Umbilical Cord SC	3 Weeks	6.77	Improved MWM
Mahmood A	2003	12943585	CCI	hMSC	4 Weeks	0.5 - 0.6	Improved Rotarod, mNSS
Wennersten A	2006	16490195	FPI	Fetal hNSC	3 or 6 Weeks, 6 Months	0.2	N.A.
Hagen M	2003	14623128	CCI	Fetal hNSC	6 Days	0.12 - 0.95	N.A.
Wennersten A	2006	16490195	WDIA	Fetal hNSC	6 Weeks	0.03	N.A.
Lu D	2002	12075993	CCI	Human Umbilical Cord Blood Cells	1 month	Not Reported	Improved Rotarod, mNSS
Watson DJ	2003	12722829	CCI	NT2N Neurons	4 Weeks	Not Reported	Improved MWM
Longhi L	2004	15684764	CCI	NT2N Neurons	4 Weeks	Not Reported	Improved MWM
Mahmood A	2005	16284572	CCI	hMSC	3 Months	Not Reported	Improved mNSS
Lu D	2007	17881974	CCI	hMSC	5 Weeks	Not Reported	Improved MWM, mNSS
Qu C	2009	19425888	CCI	hMSC	5 Weeks	Not Reported	Improved MWM
Him HJ	2010	19508155	CCI	hMSC	1, 2, 8, 15, 22, and 29 Days	Not Reported	Improved Rotarod, mNSS
Heile AM	2009	19638295	CCI	Immortalized hMSC	2, 7, and 14 Days	Not Reported	N.A.
Walker PA	2010	20637752	CCI	hMSC	3 Days	Not Reported	N.A.
Qu C	2011	21062621	CCI	hMSC	2 Weeks	Not Reported	Improved MWM, mNSS
Skardelly M	2010	21083415	CCI	Fetal hNPC	12 Weeks	Not Reported	Improved Rotarod, mNSS
Li L	2011	21275806	CCI	hMSC	6 Weeks	Not Reported	Improved MWM, mNSS
Jiang Q	2011	21432927	CCI	hMSC	6 Weeks	Not Reported	Improved MWM, mNSS
Zanier ER	2011	21725237	CCI	Human Umbilical Cord SC	5 Weeks	Not Reported	Improved MWM, mNSS, Beam Walk
Poltavtseva RA	2012	22977876	CCI	Fetal hMSC and hNSC	3 Weeks	Not Reported	Improved Limb Function
Hung CJ	2010	20230225	FPI	Immortalized hMSC	2, 7, and 14 Days	Not Reported	N.A.
Muir JK	1999	10369560	FPI	hNT Neurons	2 Weeks	Not Reported	No Differences
Philips MF	1999	10413164	FPI	NT2N Neurons	2 and 4 Weeks	Not Reported	No Differences
Zhang C	2005	16379583	FPI	NT2N Neurons	4, 8, and 12 Weeks	Not Reported	No Differences
Gao J	2006	16904107	FPI	Fetal hNSC	2 Weeks	Not Reported	Improved MWM
Wang E	2012	22077363	FPI	Fetal hNSC	4 Days	Not Reported	N.A.
Chen Z	2009	19886807	PBBI	Human Amniotic Cells	1, 2, 3, and 4 Weeks	Not Reported	N.A.
Chen Z	2011	20951684	PBBI	Human Amniotic Cells	2 Weeks	Not Reported	Improved Rotarod
Wennersten A	2004	14743917	WDIA	Fetal hNSC	2 and 6 Weeks	Not Reported	N.A.
Al Nimer F	2004	15305127	WDIA	Fetal hNSC	6 Weeks	Not Reported	N.A.
Lundberg J	2009	19562330	WDIA	hMSC	1 and 5 Days	Not Reported	N.A.

Table 2.3. Human cellular therapy studies in rodent models of TBI. 32 papers have transplanted human stem cells into a rodent model of traumatic brain injury. Only 6 of these papers reported a quantified cell survival, and none of them used immunodeficient rodent strains. Injury model, transplanted human stem cell population, length of study, quantified cell survival, and functional assessments in treated vs untreated animals is shown. Studies which looked greater than 6 weeks post-transplant are denoted in red (CCI - Controlled Cortical Impact, FPI - Fluid Percussion Injury, PBBI - Penetrating Ballistic-Like Brain Injury, WDIA - Weight Drop Impact Acceleration).

References

Adelson, P.D., Dixon, C.E., Kochanek, P.M., 2000. Long-term dysfunction following diffuse traumatic brain injury in the immature rat. Journal of neurotrauma 17, 273-282.

Albensi, B.C., Sullivan, P.G., Thompson, M.B., Scheff, S.W., Mattson, M.P., 2000. Cyclosporin ameliorates traumatic brain-injury-induced alterations of hippocampal synaptic plasticity. Experimental neurology 162, 385-389.

Allen, I.V., Scott, R., Tanner, J.A., 1982. Experimental high-velocity missile head injury. Injury 14, 183-193.

Anderson, A.J., Haus, D.L., Hooshmand, M.J., Perez, H., Sontag, C.J., Cummings, B.J., 2011. Achieving stable human stem cell engraftment and survival in the CNS: is the future of regenerative medicine immunodeficient? Regenerative medicine 6, 367-406.

Andres, R.H., Horie, N., Slikker, W., Keren-Gill, H., Zhan, K., Sun, G., Manley, N.C., Pereira, M.P., Sheikh, L.A., McMillan, E.L., Schaar, B.T., Svendsen, C.N., Bliss, T.M., Steinberg, G.K., 2011. Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. Brain : a journal of neurology 134, 1777-1789.

Andriessen, T.M., Jacobs, B., Vos, P.E., 2010. Clinical characteristics and pathophysiological mechanisms of focal and diffuse traumatic brain injury. Journal of cellular and molecular medicine 14, 2381-2392.

Bao, F., Shultz, S.R., Hepburn, J.D., Omana, V., Weaver, L.C., Cain, D.P., Brown, A., 2012. A CD11d monoclonal antibody treatment reduces tissue injury and improves neurological outcome after fluid percussion brain injury in rats. Journal of neurotrauma 29, 2375-2392.

Baranova, A.I., Whiting, M.D., Hamm, R.J., 2006. Delayed, post-injury treatment with aniracetam improves cognitive performance after traumatic brain injury in rats. Journal of neurotrauma 23, 1233-1240.

Baratz, R., Rubovitch, V., Frenk, H., Pick, C.G., 2010. The influence of alcohol on behavioral recovery after mTBI in mice. Journal of neurotrauma 27, 555-563.

Barnes, C.A., 1979. Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. Journal of comparative and physiological psychology 93, 74-104.

Barth, T.M., Jones, T.A., Schallert, T., 1990. Functional subdivisions of the rat somatic sensorimotor cortex. Behavioural brain research 39, 73-95.

Barzo, P., Marmarou, A., Fatouros, P., Corwin, F., Dunbar, J., 1996. Magnetic resonance imaging-monitored acute blood-brain barrier changes in experimental traumatic brain injury. Journal of neurosurgery 85, 1113-1121.

Borlongan, C.V., Randall, T.S., Cahill, D.W., Sanberg, P.R., 1995. Asymmetrical motor behavior in rats with unilateral striatal excitotoxic lesions as revealed by the elevated body swing test. Brain research 676, 231-234.

Bradwell, D.J., Kim, H., Sirk, A.H., Sadoway, D.R., 2012. Magnesium-antimony liquid metal battery for stationary energy storage. Journal of the American Chemical Society 134, 1895-1897.

Brody, D.L., Mac Donald, C., Kessens, C.C., Yuede, C., Parsadanian, M., Spinner, M., Kim, E., Schwetye, K.E., Holtzman, D.M., Bayly, P.V., 2007. Electromagnetic controlled cortical impact device for precise, graded experimental traumatic brain injury. Journal of neurotrauma 24, 657-673.

Browne, K.D., Iwata, A., Putt, M.E., Smith, D.H., 2006. Chronic ibuprofen administration worsens cognitive outcome following traumatic brain injury in rats. Experimental neurology 201, 301-307.

Browne, K.D., Leoni, M.J., Iwata, A., Chen, X.H., Smith, D.H., 2004. Acute treatment with MgSO4 attenuates long-term hippocampal tissue loss after brain trauma in the rat. Journal of neuroscience research 77, 878-883.

Buki, A., Povlishock, J.T., 2006. All roads lead to disconnection?--Traumatic axonal injury revisited. Acta Neurochir (Wien) 148, 181-193; discussion 193-184.

Byrnes, K.R., Loane, D.J., Stoica, B.A., Zhang, J., Faden, A.I., 2012. Delayed mGluR5 activation limits neuroinflammation and neurodegeneration after traumatic brain injury. Journal of neuroinflammation 9, 43.

Cahill, L., 2000. Neurobiological mechanisms of emotionally influenced, long-term memory, in: Uylings, H.B.M., van Eden, G.G., de Bruin, J.P.C., Feenstra, M.G.P., Pennartz, C.M.A. (Eds.), Progress in brain research. Elsevier, pp. 29-37.

Carballosa Gonzalez, M.M., Blaya, M.O., Alonso, O.F., Bramlett, H.M., Hentall, I.D., 2013. Midbrain raphe stimulation improves behavioral and anatomical recovery from fluid-percussion brain injury. Journal of neurotrauma 30, 119-130.

Carbonell, W.S., Maris, D.O., McCall, T., Grady, M.S., 1998. Adaptation of the fluid percussion injury model to the mouse. Journal of neurotrauma 15, 217-229.

Carey, M.E., Sarna, G.S., Farrell, J.B., Happel, L.T., 1989. Experimental missile wound to the brain. Journal of neurosurgery 71, 754-764.

Cernak, I., Wang, Z., Jiang, J., Bian, X., Savic, J., 2001. Ultrastructural and functional characteristics of blast injury-induced neurotrauma. The Journal of trauma 50, 695-706.

Chauhan, N.B., Gatto, R., 2010. Synergistic benefits of erythropoietin and simvastatin after traumatic brain injury. Brain research 1360, 177-192.

Chauhan, N.B., Gatto, R., 2011. Restoration of cognitive deficits after statin feeding in TBI. Restorative neurology and neuroscience 29, 23-34.

Chen, J., Li, Y., Wang, L., Lu, M., Zhang, X., Chopp, M., 2001a. Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. Journal of the neurological sciences 189, 49-57.

Chen, J., Li, Y., Wang, L., Zhang, Z., Lu, D., Lu, M., Chopp, M., 2001b. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. Stroke; a journal of cerebral circulation 32, 1005-1011.

Chen, J., Sanberg, P.R., Li, Y., Wang, L., Lu, M., Willing, A.E., Sanchez-Ramos, J., Chopp, M., 2001c. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. Stroke; a journal of cerebral circulation 32, 2682-2688.

Chen, Y., Lomnitski, L., Michaelson, D.M., Shohami, E., 1997. Motor and cognitive deficits in apolipoprotein E-deficient mice after closed head injury. Neuroscience 80, 1255-1262.

Cheng, J.P., Shaw, K.E., Monaco, C.M., Hoffman, A.N., Sozda, C.N., Olsen, A.S., Kline, A.E., 2012. A relatively brief exposure to environmental enrichment after experimental traumatic brain injury confers long-term cognitive benefits. Journal of neurotrauma 29, 2684-2688.

Colombel, C., Lalonde, R., Caston, J., 2002. The effects of unilateral removal of the cerebellar hemispheres on motor functions and weight gain in rats. Brain research 950, 231-238.

Combat Casualty Care Research Program, 2013. https://ccc.amedd.army.mil/task-areas/neuroprotection-research.jspx Accessed March 28, 2013

Coronado, V.G., McGuire, L.C., Faul, M.D., Sugerman, D.E., Peaerson, W.S., 2012. The Epidemiology and Prevention of TBI, Perspectives on Clinical Care, Public Health, and Research.

Coronado, V.G., Xu, L., Basavaraju, S.V., McGuire, L.C., Wald, M.M., Faul, M.D., Guzman, B.R., Hemphill, J.D., 2011. Surveillance for traumatic brain injury-related deaths--United States, 1997-2007. MMWR Surveill Summ 60, 1-32.

Corrigan, J.D., Selassie, A.W., Orman, J.A., 2010. The epidemiology of traumatic brain injury. The Journal of head trauma rehabilitation 25, 72-80.

Cortez, S.C., McIntosh, T.K., Noble, L.J., 1989. Experimental fluid percussion brain injury: vascular disruption and neuronal and glial alterations. Brain research 482, 271-282.

Critchley, M., 1957. Medical aspects of boxing, particularly from a neurological standpoint. British medical journal 1, 357-362.

Cummings, B.J., Uchida, N., Tamaki, S.J., Salazar, D.L., Hooshmand, M., Summers, R., Gage, F.H., Anderson, A.J., 2005. Human neural stem cells differentiate and promote locomotor

recovery in spinal cord-injured mice. Proceedings of the National Academy of Sciences of the United States of America 102, 14069-14074.

D'Hooge, R., De Deyn, P.P., 2001. Applications of the Morris water maze in the study of learning and memory. Brain research. Brain research reviews 36, 60-90.

De Feo, D., Merlini, A., Laterza, C., Martino, G., 2012. Neural stem cell transplantation in central nervous system disorders: from cell replacement to neuroprotection. Current opinion in neurology 25, 322-333.

De Ryck, M., Van Reempts, J., Duytschaever, H., Van Deuren, B., Clincke, G., 1992. Neocortical localization of tactile/proprioceptive limb placing reactions in the rat. Brain research 573, 44-60.

Department of Defense, 2013. http://www.dvbic.org/dod-worldwide-numbers-tbi Accessed March 28, 2013

Dixon, C.E., Clifton, G.L., Lighthall, J.W., Yaghmai, A.A., Hayes, R.L., 1991. A controlled cortical impact model of traumatic brain injury in the rat. Journal of neuroscience methods 39, 253-262.

Dixon, C.E., Hamm, R.J., Taft, W.C., Hayes, R.L., 1994. Increased anticholinergic sensitivity following closed skull impact and controlled cortical impact traumatic brain injury in the rat. Journal of neurotrauma 11, 275-287.

Dixon, C.E., Kochanek, P.M., Yan, H.Q., Schiding, J.K., Griffith, R.G., Baum, E., Marion, D.W., DeKosky, S.T., 1999. One-year study of spatial memory performance, brain morphology, and cholinergic markers after moderate controlled cortical impact in rats. Journal of neurotrauma 16, 109-122.

Dixon, C.E., Lyeth, B.G., Povlishock, J.T., Findling, R.L., Hamm, R.J., Marmarou, A., Young, H.F., Hayes, R.L., 1987. A fluid percussion model of experimental brain injury in the rat. Journal of neurosurgery 67, 110-119.

Edut, S., Rubovitch, V., Schreiber, S., Pick, C.G., 2011. The intriguing effects of ecstasy (MDMA) on cognitive function in mice subjected to a minimal traumatic brain injury (mTBI). Psychopharmacology 214, 877-889.

Ennaceur, A., Neave, N., Aggleton, J.P., 1996. Neurotoxic lesions of the perirhinal cortex do not mimic the behavioural effects of fornix transection in the rat. Behavioural brain research 80, 9-25.

Esmaeili, A., Dadkhahfar, S., Fadakar, K., Rezaei, N., 2012. Post-stroke immunodeficiency: effects of sensitization and tolerization to brain antigens. International reviews of immunology 31, 396-409.

Faden, A.I., Demediuk, P., Panter, S.S., Vink, R., 1989. The role of excitatory amino acids and

NMDA receptors in traumatic brain injury. Science 244, 798-800.

Faul, M., Xu, L., Wald, M.M., Coronado, V.G., 2010. Traumatic brain injury in the United States: emergency department visits, hospitalizations, and deaths. Atlanta (GA): Centers for Disease Control and Prevention. National Center for Injury Prevention and Control.

Finkelstein, E., Corso, P., Miller, T., Assiciates, a., 2006. The Incidence and Economic Burden of Injuries in the United States. Oxford University Press, New York.

Fisher, M., Feuerstein, G., Howells, D.W., Hurn, P.D., Kent, T.A., Savitz, S.I., Lo, E.H., Group, S., 2009. Update of the stroke therapy academic industry roundtable preclinical recommendations. Stroke; a journal of cerebral circulation 40, 2244-2250.

Floresco, S.B., Seamans, J.K., Phillips, A.G., 1997. Selective roles for hippocampal, prefrontal cortical, and ventral striatal circuits in radial-arm maze tasks with or without a delay. The Journal of neuroscience : the official journal of the Society for Neuroscience 17, 1880-1890.

Floyd, C.L., Golden, K.M., Black, R.T., Hamm, R.J., Lyeth, B.G., 2002. Craniectomy position affects morris water maze performance and hippocampal cell loss after parasagittal fluid percussion. Journal of neurotrauma 19, 303-316.

Foda, M.A., Marmarou, A., 1994. A new model of diffuse brain injury in rats. Part II: Morphological characterization. Journal of neurosurgery 80, 301-313.

Fox, G.B., Faden, A.I., 1998. Traumatic brain injury causes delayed motor and cognitive impairment in a mutant mouse strain known to exhibit delayed Wallerian degeneration. Journal of neuroscience research 53, 718-727.

Fox, G.B., Fan, L., LeVasseur, R.A., Faden, A.I., 1998a. Effect of traumatic brain injury on mouse spatial and nonspatial learning in the Barnes circular maze. Journal of neurotrauma 15, 1037-1046.

Fox, G.B., Fan, L., Levasseur, R.A., Faden, A.I., 1998b. Sustained sensory/motor and cognitive deficits with neuronal apoptosis following controlled cortical impact brain injury in the mouse. Journal of neurotrauma 15, 599-614.

Fox, G.B., LeVasseur, R.A., Faden, A.I., 1999. Behavioral responses of C57BL/6, FVB/N, and 129/SvEMS mouse strains to traumatic brain injury: implications for gene targeting approaches to neurotrauma. Journal of neurotrauma 16, 377-389.

Gennarelli, T.A., Thibault, L.E., Adams, J.H., Graham, D.I., Thompson, C.J., Marcincin, R.P., 1982. Diffuse axonal injury and traumatic coma in the primate. Annals of neurology 12, 564-574.

Germano, A.F., Dixon, C.E., d'Avella, D., Hayes, R.L., Tomasello, F., 1994. Behavioral deficits following experimental subarachnoid hemorrhage in the rat. Journal of neurotrauma 11, 345-353.

Giza, C.C., Griesbach, G.S., Hovda, D.A., 2005. Experience-dependent behavioral plasticity is disturbed following traumatic injury to the immature brain. Behavioural brain research 157, 11-22.

Graham, D.I., Adams, J.H., Nicoll, J.A., Maxwell, W.L., Gennarelli, T.A., 1995. The nature, distribution and causes of traumatic brain injury. Brain Pathol 5, 397-406.

Griesbach, G.S., Hovda, D.A., Molteni, R., Wu, A., Gomez-Pinilla, F., 2004. Voluntary exercise following traumatic brain injury: brain-derived neurotrophic factor upregulation and recovery of function. Neuroscience 125, 129-139.

Hall, E.D., Sullivan, P.G., Gibson, T.R., Pavel, K.M., Thompson, B.M., Scheff, S.W., 2005a. Spatial and temporal characteristics of neurodegeneration after controlled cortical impact in mice: more than a focal brain injury. Journal of neurotrauma 22, 252-265.

Hall, R.C., Hall, R.C., Chapman, M.J., 2005b. Definition, diagnosis, and forensic implications of postconcussional syndrome. Psychosomatics 46, 195-202.

Hallam, T.M., Floyd, C.L., Folkerts, M.M., Lee, L.L., Gong, Q.Z., Lyeth, B.G., Muizelaar, J.P., Berman, R.F., 2004. Comparison of behavioral deficits and acute neuronal degeneration in rat lateral fluid percussion and weight-drop brain injury models. Journal of neurotrauma 21, 521-539.

Hamm, R.J., Dixon, C.E., Gbadebo, D.M., Singha, A.K., Jenkins, L.W., Lyeth, B.G., Hayes, R.L., 1992. Cognitive deficits following traumatic brain injury produced by controlled cortical impact. Journal of neurotrauma 9, 11-20.

Hamm, R.J., Lyeth, B.G., Jenkins, L.W., O'Dell, D.M., Pike, B.R., 1993. Selective cognitive impairment following traumatic brain injury in rats. Behavioural brain research 59, 169-173.

Hamm, R.J., Pike, B.R., O'Dell, D.M., Lyeth, B.G., Jenkins, L.W., 1994. The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury. Journal of neurotrauma 11, 187-196.

Hamm, R.J., Pike, B.R., Temple, M.D., O'Dell, D.M., Lyeth, B.G., 1995. The effect of postinjury kindled seizures on cognitive performance of traumatically brain-injured rats. Experimental neurology 136, 143-148.

Han, R.Z., Hu, J.J., Weng, Y.C., Li, D.F., Huang, Y., 2009. NMDA receptor antagonist MK-801 reduces neuronal damage and preserves learning and memory in a rat model of traumatic brain injury. Neuroscience bulletin 25, 367-375.

Hanell, A., Clausen, F., Bjork, M., Jansson, K., Philipson, O., Nilsson, L.N., Hillered, L., Weinreb, P.H., Lee, D., McIntosh, T.K., Gimbel, D.A., Strittmatter, S.M., Marklund, N., 2010. Genetic deletion and pharmacological inhibition of Nogo-66 receptor impairs cognitive outcome after traumatic brain injury in mice. Journal of neurotrauma 27, 1297-1309.

Hannay, H.J., Feldman, Z., Phan, P., Keyani, A., Panwar, N., Goodman, J.C., Robertson, C.S., 1999. Validation of a controlled cortical impact model of head injury in mice. Journal of neurotrauma 16, 1103-1114.

Hayward, N.M., Immonen, R., Tuunanen, P.I., Ndode-Ekane, X.E., Grohn, O., Pitkanen, A., 2010. Association of chronic vascular changes with functional outcome after traumatic brain injury in rats. Journal of neurotrauma 27, 2203-2219.

Hicks, R., Soares, H., Smith, D., McIntosh, T., 1996. Temporal and spatial characterization of neuronal injury following lateral fluid-percussion brain injury in the rat. Acta neuropathologica 91, 236-246.

Hillary, F.G., Medaglia, J.D., Gates, K., Molenaar, P.C., Slocomb, J., Peechatka, A., Good, D.C., 2011. Examining working memory task acquisition in a disrupted neural network. Brain : a journal of neurology 134, 1555-1570.

Hoane, M.R., Becerra, G.D., Shank, J.E., Tatko, L., Pak, E.S., Smith, M., Murashov, A.K., 2004. Transplantation of neuronal and glial precursors dramatically improves sensorimotor function but not cognitive function in the traumatically injured brain. Journal of neurotrauma 21, 163-174.

Hoffman, S.W., Fulop, Z., Stein, D.G., 1994. Bilateral frontal cortical contusion in rats: behavioral and anatomic consequences. Journal of neurotrauma 11, 417-431.

Hoge, C.W., McGurk, D., Thomas, J.L., Cox, A.L., Engel, C.C., Castro, C.A., 2008. Mild traumatic brain injury in U.S. Soldiers returning from Iraq. The New England journal of medicine 358, 453-463.

Hong, S.Q., Zhang, H.T., You, J., Zhang, M.Y., Cai, Y.Q., Jiang, X.D., Xu, R.X., 2011. Comparison of transdifferentiated and untransdifferentiated human umbilical mesenchymal stem cells in rats after traumatic brain injury. Neurochemical research 36, 2391-2400.

Hooshmand, M.J., Sontag, C.J., Uchida, N., Tamaki, S., Anderson, A.J., Cummings, B.J., 2009. Analysis of host-mediated repair mechanisms after human CNS-stem cell transplantation for spinal cord injury: correlation of engraftment with recovery. PloS one 4, e5871.

Hoover, R.C., Motta, M., Davis, J., Saatman, K.E., Fujimoto, S.T., Thompson, H.J., Stover, J.F., Dichter, M.A., Twyman, R., White, H.S., McIntosh, T.K., 2004. Differential effects of the anticonvulsant topiramate on neurobehavioral and histological outcomes following traumatic brain injury in rats. Journal of neurotrauma 21, 501-512.

Horie, N., Pereira, M.P., Niizuma, K., Sun, G., Keren-Gill, H., Encarnacion, A., Shamloo, M., Hamilton, S.A., Jiang, K., Huhn, S., Palmer, T.D., Bliss, T.M., Steinberg, G.K., 2011. Transplanted stem cell-secreted vascular endothelial growth factor effects poststroke recovery, inflammation, and vascular repair. Stem Cells 29, 274-285.

Huh, J.W., Raghupathi, R., 2007. Chronic cognitive deficits and long-term histopathological

alterations following contusive brain injury in the immature rat. Journal of neurotrauma 24, 1460-1474.

Immonen, R.J., Kharatishvili, I., Grohn, H., Pitkanen, A., Grohn, O.H., 2009. Quantitative MRI predicts long-term structural and functional outcome after experimental traumatic brain injury. NeuroImage 45, 1-9.

Jenkinson, E.W., Glickstein, M., 2000. Whiskers, barrels, and cortical efferent pathways in gap crossing by rats. Journal of neurophysiology 84, 1781-1789.

Keck, C.A., Thompson, H.J., Pitkanen, A., LeBold, D.G., Morales, D.M., Plevy, J.B., Puri, R., Zhao, B., Dichter, M., McIntosh, T.K., 2007. The novel antiepileptic agent RWJ-333369-A, but not its analog RWJ-333369, reduces regional cerebral edema without affecting neurobehavioral outcome or cell death following experimental traumatic brain injury. Restorative neurology and neuroscience 25, 77-90.

Kochanek, P.M., Marion, D.W., Zhang, W., Schiding, J.K., White, M., Palmer, A.M., Clark, R.S., O'Malley, M.E., Styren, S.D., Ho, C., et al., 1995. Severe controlled cortical impact in rats: assessment of cerebral edema, blood flow, and contusion volume. Journal of neurotrauma 12, 1015-1025.

Langlois, J.A., Marr, A., Mitchko, J., Johnson, R.L., 2005. Tracking the silent epidemic and educating the public: CDC's traumatic brain injury-associated activities under the TBI Act of 1996 and the Children's Health Act of 2000. The Journal of head trauma rehabilitation 20, 196-204.

Laurer, H.L., Bareyre, F.M., Lee, V.M., Trojanowski, J.Q., Longhi, L., Hoover, R., Saatman, K.E., Raghupathi, R., Hoshino, S., Grady, M.S., McIntosh, T.K., 2001. Mild head injury increasing the brain's vulnerability to a second concussive impact. Journal of neurosurgery 95, 859-870.

Lee, L.L., Galo, E., Lyeth, B.G., Muizelaar, J.P., Berman, R.F., 2004. Neuroprotection in the rat lateral fluid percussion model of traumatic brain injury by SNX-185, an N-type voltage-gated calcium channel blocker. Experimental neurology 190, 70-78.

Lekic, T., Rolland, W., Hartman, R., Kamper, J., Suzuki, H., Tang, J., Zhang, J.H., 2011. Characterization of the brain injury, neurobehavioral profiles, and histopathology in a rat model of cerebellar hemorrhage. Experimental neurology 227, 96-103.

Lenzlinger, P.M., Shimizu, S., Marklund, N., Thompson, H.J., Schwab, M.E., Saatman, K.E., Hoover, R.C., Bareyre, F.M., Motta, M., Luginbuhl, A., Pape, R., Clouse, A.K., Morganti-Kossmann, C., McIntosh, T.K., 2005. Delayed inhibition of Nogo-A does not alter injury-induced axonal sprouting but enhances recovery of cognitive function following experimental traumatic brain injury in rats. Neuroscience 134, 1047-1056.

Li, Y., Chopp, M., Chen, J., Wang, L., Gautam, S.C., Xu, Y.X., Zhang, Z., 2000. Intrastriatal transplantation of bone marrow nonhematopoietic cells improves functional recovery after

stroke in adult mice. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 20, 1311-1319.

Lighthall, J.W., 1988. Controlled cortical impact: a new experimental brain injury model. Journal of neurotrauma 5, 1-15.

Lindgren, S., Rinder, L., 1965. Experimental studies in head injury. I. Some factors influencing results of model experiments. Biophysik 2, 320-329.

Lloyd, E., Somera-Molina, K., Van Eldik, L.J., Watterson, D.M., Wainwright, M.S., 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. Journal of neuroinflammation 5, 28.

Longa, E.Z., Weinstein, P.R., Carlson, S., Cummins, R., 1989. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke; a journal of cerebral circulation 20, 84-91.

Longhi, L., Gesuete, R., Perego, C., Ortolano, F., Sacchi, N., Villa, P., Stocchetti, N., De Simoni, M.G., 2011. Long-lasting protection in brain trauma by endotoxin preconditioning. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 31, 1919-1929.

Longhi, L., Ortolano, F., Zanier, E.R., Perego, C., Stocchetti, N., De Simoni, M.G., 2008a. Effect of traumatic brain injury on cognitive function in mice lacking p55 and p75 tumor necrosis factor receptors. Acta neurochirurgica. Supplement 102, 409-413.

Longhi, L., Perego, C., Zanier, E.R., Ortolano, F., Bianchi, P., Stocchetti, N., De Simoni, M.G., 2008b. Neuroprotective effect of C1-inhibitor following traumatic brain injury in mice. Acta neurochirurgica. Supplement 102, 381-384.

Longhi, L., Watson, D.J., Saatman, K.E., Thompson, H.J., Zhang, C., Fujimoto, S., Royo, N., Castelbuono, D., Raghupathi, R., Trojanowski, J.Q., Lee, V.M., Wolfe, J.H., Stocchetti, N., McIntosh, T.K., 2004. Ex vivo gene therapy using targeted engraftment of NGF-expressing human NT2N neurons attenuates cognitive deficits following traumatic brain injury in mice. Journal of neurotrauma 21, 1723-1736.

Lowenstein, D.H., Thomas, M.J., Smith, D.H., McIntosh, T.K., 1992. Selective vulnerability of dentate hilar neurons following traumatic brain injury: a potential mechanistic link between head trauma and disorders of the hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience 12, 4846-4853.

Lu, D., Mahmood, A., Qu, C., Hong, X., Kaplan, D., Chopp, M., 2007. Collagen scaffolds populated with human marrow stromal cells reduce lesion volume and improve functional outcome after traumatic brain injury. Neurosurgery 61, 596-602; discussion 602-593.

Lu, D., Mahmood, A., Wang, L., Li, Y., Lu, M., Chopp, M., 2001. Adult bone marrow stromal

cells administered intravenously to rats after traumatic brain injury migrate into brain and improve neurological outcome. Neuroreport 12, 559-563.

Lu, J., Goh, S.J., Tng, P.Y., Deng, Y.Y., Ling, E.A., Moochhala, S., 2009. Systemic inflammatory response following acute traumatic brain injury. Frontiers in bioscience : a journal and virtual library 14, 3795-3813.

Lyeth, B.G., Gong, Q.Z., Shields, S., Muizelaar, J.P., Berman, R.F., 2001. Group I metabotropic glutamate antagonist reduces acute neuronal degeneration and behavioral deficits after traumatic brain injury in rats. Experimental neurology 169, 191-199.

Maas, A.I., Menon, D.K., 2012. Traumatic brain injury: rethinking ideas and approaches. Lancet neurology 11, 12-13.

Mahmood, A., Lu, D., Lu, M., Chopp, M., 2003. Treatment of traumatic brain injury in adult rats with intravenous administration of human bone marrow stromal cells. Neurosurgery 53, 697-702; discussion 702-693.

Malleret, G., Hen, R., Guillou, J.L., Segu, L., Buhot, M.C., 1999. 5-HT1B receptor knock-out mice exhibit increased exploratory activity and enhanced spatial memory performance in the Morris water maze. The Journal of neuroscience : the official journal of the Society for Neuroscience 19, 6157-6168.

Management of Concussion/mTBI Working Group, 2009. VA/DoD Clinical Practice Guideline for Management of Concussion/Mild Traumatic Brain Injury. J Rehabil Res Dev 46, CP1-68.

Mannix, R.C., Zhang, J., Park, J., Zhang, X., Bilal, K., Walker, K., Tanzi, R.E., Tesco, G., Whalen, M.J., 2011. Age-dependent effect of apolipoprotein E4 on functional outcome after controlled cortical impact in mice. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 31, 351-361.

Marklund, N., Bareyre, F.M., Royo, N.C., Thompson, H.J., Mir, A.K., Grady, M.S., Schwab, M.E., McIntosh, T.K., 2007. Cognitive outcome following brain injury and treatment with an inhibitor of Nogo-A in association with an attenuated downregulation of hippocampal growth-associated protein-43 expression. Journal of neurosurgery 107, 844-853.

Marklund, N., Morales, D., Clausen, F., Hanell, A., Kiwanuka, O., Pitkanen, A., Gimbel, D.A., Philipson, O., Lannfelt, L., Hillered, L., Strittmatter, S.M., McIntosh, T.K., 2009. Functional outcome is impaired following traumatic brain injury in aging Nogo-A/B-deficient mice. Neuroscience 163, 540-551.

Marmarou, A., Foda, M.A., 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. Journal of neurosurgery 80, 291-300.

Martland, H.S., 1928. Punch drunk. J Amer Med Assoc 91, 1103-1107.

Maruichi, K., Kuroda, S., Chiba, Y., Hokari, M., Shichinohe, H., Hida, K., Iwasaki, Y., 2009. Transplanted bone marrow stromal cells improves cognitive dysfunction due to diffuse axonal injury in rats. Neuropathology : official journal of the Japanese Society of Neuropathology 29, 422-432.

Mattiasson, G.J., Philips, M.F., Tomasevic, G., Johansson, B.B., Wieloch, T., McIntosh, T.K., 2000. The rotating pole test: evaluation of its effectiveness in assessing functional motor deficits following experimental head injury in the rat. Journal of neuroscience methods 95, 75-82.

Maughan, P.H., Scholten, K.J., Schmidt, R.H., 2000. Recovery of water maze performance in aged versus young rats after brain injury with the impact acceleration model. Journal of neurotrauma 17, 1141-1153.

McIntosh, T.K., Noble, L., Andrews, B., Faden, A.I., 1987. Traumatic brain injury in the rat: characterization of a midline fluid-percussion model. Central nervous system trauma : journal of the American Paralysis Association 4, 119-134.

McIntosh, T.K., Smith, D.H., Meaney, D.F., Kotapka, M.J., Gennarelli, T.A., Graham, D.I., 1996. Neuropathological sequelae of traumatic brain injury: relationship to neurochemical and biomechanical mechanisms. Lab Invest 74, 315-342.

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

McKee, A.C., Cantu, R.C., Nowinski, C.J., Hedley-Whyte, E.T., Gavett, B.E., Budson, A.E., Santini, V.E., Lee, H.S., Kubilus, C.A., Stern, R.A., 2009. Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. Journal of neuropathology and experimental neurology 68, 709-735.

Meehan, W.P., 3rd, Zhang, J., Mannix, R., Whalen, M.J., 2012. Increasing recovery time between injuries improves cognitive outcome after repetitive mild concussive brain injuries in mice. Neurosurgery 71, 885-891.

Meng, Y., Xiong, Y., Mahmood, A., Zhang, Y., Qu, C., Chopp, M., 2011. Dose-dependent neurorestorative effects of delayed treatment of traumatic brain injury with recombinant human erythropoietin in rats. Journal of neurosurgery 115, 550-560.

Menon, D.K., Schwab, K., Wright, D.W., Maas, A.I., Demographics, Clinical Assessment Working Group of the, I., Interagency Initiative toward Common Data Elements for Research on Traumatic Brain, I., Psychological, H., 2010. Position statement: definition of traumatic brain injury. Archives of physical medicine and rehabilitation 91, 1637-1640.

Millspaugh, J., 1937. Dementia pugilistica (punch drunk). U.S. Navy Medical Bulletin 35, 297-303.

Milman, A., Rosenberg, A., Weizman, R., Pick, C.G., 2005. Mild traumatic brain injury induces persistent cognitive deficits and behavioral disturbances in mice. Journal of neurotrauma 22, 1003-1010.

Milman, A., Zohar, O., Maayan, R., Weizman, R., Pick, C.G., 2008. DHEAS repeated treatment improves cognitive and behavioral deficits after mild traumatic brain injury. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology 18, 181-187.

Morales, D.M., Marklund, N., Lebold, D., Thompson, H.J., Pitkanen, A., Maxwell, W.L., Longhi, L., Laurer, H., Maegele, M., Neugebauer, E., Graham, D.I., Stocchetti, N., McIntosh, T.K., 2005. Experimental models of traumatic brain injury: do we really need to build a better mousetrap? Neuroscience 136, 971-989.

Moretti, L., Cristofori, I., Weaver, S.M., Chau, A., Portelli, J.N., Grafman, J., 2012. Cognitive decline in older adults with a history of traumatic brain injury. Lancet neurology 11, 1103-1112.

Morganti-Kossmann, M.C., Rancan, M., Stahel, P.F., Kossmann, T., 2002. Inflammatory response in acute traumatic brain injury: a double-edged sword. Current opinion in critical care 8, 101-105.

Morganti-Kossmann, M.C., Yan, E., Bye, N., 2010. Animal models of traumatic brain injury: is there an optimal model to reproduce human brain injury in the laboratory? Injury 41 Suppl 1, S10-13.

Morris, R., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. Journal of neuroscience methods 11, 47-60.

Moscarello, J.M., Ledoux, J.E., 2013. Active avoidance learning requires prefrontal suppression of amygdala-mediated defensive reactions. The Journal of neuroscience : the official journal of the Society for Neuroscience 33, 3815-3823.

Nadel, L., Hardt, O., 2011. Update on memory systems and processes. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 36, 251-273.

Narayan, R.K., Michel, M.E., Ansell, B., Baethmann, A., Biegon, A., Bracken, M.B., Bullock, M.R., Choi, S.C., Clifton, G.L., Contant, C.F., Coplin, W.M., Dietrich, W.D., Ghajar, J., Grady, S.M., Grossman, R.G., Hall, E.D., Heetderks, W., Hovda, D.A., Jallo, J., Katz, R.L., Knoller, N., Kochanek, P.M., Maas, A.I., Majde, J., Marion, D.W., Marmarou, A., Marshall, L.F., McIntosh, T.K., Miller, E., Mohberg, N., Muizelaar, J.P., Pitts, L.H., Quinn, P., Riesenfeld, G., Robertson, C.S., Strauss, K.I., Teasdale, G., Temkin, N., Tuma, R., Wade, C., Walker, M.D., Weinrich, M., Whyte, J., Wilberger, J., Young, A.B., Yurkewicz, L., 2002. Clinical trials in head injury. J Neurotrauma 19, 503-557.

Nasonkin, I., Mahairaki, V., Xu, L., Hatfield, G., Cummings, B.J., Eberhart, C., Ryugo, D.K.,

Maric, D., Bar, E., Koliatsos, V.E., 2009. Long-term, stable differentiation of human embryonic stem cell-derived neural precursors grafted into the adult mammalian neostriatum. Stem Cells 27, 2414-2426.

Newcombe, V., Chatfield, D., Outtrim, J., Vowler, S., Manktelow, A., Cross, J., Scoffings, D., Coleman, M., Hutchinson, P., Coles, J., Carpenter, T.A., Pickard, J., Williams, G., Menon, D., 2011. Mapping traumatic axonal injury using diffusion tensor imaging: correlations with functional outcome. PLoS One 6, e19214.

Nichols, J.E., Niles, J.A., Dewitt, D., Prough, D., Parsley, M., Vega, S., Cantu, A., Lee, E., Cortiella, J., 2013. Neurogenic and neuro-protective potential of a novel subpopulation of peripheral blood-derived CD133+ ABCG2+CXCR4+ mesenchymal stem cells: development of autologous cell-based therapeutics for traumatic brain injury. Stem cell research & therapy 4, 3.

Ning, R., Xiong, Y., Mahmood, A., Zhang, Y., Meng, Y., Qu, C., Chopp, M., 2011. Erythropoietin promotes neurovascular remodeling and long-term functional recovery in rats following traumatic brain injury. Brain research 1384, 140-150.

O'Connor, C., Heath, D.L., Cernak, I., Nimmo, A.J., Vink, R., 2003. Effects of daily versus weekly testing and pre-training on the assessment of neurologic impairment following diffuse traumatic brain injury in rats. Journal of neurotrauma 20, 985-993.

Okiyama, K., Smith, D.H., Thomas, M.J., McIntosh, T.K., 1992. Evaluation of a novel calcium channel blocker, (S)-emopamil, on regional cerebral edema and neurobehavioral function after experimental brain injury. Journal of neurosurgery 77, 607-615.

Omalu, B.I., DeKosky, S.T., Minster, R.L., Kamboh, M.I., Hamilton, R.L., Wecht, C.H., 2005. Chronic traumatic encephalopathy in a National Football League player. Neurosurgery 57, 128-134; discussion 128-134.

Parker, H.L., 1934. Traumatic Encephalopathy (`Punch Drunk') of Professional Pugilists. The Journal of neurology and psychopathology 15, 20-28.

Philips, M.F., Mattiasson, G., Wieloch, T., Bjorklund, A., Johansson, B.B., Tomasevic, G., Martinez-Serrano, A., Lenzlinger, P.M., Sinson, G., Grady, M.S., McIntosh, T.K., 2001. Neuroprotective and behavioral efficacy of nerve growth factor-transfected hippocampal progenitor cell transplants after experimental traumatic brain injury. Journal of neurosurgery 94, 765-774.

Phillips, L.L., Lyeth, B.G., Hamm, R.J., Jiang, J.Y., Povlishock, J.T., Reeves, T.M., 1997. Effect of prior receptor antagonism on behavioral morbidity produced by combined fluid percussion injury and entorhinal cortical lesion. Journal of neuroscience research 49, 197-206.

Pierce, J.E., Smith, D.H., Trojanowski, J.Q., McIntosh, T.K., 1998. Enduring cognitive, neurobehavioral and histopathological changes persist for up to one year following severe experimental brain injury in rats. Neuroscience 87, 359-369.

Piltti, K.M., Salazar, D.L., Uchida, N., Cummings, B.J., Anderson, A.J., 2013. Safety of epicenter versus intact parenchyma as a transplantation site for human neural stem cells for spinal cord injury therapy. Stem cells translational medicine 2, 204-216.

Raghupathi, R., Huh, J.W., 2007. Diffuse brain injury in the immature rat: evidence for an age-at-injury effect on cognitive function and histopathologic damage. Journal of neurotrauma 24, 1596-1608.

Rau, T.F., Kothiwal, A.S., Rova, A.R., Brooks, D.M., Poulsen, D.J., 2012. Treatment with low-dose methamphetamine improves behavioral and cognitive function after severe traumatic brain injury. The journal of trauma and acute care surgery 73, S165-172.

Reid, W.M., Hamm, R.J., 2008. Post-injury atomoxetine treatment improves cognition following experimental traumatic brain injury. Journal of neurotrauma 25, 248-256.

Riess, P., Bareyre, F.M., Saatman, K.E., Cheney, J.A., Lifshitz, J., Raghupathi, R., Grady, M.S., Neugebauer, E., McIntosh, T.K., 2001. Effects of chronic, post-injury Cyclosporin A administration on motor and sensorimotor function following severe, experimental traumatic brain injury. Restorative neurology and neuroscience 18, 1-8.

Roozenbeek, B., Maas, A.I., Menon, D.K., 2013. Changing patterns in the epidemiology of traumatic brain injury. Nature reviews. Neurology.

Ross, D.T., Meaney, D.F., Sabol, M.K., Smith, D.H., Gennarelli, T.A., 1994. Distribution of forebrain diffuse axonal injury following inertial closed head injury in miniature swine. Experimental neurology 126, 291-299.

Saatman, K.E., Contreras, P.C., Smith, D.H., Raghupathi, R., McDermott, K.L., Fernandez, S.C., Sanderson, K.L., Voddi, M., McIntosh, T.K., 1997. Insulin-like growth factor-1 (IGF-1) improves both neurological motor and cognitive outcome following experimental brain injury. Experimental neurology 147, 418-427.

Salazar, D.L., Uchida, N., Hamers, F.P., Cummings, B.J., Anderson, A.J., 2010. Human neural stem cells differentiate and promote locomotor recovery in an early chronic spinal cord injury NOD-scid mouse model. PloS one 5, e12272.

Saljo, A., Bao, F., Haglid, K.G., Hansson, H.A., 2000. Blast exposure causes redistribution of phosphorylated neurofilament subunits in neurons of the adult rat brain. Journal of neurotrauma 17, 719-726.

Sanders, M.J., Dietrich, W.D., Green, E.J., 1999. Cognitive function following traumatic brain injury: effects of injury severity and recovery period in a parasagittal fluid-percussive injury model. Journal of neurotrauma 16, 915-925.

Schallert, T., Kozlowski, D.A., Humm, J.L., Cocke, R.R., 1997. Use-dependent structural events in recovery of function. Advances in neurology 73, 229-238.

Schmidt, R.H., Scholten, K.J., Maughan, P.H., 1999. Time course for recovery of water maze performance and central cholinergic innervation after fluid percussion injury. Journal of neurotrauma 16, 1139-1147.

Schutz, C., Stover, J.F., Thompson, H.J., Hoover, R.C., Morales, D.M., Schouten, J.W., McMillan, A., Soltesz, K., Motta, M., Spangler, Z., Neugebauer, E., McIntosh, T.K., 2006. Acute, transient hemorrhagic hypotension does not aggravate structural damage or neurologic motor deficits but delays the long-term cognitive recovery following mild to moderate traumatic brain injury. Critical care medicine 34, 492-501.

Scudellari, M., 2010. Brain, Interrupted. Scientist 24, 36-41.

Shapira, Y., Shohami, E., Sidi, A., Soffer, D., Freeman, S., Cotev, S., 1988. Experimental closed head injury in rats: mechanical, pathophysiologic, and neurologic properties. Critical care medicine 16, 258-265.

Sharp, J., Frame, J., Siegenthaler, M., Nistor, G., Keirstead, H.S., 2010. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. Stem Cells 28, 152-163.

Shear, D.A., Lu, X.C., Pedersen, R., Wei, G., Chen, Z., Davis, A., Yao, C., Dave, J., Tortella, F.C., 2011a. Severity profile of penetrating ballistic-like brain injury on neurofunctional outcome, blood-brain barrier permeability, and brain edema formation. Journal of neurotrauma 28, 2185-2195.

Shear, D.A., Tate, C.C., Tate, M.C., Archer, D.R., LaPlaca, M.C., Stein, D.G., Dunbar, G.L., 2011b. Stem cell survival and functional outcome after traumatic brain injury is dependent on transplant timing and location. Restorative neurology and neuroscience 29, 215-225.

Shelton, S.B., Pettigrew, D.B., Hermann, A.D., Zhou, W., Sullivan, P.M., Crutcher, K.A., Strauss, K.I., 2008. A simple, efficient tool for assessment of mice after unilateral cortex injury. Journal of neuroscience methods 168, 431-442.

Shigaki, C.L., Johnstone, B., Schopp, L.H., 2009. Financial and vocational outcomes 2 years after traumatic brain injury. Disability and rehabilitation 31, 484-489.

Shohami, E., Novikov, M., Bass, R., 1995. Long-term effect of HU-211, a novel non-competitive NMDA antagonist, on motor and memory functions after closed head injury in the rat. Brain research 674, 55-62.

Shultz, S.R., Bao, F., Omana, V., Chiu, C., Brown, A., Cain, D.P., 2012. Repeated mild lateral fluid percussion brain injury in the rat causes cumulative long-term behavioral impairments, neuroinflammation, and cortical loss in an animal model of repeated concussion. Journal of neurotrauma 29, 281-294.

Shultz, S.R., Bao, F., Weaver, L.C., Cain, D.P., Brown, A., 2013. Treatment with an anti-CD11d integrin antibody reduces neuroinflammation and improves outcome in a rat model of

repeated concussion. Journal of neuroinflammation 10, 26.

Sidaros, A., Skimminge, A., Liptrot, M.G., Sidaros, K., Engberg, A.W., Herning, M., Paulson, O.B., Jernigan, T.L., Rostrup, E., 2009. Long-term global and regional brain volume changes following severe traumatic brain injury: a longitudinal study with clinical correlates. NeuroImage 44, 1-8.

Singleton, R.H., Yan, H.Q., Fellows-Mayle, W., Dixon, C.E., 2010. Resveratrol attenuates behavioral impairments and reduces cortical and hippocampal loss in a rat controlled cortical impact model of traumatic brain injury. Journal of neurotrauma 27, 1091-1099.

Sinson, G., Perri, B.R., Trojanowski, J.Q., Flamm, E.S., McIntosh, T.K., 1997. Improvement of cognitive deficits and decreased cholinergic neuronal cell loss and apoptotic cell death following neurotrophin infusion after experimental traumatic brain injury. Journal of neurosurgery 86, 511-518.

Sinson, G., Voddi, M., McIntosh, T.K., 1995. Nerve growth factor administration attenuates cognitive but not neurobehavioral motor dysfunction or hippocampal cell loss following fluid-percussion brain injury in rats. Journal of neurochemistry 65, 2209-2216.

Skandsen, T., Kvistad, K.A., Solheim, O., Strand, I.H., Folvik, M., Vik, A., 2010. Prevalence and impact of diffuse axonal injury in patients with moderate and severe head injury: a cohort study of early magnetic resonance imaging findings and 1-year outcome. Journal of neurosurgery 113, 556-563.

Skardelly, M., Gaber, K., Burdack, S., Scheidt, F., Hilbig, H., Boltze, J., Forschler, A., Schwarz, S., Schwarz, J., Meixensberger, J., Schuhmann, M.U., 2011. Long-term benefit of human fetal neuronal progenitor cell transplantation in a clinically adapted model after traumatic brain injury. Journal of neurotrauma 28, 401-414.

Smith, D.H., Okiyama, K., Thomas, M.J., McIntosh, T.K., 1993. Effects of the excitatory amino acid receptor antagonists kynurenate and indole-2-carboxylic acid on behavioral and neurochemical outcome following experimental brain injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 13, 5383-5392.

Smith, D.H., Soares, H.D., Pierce, J.S., Perlman, K.G., Saatman, K.E., Meaney, D.F., Dixon, C.E., McIntosh, T.K., 1995. A model of parasagittal controlled cortical impact in the mouse: cognitive and histopathologic effects. Journal of neurotrauma 12, 169-178.

Soares, H.D., Thomas, M., Cloherty, K., McIntosh, T.K., 1992. Development of prolonged focal cerebral edema and regional cation changes following experimental brain injury in the rat. Journal of neurochemistry 58, 1845-1852.

Stern, S.A., Zink, B.J., Mertz, M., Wang, X., Dronen, S.C., 2000. Effect of initially limited resuscitation in a combined model of fluid-percussion brain injury and severe uncontrolled hemorrhagic shock. Journal of neurosurgery 93, 305-314.

Steru, L., Chermat, R., Thierry, B., Simon, P., 1985. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology 85, 367-370.

Stroke Therapy Academic Industry, R., 1999. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. Stroke; a journal of cerebral circulation 30, 2752-2758.

Sullivan, H.G., Martinez, J., Becker, D.P., Miller, J.D., Griffith, R., Wist, A.O., 1976. Fluid-percussion model of mechanical brain injury in the cat. Journal of neurosurgery 45, 521-534.

Sullivan, P.G., Rabchevsky, A.G., Hicks, R.R., Gibson, T.R., Fletcher-Turner, A., Scheff, S.W., 2000. Dose-response curve and optimal dosing regimen of cyclosporin A after traumatic brain injury in rats. Neuroscience 101, 289-295.

Sun, D., McGinn, M.J., Zhou, Z., Harvey, H.B., Bullock, M.R., Colello, R.J., 2007. Anatomical integration of newly generated dentate granule neurons following traumatic brain injury in adult rats and its association to cognitive recovery. Experimental neurology 204, 264-272.

Tanaka, K., Yagi, T., Shimakoshi, R., Azuma, K., Nanba, T., Ogo, H., Tamura, A., Asanuma, M., 2009. Effects of galantamine on L-NAME-induced behavioral impairment in Y-maze task in mice. Neuroscience letters 462, 235-238.

Thompson, H.J., LeBold, D.G., Marklund, N., Morales, D.M., Hagner, A.P., McIntosh, T.K., 2006. Cognitive evaluation of traumatically brain-injured rats using serial testing in the Morris water maze. Restorative neurology and neuroscience 24, 109-114.

Thurman, D.J., Alverson, C., Dunn, K.A., Guerrero, J., Sniezek, J.E., 1999. Traumatic brain injury in the United States: A public health perspective. The Journal of head trauma rehabilitation 14, 602-615.

Tomasevic, G., Laurer, H.L., Mattiasson, G., Steeg, H., Wieloch, T., McIntosh, T.K., 2012. Delayed neuromotor recovery and increased memory acquisition dysfunction following experimental brain trauma in mice lacking the DNA repair gene XPA. Journal of neurosurgery.

Tupper, D.E., Wallace, R.B., 1980. Utility of the neurological examination in rats. Acta neurobiologiae experimentalis 40, 999-1003.

Uryu, K., Laurer, H., McIntosh, T., Pratico, D., Martinez, D., Leight, S., Lee, V.M., Trojanowski, J.Q., 2002. Repetitive mild brain trauma accelerates Abeta deposition, lipid peroxidation, and cognitive impairment in a transgenic mouse model of Alzheimer amyloidosis. The Journal of neuroscience : the official journal of the Society for Neuroscience 22, 446-454.

Vink, R., Mullins, P.G., Temple, M.D., Bao, W., Faden, A.I., 2001. Small shifts in craniotomy position in the lateral fluid percussion injury model are associated with differential lesion development. Journal of neurotrauma 18, 839-847.

Vink, R., O'Connor, C.A., Nimmo, A.J., Heath, D.L., 2003. Magnesium attenuates persistent functional deficits following diffuse traumatic brain injury in rats. Neuroscience letters 336, 41-44.

Vorhees, C.V., Williams, M.T., 2006. Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nature protocols 1, 848-858.

Wahl, F., Grosjean-Piot, O., Bareyre, F., Uzan, A., Stutzmann, J.M., 2000. Enoxaparin reduces brain edema, cerebral lesions, and improves motor and cognitive impairments induced by a traumatic brain injury in rats. Journal of neurotrauma 17, 1055-1065.

Wang, S., Bates, J., Li, X., Schanz, S., Chandler-Militello, D., Levine, C., Maherali, N., Studer, L., Hochedlinger, K., Windrem, M., Goldman, S.A., 2013. Human iPSC-derived oligodendrocyte progenitor cells can myelinate and rescue a mouse model of congenital hypomyelination. Cell stem cell 12, 252-264.

Washington, P.M., Forcelli, P.A., Wilkins, T., Zapple, D., Parsadanian, M., Burns, M.P., 2012. The Effect of Injury Severity on Behavior: A phenotypic study of cognitive and emotional deficits after mild, moderate and severe controlled cortical impact injury in mice. Journal of neurotrauma.

Weick, J.P., Liu, Y., Zhang, S.C., 2011. Human embryonic stem cell-derived neurons adopt and regulate the activity of an established neural network. Proceedings of the National Academy of Sciences of the United States of America 108, 20189-20194.

Wennersten, A., Holmin, S., Al Nimer, F., Meijer, X., Wahlberg, L.U., Mathiesen, T., 2006. Sustained survival of xenografted human neural stem/progenitor cells in experimental brain trauma despite discontinuation of immunosuppression. Experimental neurology 199, 339-347.

Williams, A.J., Hartings, J.A., Lu, X.C., Rolli, M.L., Dave, J.R., Tortella, F.C., 2005. Characterization of a new rat model of penetrating ballistic brain injury. Journal of neurotrauma 22, 313-331.

Williams, A.J., Hartings, J.A., Lu, X.C., Rolli, M.L., Tortella, F.C., 2006a. 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.

Williams, A.J., Ling, G.S., Tortella, F.C., 2006b. Severity level and injury track determine outcome following a penetrating ballistic-like brain injury in the rat. Neuroscience letters 408, 183-188.

Williams, A.J., Wei, H.H., Dave, J.R., Tortella, F.C., 2007. Acute and delayed neuroinflammatory response following experimental penetrating ballistic brain injury in the rat. Journal of neuroinflammation 4, 17.

Woodlee, M.T., Asseo-Garcia, A.M., Zhao, X., Liu, S.J., Jones, T.A., Schallert, T., 2005. Testing

forelimb placing "across the midline" reveals distinct, lesion-dependent patterns of recovery in rats. Experimental neurology 191, 310-317.

Xiong, Y., Lu, D., Qu, C., Goussev, A., Schallert, T., Mahmood, A., Chopp, M., 2008a. Effects of erythropoietin on reducing brain damage and improving functional outcome after traumatic brain injury in mice. Journal of neurosurgery 109, 510-521.

Xiong, Y., Mahmood, A., Chopp, M., 2013. Animal models of traumatic brain injury. Nature reviews. Neuroscience 14, 128-142.

Xiong, Y., Mahmood, A., Lu, D., Qu, C., Kazmi, H., Goussev, A., Zhang, Z.G., Noguchi, C.T., Schallert, T., Chopp, M., 2008b. Histological and functional outcomes after traumatic brain injury in mice null for the erythropoietin receptor in the central nervous system. Brain research 1230, 247-257.

Xiong, Y., Mahmood, A., Meng, Y., Zhang, Y., Qu, C., Schallert, T., Chopp, M., 2010. Delayed administration of erythropoietin reducing hippocampal cell loss, enhancing angiogenesis and neurogenesis, and improving functional outcome following traumatic brain injury in rats: comparison of treatment with single and triple dose. Journal of neurosurgery 113, 598-608.

Xiong, Y., Mahmood, A., Meng, Y., Zhang, Y., Zhang, Z.G., Morris, D.C., Chopp, M., 2011a. Treatment of traumatic brain injury with thymosin beta(4) in rats. Journal of neurosurgery 114, 102-115.

Xiong, Y., Mahmood, A., Zhang, Y., Meng, Y., Zhang, Z.G., Qu, C., Sager, T.N., Chopp, M., 2011b. Effects of posttraumatic carbamylated erythropoietin therapy on reducing lesion volume and hippocampal cell loss, enhancing angiogenesis and neurogenesis, and improving functional outcome in rats following traumatic brain injury. Journal of neurosurgery 114, 549-559.

Xiong, Y., Zhang, Y., Mahmood, A., Meng, Y., Qu, C., Chopp, M., 2011c. Erythropoietin Mediates Neurobehavioral Recovery and Neurovascular Remodeling Following Traumatic Brain Injury in Rats by Increasing Expression of Vascular Endothelial Growth Factor. Translational stroke research 2, 619-632.

Xiong, Y., Zhang, Y., Mahmood, A., Meng, Y., Zhang, Z.G., Morris, D.C., Chopp, M., 2012. Neuroprotective and neurorestorative effects of thymosin beta4 treatment initiated 6 hours after traumatic brain injury in rats. Journal of neurosurgery 116, 1081-1092.

Yamaki, T., Murakami, N., Iwamoto, Y., Sakakibara, T., Kobori, N., Ueda, S., Kikuchi, T., Uwahodo, Y., 1997. Evaluation of learning and memory dysfunction and histological findings in rats with chronic stage contusion and diffuse axonal injury. Brain research 752, 151-160.

Yamaki, T., Murakami, N., Iwamoto, Y., Sakakibara, T., Kobori, N., Ueda, S., Uwahodo, Y., Kikuchi, T., 1998. Cognitive dysfunction and histological findings in rats with chronic-stage

contusion and diffuse axonal injury. Brain research. Brain research protocols 3, 100-106.

Yasuhara, T., Matsukawa, N., Hara, K., Yu, G., Xu, L., Maki, M., Kim, S.U., Borlongan, C.V., 2006. Transplantation of human neural stem cells exerts neuroprotection in a rat model of Parkinson's disease. The Journal of neuroscience : the official journal of the Society for Neuroscience 26, 12497-12511.

Zhang, C., Saatman, K.E., Royo, N.C., Soltesz, K.M., Millard, M., Schouten, J.W., Motta, M., Hoover, R.C., McMillan, A., Watson, D.J., Lee, V.M., Trojanowski, J.Q., McIntosh, T.K., 2005. Delayed transplantation of human neurons following brain injury in rats: a long-term graft survival and behavior study. Journal of neurotrauma 22, 1456-1474.

Zhang, Y., Chopp, M., Mahmood, A., Meng, Y., Qu, C., Xiong, Y., 2012. Impact of inhibition of erythropoietin treatment-mediated neurogenesis in the dentate gyrus of the hippocampus on restoration of spatial learning after traumatic brain injury. Experimental neurology 235, 336-344.

Zhang, Y., Xiong, Y., Mahmood, A., Meng, Y., Qu, C., Schallert, T., Chopp, M., 2009. Therapeutic effects of erythropoietin on histological and functional outcomes following traumatic brain injury in rats are independent of hematocrit. Brain research 1294, 153-164.

Zhao, H., Yao, R., Cao, X., Wu, G., 2011. Neuroimmune modulation following traumatic stress in rats: evidence for an immunoregulatory cascade mediated by c-Src, miRNA222 and PAK1. Journal of neuroinflammation 8, 159.

Zhao, Z., Loane, D.J., Murray, M.G., 2nd, Stoica, B.A., Faden, A.I., 2012. Comparing the predictive value of multiple cognitive, affective, and motor tasks after rodent traumatic brain injury. Journal of neurotrauma 29, 2475-2489.

Zink, B.J., Walsh, R.F., Feustel, P.J., 1993. Effects of ethanol in traumatic brain injury. Journal of neurotrauma 10, 275-286.

Zohar, O., Getslev, V., Miller, A.L., Schreiber, S., Pick, C.G., 2006. Morphine protects for head trauma induced cognitive deficits in mice. Neuroscience letters 394, 239-242.

Zohar, O., Schreiber, S., Getslev, V., Schwartz, J.P., Mullins, P.G., Pick, C.G., 2003. Closed-head minimal traumatic brain injury produces long-term cognitive deficits in mice. Neuroscience 118, 949-955.

CHAPTER 3

Repeated Mild Closed Head Injuries Induce Long-Term White Matter Pathology And Neuronal Loss That Are Correlated With Behavioral Deficits

ABSTRACT

An estimated 5.3 million Americans are currently living with a disability from a traumatic brain injury (TBI). Of growing concern is the emerging evidence of the detrimental effects from suffering several mild TBIs (mTBI). Repeated mTBI (rmTBI) manifests its own unique set of behavioral consequences as well as neuropathological changes, defined post-mortem as chronic traumatic encephalopathy (CTE). We have combined components of two classic rodent models of TBI, the controlled cortical impact (CCI) model and the weight drop model, to develop a repeated mild closed head injury (rmCHI) model that produces long-term deficits in several behavioral that were correlated with neuropathological changes. Mice receiving rmCHI performed differently from 1-hit or sham controls on the elevated plus maze at 1 month post injury (mpi), spending more time in open arms, as well as exhibiting hyperlocomotion; these deficits persist up to 6mpi. rmCHI mice performed significantly worse than 1-hit and control sham mice at 2mpi and 6mpi in a classic learning task, the Morris water Maze. In addition to behavioral changes following rmCHI, we observed significant white matter tract pathology similar to clinical presentations of rmTBI. Mice receiving rmCHI exhibited myelin changes in corpus callosum, as well as significant atrophy of the corpus callosum at both 2mpi and 6mpi (33% and 35%, respectively) as assessed by stereological volume analysis. In addition, although there was no significant decrease in total $NG2^+$ oligodendrocyte precursors in corpus callosum, there was a strong correlation of $NG2^+$ cells to corpus callosum volume across all injured groups

at 6MPI. Stereological analysis also revealed significant loss of cortical neurons in comparison to 1-hit and controls. Moreover, both of these pathological changes correlate with behavioral impairments. This rmCHI model suggests that strategies to restore myelination or reduce neuronal loss may ameliorate the behavioral deficits observed following rmCHI.

INTRODUCTION

In the United States, an estimated 5.3 million people are living with a traumatic brain injury (TBI) related disability (Langlois and Sattin, 2005) resulting in direct and indirect costs of 56-221 billion USD every year (Coronado et al., 2012). Over 1.7 million Americans a year suffer a TBI leading to a hospitalization (Faul et al., 2010), wherein *mild* TBI (mTBI) accounts for 75% of all TBI (Gerberding and Binder, 2003). Yet, 43% of patients discharged after acute TBI hospitalization develop a long-term disability (Rutland-Brown et al., 2006). Concussion, or mTBI, is defined as a pathophysiological process affecting the brain induced by direct or indirect biomechanical forces that may lead to acute neurological deficits that typically resolve without structural injuries (Herring et al., 2011), leading to 1.4 - 3.8 million concussions annually in the United States (Faul et al., 2010). Single and/or multiple, mild head injuries can lead to long-lasting cognitive and emotional deficits, as well as neuropathologies including chronic neuroinflammation (Johnson et al., 2013a; Ramlackhansingh et al., 2011) and tauopathies such as chronic traumatic encephalopathy (CTE) (McKee et al., 2009). Epidemiological studies that include those with mild or concussive injuries that do not lead to a hospitalization, put the estimate of all TBI incidences closer to 3.8 million per year (Laskowski et al., 2015). Clearly, TBI is a major health problem with limitations to definitive incident reporting.

Primary injury associated with TBI can result in acute axonal swelling and shearing (Johnson et al., 2013b), microglial activation (Johnson et al., 2013a; Ramlackhansingh et al., 2011), neuronal loss (Raghupathi, 2004), hemorrhage and blood brain barrier disruption (Baskaya et al., 1997; Greve and Zink, 2009). Secondary injury cascades associated with these events include white matter atrophy, chronic and dynamic neuroinflammation, and neuronal loss. Of particular interest, disruptions of major white matter tracts following TBI have also been observed in both humans and rodents. In humans with a single traumatic brain injury, 25% atrophy of the corpus callosum is observed chronically, concurrent with activated microglia within the corpus callosum (Johnson et al., 2013a). White matter abnormalities have been observed in humans following multiple concussions or traumatic brain injuries, especially in athletes (Johnson et al., 2013a; Multani et al., 2016; Tremblay et al., 2014). In a rodent model of repeated mTBI, a sustained inflammatory response was observed in corpus callosum, up to 7 weeks post injury (Shitaka et al., 2011), and in a study at more chronic timepoints of 6- and 12- months post-injury, 10-20% corpus callosum thinning occurred, as measured by thickness in 2D sections, not total volume, in combination with increased astroglial and microglial activation (Mouzon et al., 2014).

Rodent models are often utilized to mimic repeated mild traumatic brain injury (rmTBI) to investigate mechanisms underlying pathological and cognitive changes following injury. In acute TBI, correlates of hippocampal neuropathology and memory performance have been reported (Haus et al., 2016; Hicks et al., 1993). However, few rodent models of *rmTBI* have quantified neuropathology, and to our knowledge, none have correlated these measures

with assessments of behavior and cognition. rmTBI can be caused by a wide range of insults followed by heterogeneous symptoms and pathological outcomes. A single animal model of rmTBI is unlikely to be sufficient to understand all of the possible pathologies associated with injury. Indeed, modeling human TBI in rodents will always lack some components of the human condition, due to size differences, differences in rotational forces, a lack of one common mechanism for human injury, and because rodents are lissencephalic. Some current rodent models of rmTBI include impacting a mouse skull directly using a home-made or commercially available pneumatic device (Laurer et al., 2001; Prins et al., 2010; Shitaka et al., 2011; Uryu et al., 2002), dropping a weight down a tube onto a closed head of a suspended mouse on a kimwipe (Meehan et al., 2012) or aluminum foil (Kane et al., 2012), and a newly developed closed-head impact model of engineered rotational acceleration (CHIMERA) focusing on precise control of injuries with unrestricted head movement allowing for coup-countrecoup injuries (Namjoshi et al., 2014). Models that do not resect the skin to impact the skull or dura directly are commonly referred to as repeated mild closed head injuries (rmCHI), a subset of rmTBI. We sought to utilize commercially available components from these models to allow for robust, high-throughput rmCHI that can be easily replicated across laboratories with precise control over injury parameters. Further, we sought to develop a model of closed head injury with components of acceleration-deceleration forces in an unrestrained head that produced white matter tract neuropathology representative of that observed in the clinical setting. Finally, we sought a model that demonstrates external validity, resulting in cognitive and behavioral deficits in variety of tasks that are sustained chronically (Henderson et al., 2013; Malkesman et al., 2013).

We hypothesize that repeated *mild* closed head injuries (rmCHI), but not a single injury, will produce chronic behavioral changes as well as pathological abnormalities. We predicted that the extent of behavioral impairment would correlate with underlying levels of pathology. Critically, to our knowledge, there are no reports comparing the relationship of white matter or cortical pathology in rmCHI rodents with multiple measures of behavioral function. We focus on investigating corpus callosum neuropathology following rmTBI given the common finding of white matter damage in humans, and focused in the relationship between behavioral changes post-injury and the underlying pathology. We also assessed cortical neuron number below the site of impact. We present a rodent injury model of rmCHI that allows for high experimental throughput of mice, and report on the association/correlation of robust long-term behavioral changes and learning deficits with corpus callosum atrophy, long-term neuroinflammation, and cortical neuronal loss.

MATERIAL AND METHODS

Animals and Injury Paradigm

All procedures were carried out under the approval of the UCI IACUC committee (#2010-2945) in an AAALAC accredited vivarium (#000238). Components of both the controlled cortical impact (CCI) device (TBI-0310 Head Impactor, Precision Systems and Instrumentation, LLC, Fairfax Station, VA, Figure 3.1A) and the weight drop model were combined to produce this novel model of repeated mild closed head injury (rmCHI). Using the CCI device as intended, a stereotaxic frame holding a rodent is positioned on the stage and the impactor tip is centered over a craniotomy exposing the brain and intact dura

below, whereby the piston can impact the brain directly. Rather than using a stereotaxic frame and performing a craniotomy, we made a foam bed modified from 'Marmarou foam' (Type E Bed, Foam to Size, Inc., Ashland, VA), the standard foam commonly used in weight drop models of TBI. The foam was modified to fit on the stage of the CCI device (5.25" x 2.75" x 17.25") and a trench was cut out to position tubing and a nose cone to keep the animal under anesthesia for the duration of the procedure.

Prior to all procedures, 9-10 week old male C57Bl/6J mice were acclimated to the vivarium for 1 week and handled for 3 days, 5 minutes per day. The sequence of events leading up to the moment of impact is tightly controlled to ensure the same minimal, precise amount of time under anesthesia. Animals are first placed into a pre-filled isoflurane knockdown chamber at 2.5% isoflurane for 2 minutes. Mice are then moved to a prep station under 2.5% isoflurane, where tails are marked for animal identification, ophthalmic ointment is applied, and their head is shaved to allow for a clean, smooth surface to zero the impactor tip. Mice stay at this station for 2 minutes, regardless of when all necessary procedures are completed. The animal is then placed onto the modified Marmarou foam and positioned to breath 1.5% isoflurane from a nose cone. Standard lab tape was lightly applied to the tips of mice's ears and lightly fixed to the foam to help keep head movement at a standstill (Figure 3.1B). The mouse is in the CCI station for a total of 3 minutes until the device delivers an impact with speed 5.0m/s, 1.0mm depth, and 50ms dwell time (Slow-motion video of impact shows displacement of head into supporting foam pad, Figure 3.1C). In total, mice are under anesthesia for 7 minutes from knockdown to TBI impact. Isoflurane percentages were optimized to minimize head movement from respiration while zeroing

the piston and during impact, while still keeping the animal unresponsive to a toe pinch reflex.

This short procedure allows for high throughput and large animal cohorts for experiments, becoming very useful when requiring large group sizes for behavioral assessments. An experienced experimenter could solely run this procedure on 10 mice per hour. Following impact, animals were moved out of the apparatus and onto a recovery area where righting time was recorded as a measure of loss of consciousness. Sham animals underwent the same 7 minute procedure with anesthesia, but were not impacted. Animals were randomly assigned to treatment groups and observers were blind to group during all behavioral and histological evaluations.

Experiment 1 Timeline - 2 Month Study, Sham, 1 Hit, 5 Hit, and 10 Hit groups over 10 Days

C57BL/6J male mice were grouped into four different conditions. One group received a single impact (n=11). Two groups received multiple impacts, either 10 hits over 10 days (once every 24hrs, n=13) or 5 hits over 10 days (once every 48hrs, n=9), Figure 3.1D. Two sham control groups were included in the studies to parallel 7 minutes of isoflurane exposure, but receiving no impact at the conclusion of 7 minutes. The two sham groups received either 10 sham procedures over 10 days, or 5 procedures over 10 days (once every 48hrs). Sham groups were ultimately combined in all statistical analysis, as there were no differences in any behavioral measurement between the two sham groups (n=14).

Single impact animals received an impact on the final day of hits of both 5 and 10 hit groups to align all groups at 0 days post injury (dpi).

An array of behavioral tasks was chosen (see below) to assess motor function as well as cognitive and/or emotional function. Experimental conditions were randomized prior to injury or behavioral testing, and all behavioral testing was done blind to experimental condition. No pre-injury baseline behavioral testing was conducted. Animals were sacrificed at 9 weeks post injury, perfused with 4% paraformaldehyde. Brains were excised, immersed in 30% sucrose/4% paraformaldehyde overnight, and snap-frozen in -50°C isopentane.

Experiment 2 Timeline - 6 Month Study, Sham, 1 Hit, and 10 Hit

9-10 week old C57BL/6J male mice were grouped into three conditions: Sham (n=7), 1 Hit (n=11), or 10 hits over 10 days (once every 24 hrs, n=9). Mice were tested on elevated plus maze at 1MPI and 6MPI, a single forced swim test at 2MPI and 6MPI, and Morris water maze at 6MPI. 2 animals from each group were sacrificed at 2MPI for pilot data for another experiment.

Rotarod

Mice were tested for motor function, control, and balance using the rotarod task at 1, 4, 7, 14, 21, and 35 days post-injury. Mice were placed onto a rota-rod device (Economex Rota-Rod, Columbus Instruments, Columbus, Ohio) first for a minute to acclimate, followed by 2 minutes of learning/acclimation at a steady rate of 5 revolutions per minute (rpm).

Animals falling off the beam were immediately re-positioned atop the beam during this 2 minute learning phase. Following 2 minutes of acclimation to the task, two training trials of were performed, followed by 5 testing trials. Daily latency to fall measurements were an average of the 5 testing trials. Both training and testing trials began at 3rpm, accelerating at 2.0 rpm/sec for the duration of each trial. Between each training and testing trial, any urine left on the beam was removed and the beam was cleaned and dried.

Horizontal Ladder Beam

To assess motor function, mice were tested using the horizontal ladderbeam. Foot-faults, or mis-steps, were tabulated from video recordings of animals traversing the horizontal ladder with variable rung spacing (Cummings et al., 2007). Testing was performed in a dark room with a light source placed at the starting end of the ladder to motivate movement away from the light, into a closed dark cage at the end of the ladder. An enclosure, just bigger than the mouse, sits atop the ladder to allow a track for mice to walk in a line to the opposite end. Three successfully traversed trials in total were scored, using total forepaw missteps over 3 trials as the outcome measure.

Tail Suspension Test

The tail suspension test is a common, simple task to assess depression in mice. Mice were hung from a beam raised 12" off of a surface, affixed by a piece of tape at the tip of their tail. Mice tried to grab their tails and climb up to right themselves. Assessment of depression-like behavior was measured by scoring time immobile, or time not struggling to right

themselves. More depressive-like behavior is indicated by more time immobile. A single 5 minute trial was conducted under normal ambient light conditions.

Elevated Plus Maze

Animals were placed at the center point of the elevated plus maze apparatus (Noldus Information Technology, Leesburg, VA) 29" off of the ground, facing an open arm extending 13.5" from the center. Mice could move freely between unenclosed and enclosed closed arms, 7.5" high walls. Trials lasted 5 minutes and were analyzed by EthoVision XT software (Noldus Information Technology, Leesburg, VA). Interpreting different outcome measures can give a better understanding of general locomotor activity, as well as anxiety-like behavior. Simply, overall locomotion can be analyzed for general activity levels. Anxiety-like behavior was assessed by comparing time spent in open arms, or by amount of entries into open arms. An animal spending more time in closed arms was considered to have elevated anxiety. Administering anxiolytic drugs to rodents will increase their time spent in open arms (Pellow and File, 1986). However, risk-like behavior could also be an interpretation from the data where more time spent in open arms is indicative of increased risk-like behavior and less risk aversion. Animals in Experiment 1 were tested under ambient light conditions, while Experiment 2 was conducted in a dark room with near-infrared illumination under the maze arms to allow for contrast and camera detection. Outcome measures gathered were total distance moved, entries into open and closed arms, and time spent in open/closed arms.

Morris Water Maze

The Morris water maze is a well-characterized task to assess spatial learning and memory. The maze consists of a circular metal pool (44" diameter) filled with water 3 inches from the top. White paint was used to dye the water and hide a platform (5" diameter) 1cm below the surface and out of the animal's sightline. Water was maintained at 25-30°C. Mice underwent 4 trials, 60s max, per day over 5 days of testing. The platform remained stationary in a particular quadrant, however, the entry point order changed each day, as well as within a given day. Mice used cues outside the maze (various poster-board shapes fixed to the testing room walls), to spatially navigate to the 'hidden' platform. EthoVision XT software (Noldus Information Technology, Leesburg, VA) recorded each trial, quantifying latency to reach platform and time spent in each quadrant.

Forced Swim Test

The forced swim test is a measure of depressive-like behavior. Mice were placed into a 2000mL glass beaker filled with 7-8 cm of water (25°C) for six minutes. Trials were video recorded and analyzed using EthoVision XT software (Noldus Information Technology, Leesburg, VA). EthoVision software allowed for simple quantification of time moving vs not moving, but also a measurement of relative mobility. Moving was determined by calculating the duration for which the center point of the animal was changing location with a start velocity set at 1.25cm/s and a stop velocity set at 1.0cm/s. Mobility was quantified using changes in pixel area of the subject between samples collected. Standard software thresholds were used to segment mobility into 'immobile', 'mobile', and high mobility' levels, where the immobile threshold was set to 6% and the high mobility

threshold was set to 18%. Mice were tested on the forced swim test in Experiment 2 at 2 and 6 months post-injury. A single exposure was used, not the 5-day repetitive variant of the forced swim test, which has a learning component (Mul et al., 2016).

Immunohistochemistry

Personnel blind to experimental group performed all histological analyses. Serial 30um coronal brain sections were collected from mice sacrificed at 9 weeks post injury. Tissue was stained in free floating wells. Endogenous peroxide activity was quenched with hydrogen peroxide/methanol for 20 minutes, followed by cell permeabilization in 0.1% Triton-X. Tissue was blocked in fetal bovine serum and donkey serum for an hour prior to primary antibody incubation. Primary antibodies were incubated overnight at room temperature and include rat anti-myelin basic protein (Millipore, #MAB386, 1:800), rabbit anti-Iba1 (Wako, #019-19741, 1:1000), rabbit anti-GFAP (Dako, #Z0334, 1:10,000), mouse anti-NeuN (Millipore, #MAB377, 1:1000), and rabbit anti-NG2 (Millipore, #AB5320, 1:800). Tissue was incubated with secondary antibodies for an hour at room temperature. Secondary antibodies used include donkey anti-rabbit $F(ab')_2$ biotin conjugated (Jackson ImmunoResearch Laboratories, #711-066-152, 1:500), donkey anti-mouse $F(ab')_2$ biotin conjugated (Jackson ImmunoResearch Laboratories, #715-066-151, 1:500), donkey anti-rat $F(ab')_2$ biotin conjugated (Jackson ImmunoResearch Laboratories, #712-066-153, 1:500), and donkey anti-rabbit $F(ab')_2$ AF647 conjugated (JacksonImmunoResearch Laboratories, #711-606-152, 1:500) for NG2 immunofluorescent labeling. Following incubation with ABC Kit (Vector Labs, #PK-6100), DAB substrate was added to visualize antigens (Vector Labs, SK-4100). Tissue was mounted onto gelatin-coated slides and

counterstained with methyl green to visualize nuclei. NG2 immunofluorescent sections were counterstained with Hoechst.

Corpus Callosum Volume Quantification

30μm sections were stained with Cresyl violet for quantification of the corpus callosum. Contours of the corpus callosum were drawn using StereoInvestigator software v11.08.01 (MicroBrightField, Inc., Williston, VT) on every 12th section to evaluate estimated volume in a systematic, non-biased, design-based approach. Anterior and posterior limits of corpus callosum were bound by sections that contained intact corpus callosum connecting the two hemispheres in the same coronal plane (Figure 3.4B). A 100um grid was laid down to assess corpus callosum volume and coefficient of error for each animal was less than 0.10.

NeuN$^+$ Neuron Stereological Quantification

The optical fractionator probe was used to quantify NeuN immunopositive cortical neurons. From 30um serial coronal sections, every 12th section was assessed. Anterior and posterior borders were determined by where corpus callosum was intact between both hemispheres. NeuN$^+$ neurons were counted bilaterally, and borders were contoured based on relative anatomy as follows: a ventral limit was established by contouring a horizontal line form the most dorsal point of both thalamus, extending to the most lateral aspect of the cortex. The corpus callosum was used as the ventral limit, until intersecting with the extended thalamic border. On each hemisphere, a medial limit was drawn extending from the point at which the cingulate gyrus extended into the cortex (Figure 3.5B). Using a 750 x 750um grid with a 30 x 30um counting frame, a 10um optical fractionator probe was used

to count NeuN$^+$ cells (MicroBrightField, Inc., Williston, VT). A 100um grid was laid down to assess cortical volume using Cavalieri estimator.

NG2$^+$ Oligodendrocyte Precursor Stereological Quantification

The optical fractionator probe was used to quantify NG2$^+$ immunopositive oligodendrocyte precursors. From 30um serial coronal sections, every 12th section was assessed. Corpus callosum borders were drawn as previously mentioned. Anterior and posterior limits of corpus callosum were bound by sections that contained intact corpus callosum connecting the two hemispheres in the same coronal plane. Using a 200 x 200um grid with a 50 x 50um counting frame, a 12um optical fractionator probe was used to count NG2$^+$ cells (MicroBrightField, Inc., Williston, VT). CE values are reported in results section.

Statistical Analyses

Prior to statistical analysis, and blind to treatment group, a Grubbs' test was performed to identify potential outliers from each group ($\alpha = 0.05$) and removed no more than a single identified outlier, as defined by the Grubbs' test. Final n are shown in graphs as individual data points, or reported in the F statistic. For behavioral measures, one-way analysis of variance was performed for single time point assessments and repeated measures two-way analysis of variance was performed for multiple time point assessments (rotarod and Morris water maze tasks). Statistical significance was determined as $p < 0.05$. Error bars in figures are SEM.

RESULTS

Experiment 1 - 2 Month Study, Sham, 1 Hit, 5 Hits, or 10 Hits over 10 Days

Rotarod

There was a significant interaction between injury group and latency to fall over time (2-Way repeated measures ANOVA, $F_{(12,172)} = 2.354$, $p = 0.008$, Figure 3.2B). Specifically, mice that received 10 hits over 10 days showed significant impairments compared to sham animals at 1dpi (Tukey post-hoc, $p < 0.01$). There was a main effect of days post-injury on latency to fall ($F_{(4,172)} = 26.81$, $p < 0.0001$) as well as a main effect of group ($F_{(3,43)} = 4.492$, $p = 0.0079$). By 4dpi, there were no significant differences between any group and shams.

Horizontal Ladderbeam

Mice underwent testing on the horizontal ladder beam at 1 month post injury to assess motor function and control. There were no significant effects of group on number of forepaw misses (1-Way ANOVA, $F_{(3,41)} = 1.945$, $p = 0.1375$, Figure 3.2C) as assessed by total number of forepaw foot-faults or misses over three trials along beam rungs.

Elevated Plus Maze

Anxiety-like behavior was assessed using the elevated plus maze task at 1 month post injury. There was a significant effect of group on time spent in open arms (1-Way ANOVA, $F_{(3,42)} = 8.301$, $p = 0.0001$, Figure 3.3B). Specifically, 10 hit mice spent more time in open arms compared to both sham and 1 hit groups (Tukey post-hoc, $p < 0.01$). 5 hit mice also spent significantly more time in open arms compared to sham and 1 hit mice (Tukey post-hoc, $p < 0.05$). There was a significant effect of group on total distance traveled during the

task (1-Way ANOVA, $F_{(3,42)}$ = 6.800, p = 0.0008, Figure 3.3C). Specifically, 10 hit mice traveled more compared to both sham and 1 hit groups (Tukey post-hoc, $p < 0.01$ and $p < 0.05$, respectively). 5 hit mice also traveled more compared to sham and 1 hit mice (Tukey post-hoc, $p < 0.05$). Moreover, there was a significant effect of group on number of entries into open arms (1-Way ANOVA, $F_{(3,43)}$ = 9.327, $p < 0.0001$, Figure 3.3D). Specifically, 10 hit mice had more entries into open arms compared to both sham and 1 hit groups (Tukey post-hoc, $p < 0.01$). 5 hit mice also had more entries into open arms compared to sham and 1 hit mice (Tukey post-hoc, $p < 0.01$). There was no significant effect of group on number of closed arm entries ($F_{(3,41)}$ = 0.3840, p = 0.7651, data not shown).

Morris Water Maze

Mice were tested on the Morris water maze task at 2 months post-injury to assess learning and memory. There was a significant interaction between group and latency to find the platform over 5 days of testing (2-way repeated measures ANOVA, $F_{(12,172)}$ = 1.943, p = 0.0324, Figure 3.3E). There was a main effect of time on time to reach platform ($F_{(4,172)}$ = 17.42, $p < 0.0001$) as well as a main effect of group ($F_{(3,43)}$ = 7.778, p = 0.0003). Mice receiving 10 hits exhibited a significantly longer latency to reach the platform on testing day 4 compared to 1 hit mice (Tukey post-hoc, $p < 0.01$) and on day 5 compared to 1 hit ($p < 0.001$) and sham ($p < 0.05$) mice. Mice receiving 5 hits exhibited a significantly longer latency to reach the platform on testing day 4 compared to 1 hit mice ($p < 0.05$) and on testing day 5 compared to 1 hit mice ($p < 0.01$). On day 6, a probe trial was completed without the platform in the tank. There was a significant effect of group on this probe task (1-Way ANOVA, $F_{(3,43)}$ = 5.898, p =0.0018), where 10-hit mice spent less time in the target

quadrant than 1-hit mice (Tukey post-hoc, $p < 0.01$), and 5-hit mice spent less time in the target quadrant compared to 1-hit (Tukey post-hoc, $p < 0.01$) and sham mice (Tukey post-hoc, $p < 0.05$).

Tail Suspension Test

Depression-like behavior was assessed using the tail suspension test at 1 month post injury. There was no significant difference between any group on time immobile during the task (1-Way ANOVA, $F_{(3,43)} = 2.350$, $p = 0.0857$, data not shown).

Corpus Callosum Volume Quantification

To assess white matter integrity, volumetric analysis was performed using a Cavalieri probe following principles of stereology. There was a significant effect of injury condition on corpus callosum volume (1-Way ANOVA, $F_{(3,20)} = 23.35$, $p < 0.0001$). Mice that received 10 hits and 5 hits had a significantly smaller corpus callosum compared to both sham and 1 hit mice (Tukey post-hoc, $p < 0.001$ for all comparisons, Figure 3.4C). 3D reconstructions were made using StereoInvestigator software to better visualize shape and size of corpus callosum (Figure 3.4D), where noticeable atrophy, especially in the splenium of the corpus callosum, was observed.

There were several relationships between corpus callosum volume and performance on behavioral tasks. Corpus callosum volume was negatively correlated with several measures in the elevated plus maze task at 1 month post injury: time spent in open arms (Pearson correlation, $r^2 = 0.345$, $p = 0.003$, Figure 3.4E), distance traveled ($r^2 = 0.406$, $p = 0.003$, data

not shown), and open arm entries ($r^2 = 0.306$, $p = 0.005$, data not shown). Corpus callosum volume was also negatively correlated with Day 5 latency to platform in the Morris water maze task ($r^2 = 0.185$, $p = 0.036$, Figure 3.4F).

Cortical NeuN$^+$ Neuron Quantification

To assess neuronal loss following rmCHI, an optical fractionator was employed following the principles of stereology to quantify NeuN$^+$ neurons in the cortex. There was a significant effect of group on NeuN$^+$ cortical neurons (1-Way ANOVA, $F_{(3,20)} = 5.693$, $p = 0.0055$, Figure 3.5C). Specifically, 10 hit mice had significantly less NeuN$^+$ neurons compared to both sham and 1 hit groups (Tukey post-hoc, $p < 0.01$ and $p < 0.05$, respectively). NeuN$^+$ neurons were reduced 25% in comparison to controls at 2mpi. Using a Cavalieri probe to quantify the volume assessed as cortex, a significant effect of group on cortical volume was also observed (1-Way ANOVA, $F_{(3,20)} = 9.514$, $p = 0.0004$, data not shown). 10 hit mice had smaller cortical volume compared to both sham and 1 hit controls (Tukey post-hoc, $p < 0.01$). Similarly, 5 hit mice had smaller cortical volumes compared to both sham and 1 hit mice (Tukey post-hoc, $p < 0.01$ and $p < 0.05$, respectively). Normalizing neuronal count to cortical volume reveals no significant effect of group on NeuN+ neurons per mm^3 (1-Way ANOVA, $F_{(3,20)} = 2.384$, $p = 0.0996$, data not shown). NeuN+ neuronal count was negatively correlated with time spent in open arms during the elevated plus maze task at 1 month post injury (Pearson correlation, $r^2 = 0.2779$, $p = 0.0041$, Figure 3.5D) and also latency to platform on day 5 of Morris water maze testing at 2 months post injury ($r^2 = 0.2595$, $p = 0.0055$, Figure 3.5E).

Experiment 2 - 6 Month Study, Sham, 1 Hit, 10 Hit

Elevated Plus Maze

Anxiety-like behavior was assessed using the elevated plus maze task, first at 1 month post-injury (mpi) to replicate data from Experiment 1 and later 6mpi. There was a significant effect of group on time spent in open arms (1-Way ANOVA, $F_{(2,25)} = 18.31$, $p < 0.0001$, Figure 3.6B). Specifically, 10 hit mice spent more time in open arms compared to both sham and 1 hit groups (Tukey post-hoc, $p < 0.0001$). Moreover, there was a significant effect of group on number of entries into open arms (1-Way ANOVA, $F_{(2,25)} = 6.218$, $p = 0.0064$, data not shown). Specifically, 10 hit mice had more entries into open arms compared to both sham and 1 hit groups (Tukey post-hoc, $p < 0.05$ and $p < 0.01$, respectively). There was also a significant effect of group on number of closed arm entries ($F_{(2,25)} = 4.202$, $p = 0.0267$, data not shown). 10 hit mice had fewer entries into closed arms compared to sham mice (Tukey post-hoc, $p < 0.05$). At one-month post-injury, there was no significant effect of group on total distance traveled during the task (1-Way ANOVA, $F_{(2, 25)} = 1.932$, $p = 0.1658$, Figure 3.6C).

Mice were again tested on the elevated plus maze task at 6mpi. There was a significant effect of group on time spent in open arms (1-Way ANOVA, $F_{(2,19)} = 9.960$, $p = 0.0011$, Figure 3.6B). Specifically, 10 hit mice spent more time in open arms compared to both sham and 1 hit groups (Tukey post-hoc, $p < 0.01$). Moreover, there was a significant effect of group on number of entries into open arms (1-Way ANOVA, $F_{(2,18)} = 26.48$, $p < 0.0001$, data not shown). Specifically, 10 hit mice had more entries into open arms compared to both sham and 1 hit groups (Tukey post-hoc, $p < 0.001$ and $p < 0.0001$, respectively).

However, there was no effect of group on number of closed arm entries ($F_{(2,19)} = 0.2867$, $p = 0.7539$, data not shown). In contrast to the 1mpi data, there was a significant effect of group on total distance traveled during the task (1-Way ANOVA, $F_{(2, 19)} = 10.26$, $p = 0.0010$, Figure 3.6C). Specifically, 10 hit mice traveled more during the task compared to both sham and 1 hit groups (Tukey post-hoc, $p < 0.01$).

Forced Swim Test

Mice were tested on the forced swim test to assess depressive-like behavior at, and again at 6mpi. There were no significant effects of group on time not moving (1-Way ANOVA, $F_{(2,25)} = 2.107$, $p = 0.1426$, Figure 3.6D), 'immobile' time (1-Way ANOVA, $F_{(2,25)} = 3.375$, $p < 0.0504$, data not shown), or 'mobile' time (1-Way ANOVA, $F_{(2,25)} = 2.114$, $p = 0.1418$, data not shown) at 2mpi. However, there was a significant effect of group on 'high mobility' time (1-Way ANOVA, $F_{(2,25)} = 3.542$, $p = 0.0442$, Figure 3.6E), where 10 hit mice had more time spent as 'high mobility' compared to sham mice (Tukey post-hoc, $p < 0.05$) at 2mpi.

Mice were again tested on the forced swim test to assess depressive-like behaviors at 6 months post-injury. At this later timepoint, there was a significant effect of group on time not moving (1-Way ANOVA, $F_{(2,19)} = 15.62$, $p < 0.0001$, Figure 3.6D), where 10 hit mice spent less time 'not moving' than both sham and 1 hit groups (Tukey post-hoc, $p < 0.001$). There was a significant effect of group on 'immobile' time (1-Way ANOVA, $F_{(2,19)} = 24.01$, $p < 0.0001$, data not shown), where 10 hit mice spent less time 'immobile' than both sham and 1 hit groups (Tukey post-hoc, $p < 0.0001$). There was a significant effect of group on 'mobile' time (1-Way ANOVA, $F_{(2,19)} = 19.76$, $p < 0.0001$, data not shown), where 10 hit mice

spent more time 'mobile' than both sham and 1 hit groups (Tukey post-hoc, $p < 0.0001$). There was a significant effect of group on 'high mobility' time (1-Way ANOVA, $F_{(2,17)} = 15.21$, $p = 0.0002$, Figure 3.6E), where 10 hit mice spent more time having 'high mobility' than both sham and 1 hit groups (Tukey post-hoc, $p < 0.01$ and $p < 0.001$, respectively).

Morris Water Maze

Mice were tested on the Morris water maze task at 6 months post-injury to assess long-term effects of injury on learning and memory. There was a significant interaction between group and latency to find the platform over 5 days of testing (2-way repeated measures ANOVA, $F_{(8,76)} = 2.858$, $p = 0.0078$, Figure 3.6F). There was a main effect of time on time to reach platform ($F_{(4,76)} = 25.27$, $p < 0.0001$) as well as a main effect of group ($F_{(2,19)} = 5.264$, $p = 0.0152$). Mice receiving 10 hits had significantly longer latency to reach platform on testing day 3 compared to sham mice (Tukey post-hoc, $p < 0.01$), on testing day 4 compared to sham ($p < 0.05$) and 1 hit ($p < 0.01$) mice, and on day 5 compared to both sham and 1 hit mice ($p < 0.01$). During the probe trial, mice with 10 hits spent less time in the target quadrant than both sham and 1 hit animals (1-Way ANOVA, $F_{(2,18)} = 8.085$, $p < 0.0031$, Tukey post-hoc, $p < 0.01$, Figure 3.6G).

Corpus Callosum Volume Quantification

To assess white matter integrity, volumetric analysis was performed using a Cavalieri probe following principles of stereology. There was a significant effect of injury condition on corpus callosum volume (1-Way ANOVA, $F_{(2,15)} = 9.617$, $p = 0.0021$, Figure 3.6H) at 6-months post injury. Mice that received 10 hits had significantly smaller corpus callosum

compared to both sham and 1 hit mice (Tukey post-hoc, $p < 0.01$, $p < 0.05$); corpus callosum volume was reduced 35% compared to sham controls. Corpus callosum volume negatively correlates with latency to time in open arms during the elevated plus maze at 2MPI ($r^2 = 0.6083$, $p = 0.0001$, Figure 3.6H).

NG2$^+$ Quantification

Stereological quantification via optical fractionator probe was performed to assess how many NG2$^+$ oligodendrocyte precursors were in the corpus callosum of injured and uninjured mice. There was no significant effect of absolute number of NG2$^+$ cells between groups (1-way ANOVA, $F_{(2,15)}=1.012$, $p=0.3871$, data not shown). However, there was a strong correlation between NG2$^+$ cells and corpus callosum volume at 6MPI (Figure 3.6I, $r^2=0.5781$, $p=0.0002$) whereby there were more NG2$^+$ cells in larger *corp**ora** collosa*. CE values for sham, 1 hit, and 10 hit groups averaged 0.13, 0.17, and 0.16 respectively.

For a summary the effects of rmCHI on a range of behavioral assessments, see Table 3.1.

Gliosis and Neuroinflammation

Following rmCHI, extensive glial activation and inflammation were observed at both 2MPI and 6MPI. Specifically, GFAP+ astrocytes (Figure 3.7) and Iba+ microglial (Figure 3.8) were observed in 10 hit animals, particularly localized to corpus callosum tracts.

DISCUSSION

Repeat mild TBI is not "one" disease. Traumatic brain injury is often used as a term to describe head injuries encompassing an extremely wide range of precipitating events, followed by an equally wide range of cognitive and pathological consequences. To better understand traumatic brain injury, many experimental animal models have been developed to recapitulate specific consequences of head injuries, depending on the model chosen. When modeling TBI and rmTBI in rodents, it is impossible to account for every injury type or every injury sequelae due to the variability of injuries. While it may seem useful to have one universal rodent model, we would be severely restricting our research efforts to standardize on one. It is therefore useful to have multiple models of traumatic brain injury that have their own characteristics relevant to a specific clinical injury phenotype – provided those models have been well characterized and recapitulate specific features of the human condition. We chose to combine a well controlled cortical impact model with a freely moving head so that rotational/axon shearing forces were combined with CCI. Our goal was to replicate *chronic* symptoms and pathologies clinically similar to that of patients with repeated closed head mild traumatic brain injuries and to correlate behavioral function with measures of pathology.

A reproducible, closed head, repeat mild concussion model of rmCHI with long-term behavioral deficits. The model of rmCHI presented here induces long-term cognitive deficits, white matter atrophy, cortical neuronal loss, demyelination and chronic neuroinflammation in the corpus callosum. These pathological changes are correlated with behavioral deficits observed up to 6 months post injury, demonstrating the chronic,

additive nature of mild head injury. This model utilizes components of commercially available equipment allowing for standardization across laboratories. The TBI-0310, controlled cortical impact device, facilitates controlled velocity, depth, and dwell time for repeatable, reproducible impacts. Avoiding use of a fixed head stereotaxic position and using a base platform of Marmarou foam allowed for rotational forces in the anterior-posterior axis and coup-countercoup intracranial impacts. Another benefit of this procedure is the reduced time under anesthesia (7 minutes), minimizing confounding neuroprotective effects of isoflurane (Statler et al., 2006; Statler et al., 2000). There is low behavioral and pathology variability within groups, high reproducibility across users and labs (unpublished), and the procedure was calibrated such that a single impact causes no readily detectable behavioral or pathological changes, while repeated impacts produce deficits.

Current rodent models of rmTBI. To mimic repeated mild traumatic brain in rodents, several models have already been developed and characterized. The most common component in these models is delivery of a blunt trauma induced by either a free falling weight (Kane et al., 2012; Mannix et al., 2013; Meehan et al., 2012) or a modified pneumatic or electromagnetic driven controlled cortical impact device (Laurer et al., 2001; Prins et al., 2010; Shitaka et al., 2011; Uryu et al., 2002). In a weight drop model, different bases of support have been tested, including a kimwipe (Meehan et al., 2012) or aluminum foil (Kane et al., 2012) to allow for rotational forces following impact. In models involving pneumatic or electromagnetic devices, animals are most often affixed in a stereotaxic frame (Mouzon et al., 2012; Mouzon et al., 2014; Shitaka et al., 2011). Many models make a skin

incision and hit the skull directly, while others affix a disk to the skull. Others opt for a more complete 'closed head injury' by impacting the skin directly without an incision. A model using unanesthestized mice placed in a restraint bag and impacted unilaterally has recently been described for repeated injuries, producing behavioral changes at 1MPI and 6MPI, however no pathology or histological data was reported in this model (Petraglia et al., 2014). Across all rodent models, the impactor tip varies widely, ranging from a 3-6mm in diameter with several top coatings including metal, rubber, and lacrosse ball rubber. Standardization of experimental design across laboratories remains elusive, but the present model offers the possibility of easy replication by others using standard and readily available commercial equipment. Furthermore, none of the studies cited above examined the relationship between behavioral outcome measures and underlying neuropathology levels.

Long-term cognitive and behavioral deficits following repeated mild closed head injury.

Clinically, repeated head injuries can result in a range of symptoms. Patients who have had a history of TBI frequently develop depression (33%) with co-morbidities including anxiety (77%) and aggressive behavior (57%) (Jorge et al., 2004). Patients have also reported deficits in working memory function (Collins et al., 1999a; Collins et al., 1999b; Iverson et al., 2006). The rmCHI model presented here results in robust behavioral changes observable at chronic time points, tested as far as 6 months post injury. Importantly, a single impact at the standardized injury parameters we report here (5m/s and 1mm depth) does not result in detectable behavioral changes, supporting the argument that *repeated*

injuries of a given mild severity can lead to impairments as the result of an additive or cumulative effect. When tested on the Morris water maze, mice receiving either 5 or 10 hits took longer to find the hidden platform over 5 days of testing at both 2 and 6 months post injury. Mice receiving a single hit were able to learn the task, but animals of both rmCHI groups were unable to learn the platform location from the surrounding spatial cues. Learning the water maze task is a dorsal hippocampus-mediated task (Moser et al., 1993). Although no gross hippocampal changes were observed in these animals, it is possible that further analysis of hippocampal neuron counts and/or synapse quantification could provide more insight for the role of these learning deficits following rmCHI in association with hippocampal anatomy.

Deficits in learning at chronic time-points post repeated head injury have been reported before in the water maze (Mannix et al., 2013; Meehan et al., 2012). Using a weight drop model of rmCHI, mice with 5 daily or 5 weekly, but not 5 monthly, head impacts had deficits in the Morris water maze (Mannix et al., 2013). Moreover, this study also observed deficits in the task persisting up to 1 year post-injury. In a modified CCI mCHI injury, where animals received 2 impacts over 2 days, deficits in learning were observed at 7 weeks post injury (Shitaka et al., 2011). None of these studies correlated water maze performance with associated neuropathologies. Models of rmCHI with long-lasting deficits are critical for future intervention studies and for understanding CTE.

We would expect an anxiety-like phenotype to be represented by less time in open arms (Lister, 1987). However, rmCHI in our model created the opposite response. Performance

on the elevated plus maze is considered to probe an animal's level of anxiety whereas the extent of movement versus immobility on forced swim test is considered a surrogate for depression (Petit-Demouliere et al., 2005). At both 1 month and 6 months post injury, mice receiving rmCHI displayed significant changes in both anxiety and depression-linked behavioral tasks; in contrast, mice with a signal hit exhibited no detectable deficits. On the elevated plus maze test, mice with rmCHI were hyperactive, spent *more* time in open arms, and had more open arm entries. This indicates an overall hyperactive state as well as a varied risk profile (e.g. based on risk assessment, decision-making, exploration, and vertical activity) (Rodgers and Johnson, 1995). Common long-term sequelae of traumatic brain injury are disinhibition and aggression (Arciniegas and Wortzel, 2014). This suggests that mice in this model of rmCHI do not display a classic anxiety-like phenotype on these tests, but rather, exhibit a hyperactive, less risk-aversion phenotype. In support of this possibility, we also observed a hyperactive state in the forced swim test at 2 and 6 months post injury (Figure 3.6). Opposite to the predicted classic depressive-like behavior of more immobile time, animals that experienced rmCHI displayed more moving time as well more 'highly mobile' time. These mice responded more aggressively to the task and tried to escape the test more so than uninjured or single hit animals. While counter-intuitive, viewed from the perspective of emotional "dyscontrol" common in individuals with TBI, the phenotype we observed in this model of rmTBI has some external validity and argues for more thorough testing of emotional and behavioral in mice post-rmTBI.

Similar changes in anxiety-like behavior on the elevated plus maze has also been reported at chronic time points of 1 and 6 months following rmCHI (Petraglia et al., 2014) where

animals spent significantly more time in open arms (as observed here), however no data on hyperactivity following rmCHI on this test has been previously reported. Important to an understanding of the development of cognitive deficits, chronic time points assessed at greater than 1 month post injury are vital to investigating disease progression and sustained or even increasing deficits over time (Gold et al., 2013). Interestingly, a slight progression of behavioral deficits was observed in the re-test of the forced swim test at 6MPI vs 2MPI, whereas deficits in performance on elevated plus maze and Morris water maze remained constant. Discovery of behavioral measures that show disease progression over time will be valuable in linking cognitive changes and with neuropathological progression.

Long-term neuropathological findings post rmCHI. Our model of repeated mild closed head injury results in reproducible behavioral and pathological consequences. Animals receiving rmCHI exhibited significant white matter atrophy of the corpus callosum. Atrophy was observed as early as 2 month post-injury, and remained 6 months following injury. This 33-35% loss of volume was associated with demyelination as well as chronic astroglial neuroinflammation and reactive microglia (Figure 3.7 and 3.8). Chronic neuroinflammation at both 6 and 12 months post injury has been previously reported in other experimental models of rmCHI as well (Mouzon et al., 2012; Shitaka et al., 2011). In addition, we observe significant cortical neuronal loss following rmCHI (25%). Loss of neurons was associated with cortical volume loss, while no changes in neuronal density were observed. Future studies should focus on quantification and assessment of a variety

of cell subpopulations, including both immature and mature oligodendrocytes, as well as cortical layer segmented analysis.

What are the underlying mechanisms leading to white matter atrophy and cortical neuron loss? One hypothesis is that cortical neurons projecting through the corpus callosum have become demyelinated, or the neurons and axonal projections through the corpus callosum have died. Importantly, we did in fact observe cortical neuronal loss in areas sending contralateral projections. Demyelination can occur due to several mechanisms as a result of injury. Mature, myelinating oligodendrocytes could be selectively more susceptible to physical trauma, or fail to remyelinate disrupted or demyelinated axons. Or multiple mechanisms may be active. Upon oligodendrocyte death, oligodendrocyte progenitor cell (OPC) populations migrate to the site, divide, and differentiate into newborn oligodendrocytes capable of repopulating lesions and subsequently remyelinating axons (Franklin and Ffrench-Constant, 2008; Kirby et al., 2006). OPCs may not remyelinate completely, but axons could be remyelinated with several looser than normal wraps of myelin sheath; such axons would be physiologically impaired. Another possibility is a dysfunction of oligodendrocyte progenitor cell populations. OPCs are evenly distributed throughout the brain, including the corpus callosum. Upon chemically-induced lesions, OPCs migrate to these sites, differentiate and remyelinate (Skripuletz et al., 2011). rmCHI could also possibly lead to failed signaling of OPC migration to required injured areas. If migration is successful in these rmCHI induced lesions, cell division and differentiation must also occur, which may also be impeded following these injuries.

$NG2^+$ oligodendrocytes have been shown to proliferate up to 7 days post traumatic brain injury in an experimental rodent model, but proliferation subsides by 21DPI (Flygt et al., 2017). To our knowledge, no $NG2^+$ quantifications have been performed in a chronic phase up to 6MPI following an experimental rodent model of rmTBI. The data presented here, showing no change in absolute $NG2^+$ oligodendrocyte precursors, but an overall correlation of $NG2^+$ cells with corpus callosum volume, provides a snapshot to the injury response in white matter tracts. At 6MPI, we demonstrate that the overall density of $NG2^+$ cells relative to volume remains constant. These data suggest that the damage occurring in white matter following this experimental model of rmTBI is not due to a lack of oligodendrocyte progenitor cells. Further experiments aimed at understanding the temporal dynamics of $NG2^+$ oligodendrocyte precursor death, proliferation, and/or migration in the corpus callosum following rmTBI in relation to axonal pathologies will help us better understand the underlying pathological sequelae.

Linking neuropathology to behavioral changes. The link between white matter disruption and cognitive/emotional impairments has yet to be clearly elucidated in either humans or rodents. Many clinical studies have focused on investigating associations between diffusion-tensor imaging (DTI), particularly in white matter tracts, with emotional and cognitive changes following TBI (Cubon et al., 2011; Kraus et al., 2007; Shenton et al., 2012). The corpus callosum is the major thoroughfare for cortical neuronal commisural fibers between hemispheres. Even a single traumatic brain injury can lead to chronic white matter inflammation and atrophy in humans (Johnson et al., 2013a). A 25% reduction in corpus callosum was observed in patients suffering from a single traumatic brain injury.

Inflammation is a consistent feature of neurotrauma (McKee and Lukens, 2016). In Rag1(-/-) mice, which lack mature T- and B-cells, there were no observed differences in injury response or neurological score in the acute phase of injury (≤7DPI) (Weckbach et al., 2012). In contrast, chronic microgliosis in humans has been observed post-TBI, especially in white matter tracts (Smith et al., 2013). The contribution of microgliosis to axonal injury has yet to be fully understood. Chronic $Iba1^+$ reactive microglia have been observed post TBI in rodents, especially in white matter tracts following two mild closed head TBIs (Shitaka et al., 2011). However, in a mouse model of rmCHI, acute administration of valganciclovir to CD11b-thymidine kinase transgenic mice depleting macrophages had no effect on chronic axonal injury (Bennett and Brody, 2014). In addition, chronic $Iba1^+$ microglia activation has been reported in white matter tracts including corpus callosum, olfactory nerve layer, optic tract and the brachium of superior colliculus in the CHIMERA model (Namjoshi et al., 2014). Future studies investigating chronic microgliosis like that observed in our experimental model may shed light on its contribution to white matter atrophy and/or cognitive deficits.

There are shortcomings of any model and experiment, the present study included. For example, we do not know what the minimum threshold for injury is such that pathological or cognitive deficits are observed after 5 or 10 hits, but not after 1? A more precise threshold for impactor velocity, depth of injury, and timing between injuries of injury severity should be defined to better classify the injury as *mild, moderate or severe* and to allow others to calibrate the level of injury. We would argue that any combination of parameters that results in undetectable functional or pathological consequences is by

definition mild. Future experiments will decrease injury parameters such as speed, depth, and number of impacts, to determine the minimal settings required to observe both cognitive and pathological consequences. In this experiment, 5 and 10 impacts were sufficient at 5.0 m/s speed and 1mm depth of impacts.

In humans, acute trauma results in one set of repercussions and secondary degeneration. But we are now learning that the consequences of concussion, and particularly repeated or multiple concussions, such as those sustained by athletes in a wide range of sports have their own sequelae of repercussions, leading to increased risk of developing chronic traumatic encephalopathy. Animal models that can mirror more facets of CTE will become increasingly valuable and enable us to explore the potential to block degenerative pathways or enhance regeneration post-concussion. This study lays the groundwork for further exploring the link between rmCHI, neuroinflammation, and the association of discrete neuropathological features with cognitive and emotional alterations.

ACKNOWLEDGEMENTS

The preceding studies were supported by a grant from the California Institute for Regenerative Medicine (CIRM): Early Translational Award TR2-01767 awarded to BJC, and from Predoctoral Fellowships from the National Institutes of Health for 'Training in the Neurobiology of Aging' (T32 AG00096-30) and for 'Training Program in Stem Cell Translational Medicine for Neurological Disorders' (T32 NS082174-01) awarded to EMG.

REFERENCES

Arciniegas, D.B., Wortzel, H.S., 2014. Emotional and behavioral dyscontrol after traumatic brain injury. Psychiatr Clin North Am 37, 31-53.

Baskaya, M.K., Rao, A.M., Dogan, A., Donaldson, D., Dempsey, R.J., 1997. The biphasic opening of the blood-brain barrier in the cortex and hippocampus after traumatic brain injury in rats. Neuroscience letters 226, 33-36.

Bennett, R.E., Brody, D.L., 2014. Acute Reduction of Microglia Does Not Alter Axonal Injury in a Mouse Model of Repetitive Concussive Traumatic Brain Injury. Journal of neurotrauma 17, 1-17.

Collins, M.W., Grindel, S.H., Lovell, M.R., Dede, D.E., Moser, D.J., Phalin, B.R., Nogle, S., Wasik, M., Cordry, D., Daugherty, K.M., Sears, S.F., Nicolette, G., Indelicato, P., McKeag, D.B., 1999a. Relationship between concussion and neuropsychological performance in college football players. JAMA 282, 964-970.

Collins, M.W., Lovell, M.R., McKeag, D.B., 1999b. Current issues in managing sports-related concussion. JAMA 282, 2283-2285.

Coronado, V.G., McGuire, L.C., Sarmiento, K., Bell, J., Lionbarger, M.R., Jones, C.D., Geller, A.I., Khoury, N., Xu, L., 2012. Trends in Traumatic Brain Injury in the U.S. and the public health response: 1995-2009. J Safety Res 43, 299-307.

Cubon, V.A., Putukian, M., Boyer, C., Dettwiler, A., 2011. A diffusion tensor imaging study on the white matter skeleton in individuals with sports-related concussion. J Neurotrauma 28, 189-201.

Cummings, B.J., Engesser-Cesar, C., Cadena, G., Anderson, A.J., 2007. Adaptation of a ladder beam walking task to assess locomotor recovery in mice following spinal cord injury. Behav Brain Res 177, 232-241.

Faul, M., Xu, L., Wald, M.M., Coronado, V.G., 2010. Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations and Deaths 2002-2006, Available: http://www.cdc.gov/traumaticbraininjury/pdf/blue_book.pdf.

Flygt, J., Clausen, F., Marklund, N., 2017. Diffuse traumatic brain injury in the mouse induces a transient proliferation of oligodendrocyte progenitor cells in injured white matter tracts. Restorative neurology and neuroscience 35, 251-263.

Franklin, R.J., Ffrench-Constant, C., 2008. Remyelination in the CNS: from biology to therapy. Nat Rev Neurosci 9, 839-855.

Gerberding, J.L., Binder, S., 2003. Report to Congress on Mild Traumatic Brain Injury in the United States: Steps to Prevent a Serious Public Health Problem. Centers for Disease Control and Prevention, Atlanta, GA.

Gold, E.M., Su, D., Lopez-Velazquez, L., Haus, D.L., Perez, H., Lacuesta, G.A., Anderson, A.J., Cummings, B.J., 2013. Functional assessment of long-term deficits in rodent models of traumatic brain injury. Regen Med 8, 483-516.

Greve, M.W., Zink, B.J., 2009. Pathophysiology of traumatic brain injury. Mt Sinai J Med 76, 97-104.

Haus, D.L., Lopez-Velazquez, L., Gold, E.M., Cunningham, K.M., Perez, H., Anderson, A.J., Cummings, B.J., 2016. Transplantation of human neural stem cells restores cognition in an immunodeficient rodent model of traumatic brain injury. Experimental neurology 281, 1-16.

Henderson, V.C., Kimmelman, J., Fergusson, D., Grimshaw, J.M., Hackam, D.G., 2013. Threats to validity in the design and conduct of preclinical efficacy studies: a systematic review of guidelines for in vivo animal experiments. PLoS Med 10, e1001489.

Herring, S.A., Cantu, R.C., Guskiewicz, K.M., Putukian, M., Kibler, W.B., Bergfeld, J.A., Boyajian-O'Neill, L.A., Franks, R.R., Indelicato, P.A., American College of Sports, M., 2011. Concussion (mild traumatic brain injury) and the team physician: a consensus statement--2011 update. Med Sci Sports Exerc 43, 2412-2422.

Hicks, R.R., Smith, D.H., Lowenstein, D.H., Saint Marie, R., McIntosh, T.K., 1993. Mild experimental brain injury in the rat induces cognitive deficits associated with regional neuronal loss in the hippocampus. J Neurotrauma 10, 405-414.

Iverson, G.L., Brooks, B.L., Collins, M.W., Lovell, M.R., 2006. Tracking neuropsychological recovery following concussion in sport. Brain Inj 20, 245-252.

Johnson, V.E., Stewart, J.E., Begbie, F.D., Trojanowski, J.Q., Smith, D.H., Stewart, W., 2013a. Inflammation and white matter degeneration persist for years after a single traumatic brain injury. Brain 136, 28-42.

Johnson, V.E., Stewart, W., Smith, D.H., 2013b. Axonal pathology in traumatic brain injury. Experimental neurology 246, 35-43.

Jorge, R.E., Robinson, R.G., Moser, D., Tateno, A., Crespo-Facorro, B., Arndt, S., 2004. Major depression following traumatic brain injury. Arch Gen Psychiatry 61, 42-50.

Kane, M.J., Angoa-Pérez, M., Briggs, D.I., Viano, D.C., Kreipke, C.W., Kuhn, D.M., 2012. A mouse model of human repetitive mild traumatic brain injury. Journal of Neuroscience Methods 203, 41-49.

Kirby, B.B., Takada, N., Latimer, A.J., Shin, J., Carney, T.J., Kelsh, R.N., Appel, B., 2006. In vivo time-lapse imaging shows dynamic oligodendrocyte progenitor behavior during zebrafish development. Nat Neurosci 9, 1506-1511.

Kraus, M.F., Susmaras, T., Caughlin, B.P., Walker, C.J., Sweeney, J.A., Little, D.M., 2007. White matter integrity and cognition in chronic traumatic brain injury: a diffusion tensor imaging study. Brain 130, 2508-2519.

Langlois, J.A., Sattin, R.W., 2005. Traumatic brain injury in the United States: research and programs of the Centers for Disease Control and Prevention (CDC). J Head Trauma Rehabil 20, 187-188.

Laskowski, R.A., Creed, J.A., Raghupathi, R., 2015. Pathophysiology of Mild TBI: Implications for Altered Signaling Pathways, in: Kobeissy, F.H. (Ed.), Brain Neurotrauma: Molecular, Neuropsychological, and Rehabilitation Aspects, Boca Raton (FL).

Laurer, H.L., Bareyre, F.M., Lee, V.M., Trojanowski, J.Q., Longhi, L., Hoover, R., Saatman, K.E., Raghupathi, R., Hoshino, S., Grady, M.S., McIntosh, T.K., 2001. Mild head injury increasing the brain's vulnerability to a second concussive impact. Journal of neurosurgery 95, 859-870.

Lister, R.G., 1987. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 92, 180-185.

Malkesman, O., Tucker, L.B., Ozl, J., McCabe, J.T., 2013. Traumatic brain injury - modeling neuropsychiatric symptoms in rodents. Front Neurol 4, 157.

Mannix, R., Meehan, W.P., Mandeville, J., Grant, P.E., Gray, T., Berglass, J., Zhang, J., Bryant, J., Rezaie, S., Chung, J.Y., Peters, N.V., Lee, C., Tien, L.W., Kaplan, D.L., Feany, M., Whalen, M., 2013. Clinical correlates in an experimental model of repetitive mild brain injury. Annals of Neurology 74, 65-75.

McKee, A.C., Cantu, R.C., Nowinski, C.J., Hedley-Whyte, E.T., Gavett, B.E., Budson, A.E., Santini, V.E., Lee, H.S., Kubilus, C.A., Stern, R.A., 2009. Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. J Neuropathol Exp Neurol 68, 709-735.

McKee, C.A., Lukens, J.R., 2016. Emerging Roles for the Immune System in Traumatic Brain Injury. Front Immunol 7, 556.

Meehan, W.P., Zhang, J., Mannix, R., Whalen, M.J., 2012. Increasing recovery time between injuries improves cognitive outcome after repetitive mild concussive brain injuries in mice. Neurosurgery 71, 885-891.

Moser, E., Moser, M.B., Andersen, P., 1993. Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions. J Neurosci 13, 3916-3925.

Mouzon, B., Chaytow, H., Crynen, G., Bachmeier, C., Stewart, J., Mullan, M., Stewart, W., Crawford, F., 2012. Repetitive mild traumatic brain injury in a mouse model produces

learning and memory deficits accompanied by histological changes. J Neurotrauma 29, 2761-2773.

Mouzon, B.C., Bachmeier, C., Ferro, A., Ojo, J.O., Crynen, G., Acker, C.M., Davies, P., Mullan, M., Stewart, W., Crawford, F., 2014. Chronic neuropathological and neurobehavioral changes in a repetitive mild traumatic brain injury model. Ann Neurol 75, 241-254.

Mul, J.D., Zheng, J., Goodyear, L.J., 2016. Validity Assessment of 5 Day Repeated Forced-Swim Stress to Model Human Depression in Young-Adult C57BL/6J and BALB/cJ Mice. eNeuro 3.

Multani, N., Goswami, R., Khodadadi, M., Ebraheem, A., Davis, K.D., Tator, C.H., Wennberg, R., Mikulis, D.J., Ezerins, L., Tartaglia, M.C., 2016. The association between white-matter tract abnormalities, and neuropsychiatric and cognitive symptoms in retired professional football players with multiple concussions. J Neurol 263, 1332-1341.

Namjoshi, D.R., Cheng, W.H., McInnes, K.A., Martens, K.M., Carr, M., Wilkinson, A., Fan, J., Robert, J., Hayat, A., Cripton, P.A., Wellington, C.L., 2014. Merging pathology with biomechanics using CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration): a novel, surgery-free model of traumatic brain injury. Mol Neurodegener 9, 55.

Pellow, S., File, S.E., 1986. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. Pharmacol Biochem Behav 24, 525-529.

Petit-Demouliere, B., Chenu, F., Bourin, M., 2005. Forced swimming test in mice: a review of antidepressant activity. Psychopharmacology (Berl) 177, 245-255.

Petraglia, A.L., Plog, B.A., Dayawansa, S., Chen, M., Dashnaw, M.L., Czerniecka, K., Walker, C.T., Viterise, T., Hyrien, O., Iliff, J.J., Deane, R., Nedergaard, M., Huang, J.H., 2014. The spectrum of neurobehavioral sequelae after repetitive mild traumatic brain injury: a novel mouse model of chronic traumatic encephalopathy. J Neurotrauma 31, 1211-1224.

Prins, M.L., Hales, A., Reger, M., Giza, C.C., Hovda, D.A., 2010. Repeat traumatic brain injury in the juvenile rat is associated with increased axonal injury and cognitive impairments. Dev Neurosci 32, 510-518.

Raghupathi, R., 2004. Cell death mechanisms following traumatic brain injury. Brain Pathol 14, 215-222.

Ramlackhansingh, A.F., Brooks, D.J., Greenwood, R.J., Bose, S.K., Turkheimer, F.E., Kinnunen, K.M., Gentleman, S., Heckemann, R.A., Gunanayagam, K., Gelosa, G., Sharp, D.J., 2011. Inflammation after trauma: microglial activation and traumatic brain injury. Ann Neurol 70, 374-383.

Rodgers, R.J., Johnson, N.J., 1995. Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacol Biochem Behav 52, 297-303.

Rutland-Brown, W., Langlois, J.A., Thomas, K.E., Xi, Y.L., 2006. Incidence of traumatic brain injury in the United States, 2003. J Head Trauma Rehabil 21, 544-548.

Shenton, M.E., Hamoda, H.M., Schneiderman, J.S., Bouix, S., Pasternak, O., Rathi, Y., Vu, M.A., Purohit, M.P., Helmer, K., Koerte, I., Lin, A.P., Westin, C.F., Kikinis, R., Kubicki, M., Stern, R.A., Zafonte, R., 2012. A review of magnetic resonance imaging and diffusion tensor imaging findings in mild traumatic brain injury. Brain imaging and behavior 6, 137-192.

Shitaka, Y., Tran, H.T., Bennett, R.E., Sanchez, L., Levy, M.A., Dikranian, K., Brody, D.L., 2011. Repetitive closed-skull traumatic brain injury in mice causes persistent multifocal axonal injury and microglial reactivity. J Neuropathol Exp Neurol 70, 551-567.

Skripuletz, T., Gudi, V., Hackstette, D., Stangel, M., 2011. De- and remyelination in the CNS white and grey matter induced by cuprizone: the old, the new, and the unexpected. Histol Histopathol 26, 1585-1597.

Smith, C., Gentleman, S.M., Leclercq, P.D., Murray, L.S., Griffin, W.S.T., Graham, D.I., Nicoll, J.A.R., 2013. The neuroinflammatory response in humans after traumatic brain injury. Neuropathology and Applied Neurobiology 39, 654-666.

Statler, K.D., Alexander, H., Vagni, V., Holubkov, R., Dixon, C.E., Clark, R.S., Jenkins, L., Kochanek, P.M., 2006. Isoflurane exerts neuroprotective actions at or near the time of severe traumatic brain injury. Brain Res 1076, 216-224.

Statler, K.D., Kochanek, P.M., Dixon, C.E., Alexander, H.L., Warner, D.S., Clark, R.S., Wisniewski, S.R., Graham, S.H., Jenkins, L.W., Marion, D.W., Safar, P.J., 2000. Isoflurane improves long-term neurologic outcome versus fentanyl after traumatic brain injury in rats. J Neurotrauma 17, 1179-1189.

Tremblay, S., Henry, L.C., Bedetti, C., Larson-Dupuis, C., Gagnon, J.F., Evans, A.C., Theoret, H., Lassonde, M., De Beaumont, L., 2014. Diffuse white matter tract abnormalities in clinically normal ageing retired athletes with a history of sports-related concussions. Brain 137, 2997-3011.

Uryu, K., Laurer, H., McIntosh, T., Praticò, D., Martinez, D., Leight, S., Lee, V.M.Y., Trojanowski, J.Q., 2002. Repetitive Mild Brain Trauma Accelerates Aβ Deposition, Lipid Peroxidation, and Cognitive Impairment in a Transgenic Mouse Model of Alzheimer Amyloidosis. The Journal of Neuroscience 22, 446-454.

Weckbach, S., Neher, M., Losacco, J.T., Bolden, A.L., Kulik, L., Flierl, M.A., Bell, S.E., Holers, V.M., Stahel, P.F., 2012. Challenging the role of adaptive immunity in neurotrauma: Rag1(-/-) mice lacking mature B and T cells do not show neuroprotection after closed head injury. J Neurotrauma 29, 1233-1242.

Behavioral Task	Timepoint	Significant Injury Effect?	Summary
Rotarod	1DPI	**Yes**	Deficits at 1DPI for 10 Hit group: 10 vs Sham
Rotarod	4DPI	No	
Rotarod	10DPI	No	
Rotarod	21DPI	No	
Rotarod	35DPI	No	
Elevated Plus Maze (Ambient Light)	1MPI	**Yes**	10 Hits & 5 Hits vs 1 and Sham: More time in Open Arm, More distance traveled, More Open Arm Entires; Closed Arm entries N.S.
Elevated Plus Maze (Dark Room)	1MPI	**Yes**	10 Hits vs 1 and Sham: More time in Open Arm, Trend for more distance traveled (N.S.)
Horizontal Ladderbeam	1MPI	No	N.S.
Tail Suspension Test	1MPI	No	N.S.
Forced Swim Test	2MPI	**Yes**	10 Hit vs Sham: More time 'High Mobility'
Morris Water Maze	2MPI	**Yes**	10 Hits & 5 Hits vs 1 and Sham: Deficits in learning platform location (latency to reach platform)
Elevated Plus Maze (Dark Room)	6MPI	**Yes**	10 Hits vs 1 and Sham: More time in Open Arm, More distance traveled
Forced Swim Test	6MPI	**Yes**	10 Hit vs 1 and Sham: Less time 'Not Moving', More time 'High Mobility'
Morris Water Maze	6MPI	**Yes**	10 Hit vs 1 and Sham: Deficits in learning platform location (latency to reach platform)

Table 3.1. Summary of deficits observed on behavioral tasks assessed following repeated mild closed head injury.

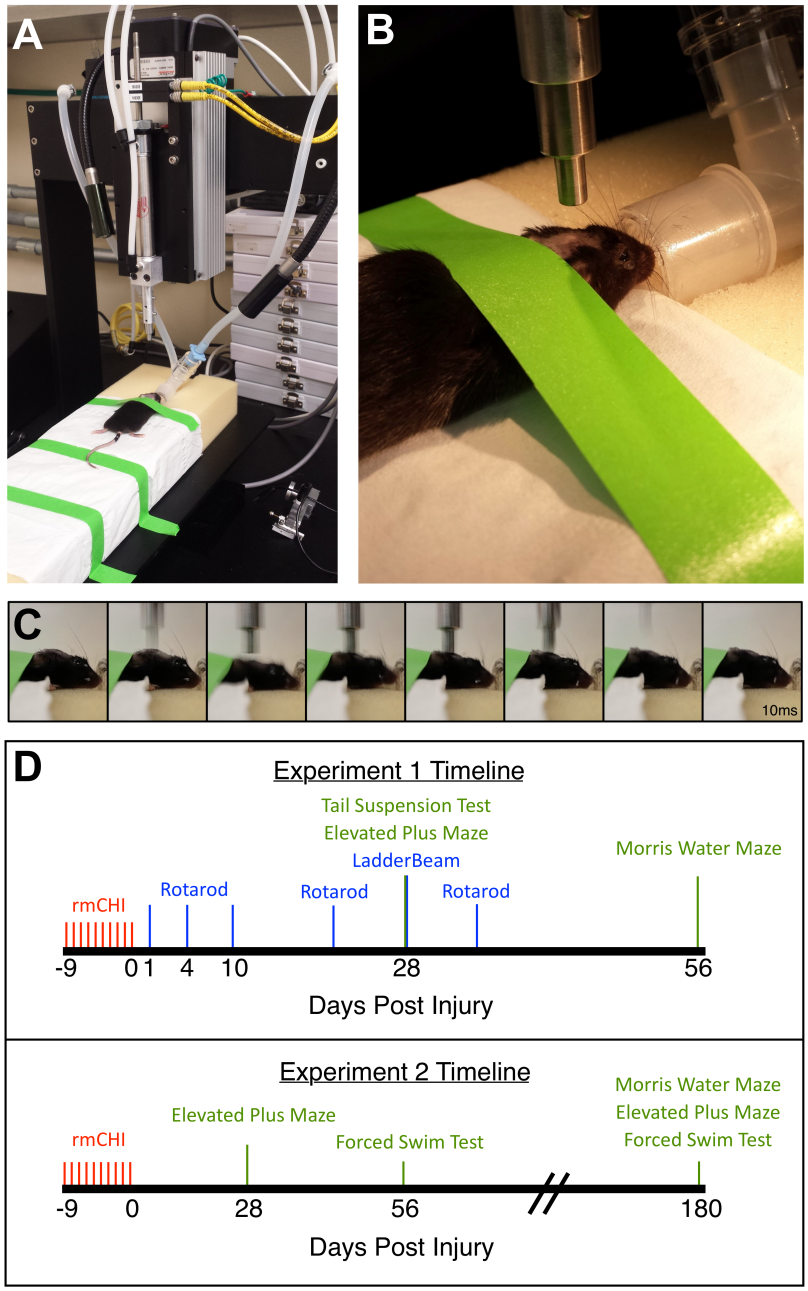

Figure 3.1. Novel model of repeated mild closed head injury used in long-term behavioral testing of mice. Following 4 minutes of isoflurane anesthesia exposure to shave and prepare the mouse head for impacts, mice are positioned atop Marmarou Foam placed in the TBI-0310 controlled cortical impactor device (A). Lab tape is lightly applied to pin the ears back and flatten the head onto the foam (B) while positioning the piston

directly above the dorsal aspect of the sagittal suture. 7 minutes after initial knockdown in anesthesia, an injury is delivered to the mouse head. Stills from a time lapse video (10fps) show the mouse receiving an impact, followed by depression and rotation into the foam, followed by a quick rebound towards the piston (C). Experiment 1 consisted of a terminal timepoint of 2 months post injury, testing both motor and cognitive tests, while Experiment 2 lasted 6 months and tested primarily cognitive function (D).

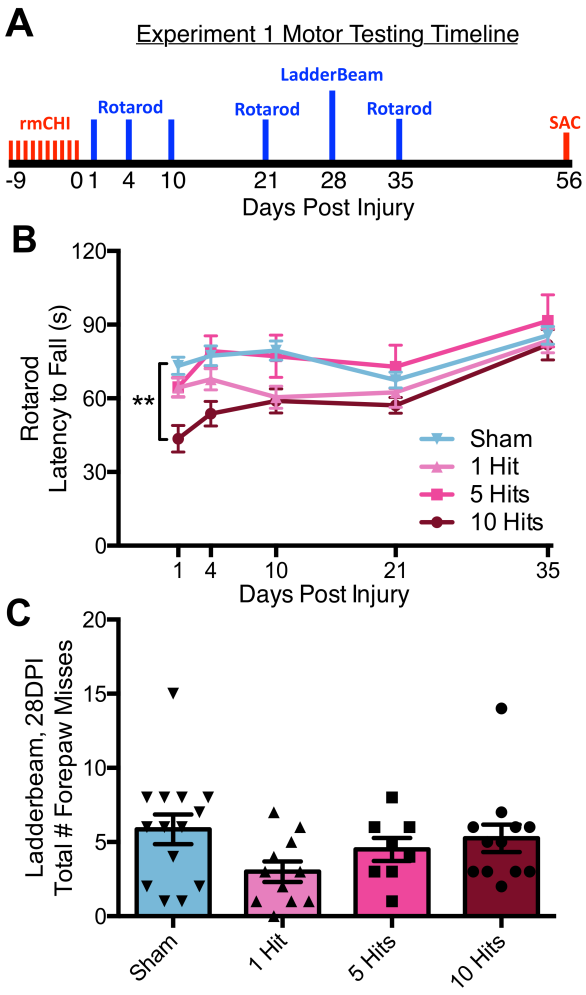

Figure 3.2. rmCHI results in minimal motor behavior deficits 1DPI, returning to control levels by 4DPI and sustaining motor function chronically. Following 0, 1, 5, or 10 rmCHIs, mice were tested on motor function using the rotarod at 1, 4, 10, 21, 28 and 35DPI and the horizontal ladderbeam at 28DPI (A). rmCHI resulted in rotarod performance deficits (2-way ANOVA, Interaction, $p=0.0080$), such that mice with 10 Hits fell quicker off the rotarod at 1DPI compared to sham controls (Tukey post-hoc, $p<0.01$) (B). No other time points of rotarod testing had significant differences between groups. rmCHI had no effect on motor performance on the horizontal ladderbeam as assessed by total forepaw misses over three trials (C, 1-way ANOVA, $F_{(3,41)}=1.945$, $p=0.1375$).

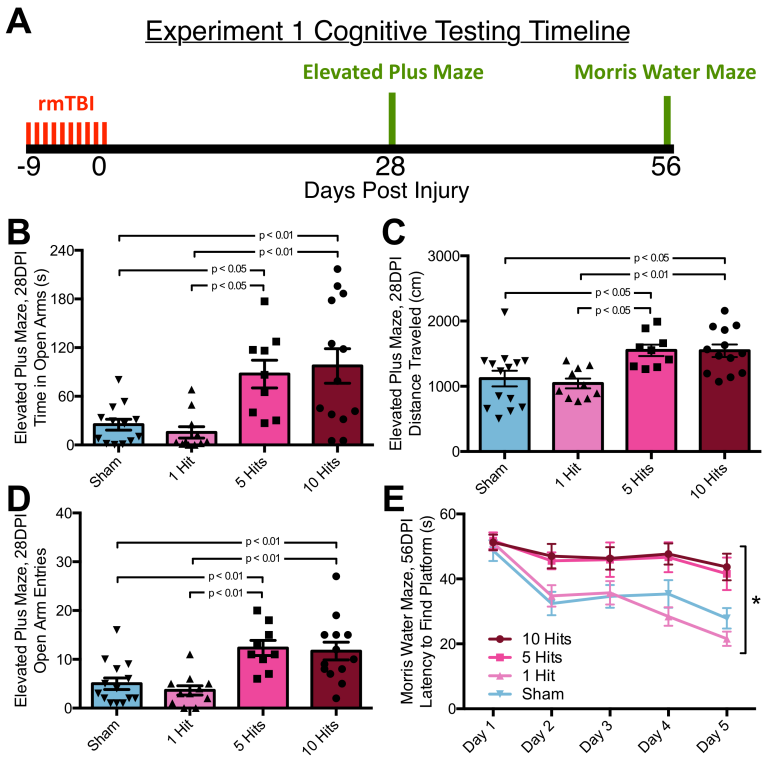

Figure 3.3. rmCHI alters cognitive function at 1 and 2 months post-injury. Following 0, 1, 5, or 10 rmCHIs, mice were tested on the elevated plus maze at 28DPI and the Morris Water Maze at 56DPI (A). rmCHI resulted in changes to performance on the elevated plus maze. Mice with 5 or 10 hits spent more time in the open arms compared to sham or 1 hit mice (B, 1-way ANOVA, $F_{(3,42)}=8.301$, p=0.0002) and also traveled more distance during the task (C, 1-way ANOVA, $F_{(3,42)}=6.800$, p=0.0008). In addition, mice with 5 or 10 hits had more open arm entries than sham or 1 hit mice (D, 1-way ANOVA, $F_{(3,42)}=9.327$, p<0.0001). 2 months following injuries, mice were tested over 5 days on the Morris Water Maze. rmCHI mice suffering either 5 and 10 hits were impaired in the ability to learn the location of the hidden platform (E, 2-way ANOVA, $F_{(12,172)}=0.0324$, * signifying p=0.0324 interaction). p-values in graphs represent Tukey post-hoc differences.

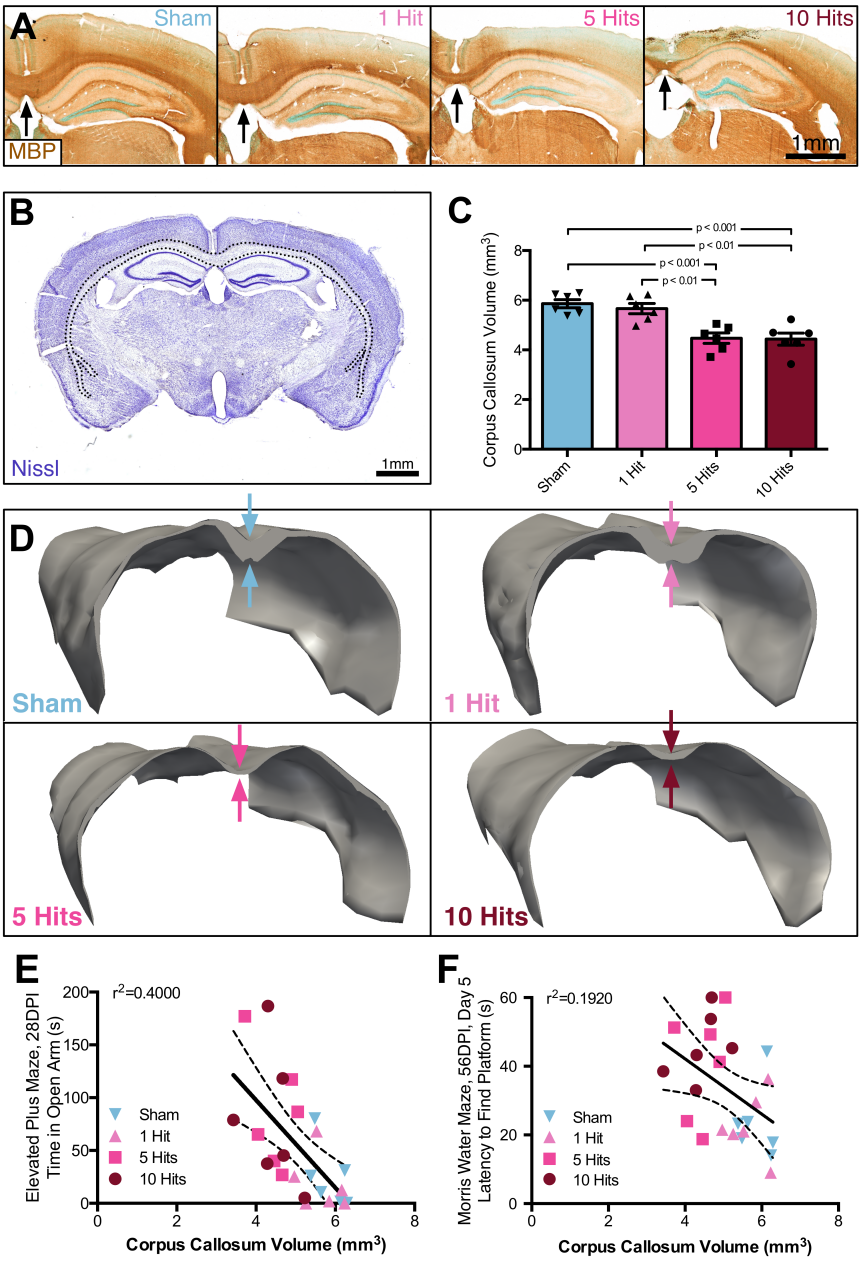

Figure 3.4. Mice suffering rmCHI have significant white matter damage and over 30% atrophy of corpus callosum at 2 months post injury. Following 5 or 10 Hits, mice developed white matter abnormalities and corpus callosum atrophy (A, myelin basic protein, black arrows - corpus callosum thinning, scale bar=1mm). Using principles of unbiased design-based stereology, volumes of corpus callosum were estimated utilizing the

Cavalieri probe. Contours tracing the corpus callosum following cresyl violet staining were made throughout the mouse brain at sections where corpus callosum was intact between the two hemispheres (B, scale bar=1mm). Sampling was conducted in a 1 in 12 series. At two months post injury, mice with rmCHI had a 33% reduction in corpus callosum volume compared to both sham and single hit groups (C, 1-way ANOVA, $F_{(3,20)}$=23.35, p<0.0001). 3D reconstructions of representative animals illustrate the atrophy especially in the splenium of the corpus callosum (D, arrows). Corpus callosum volume negatively correlates with both elevated plus maze time in open arm (E, p=0.0009), as well as latency to reach platform on day 5 of Morris water maze testing (F, p=0.0322). p-values in graphs represent Tukey post-hoc differences.

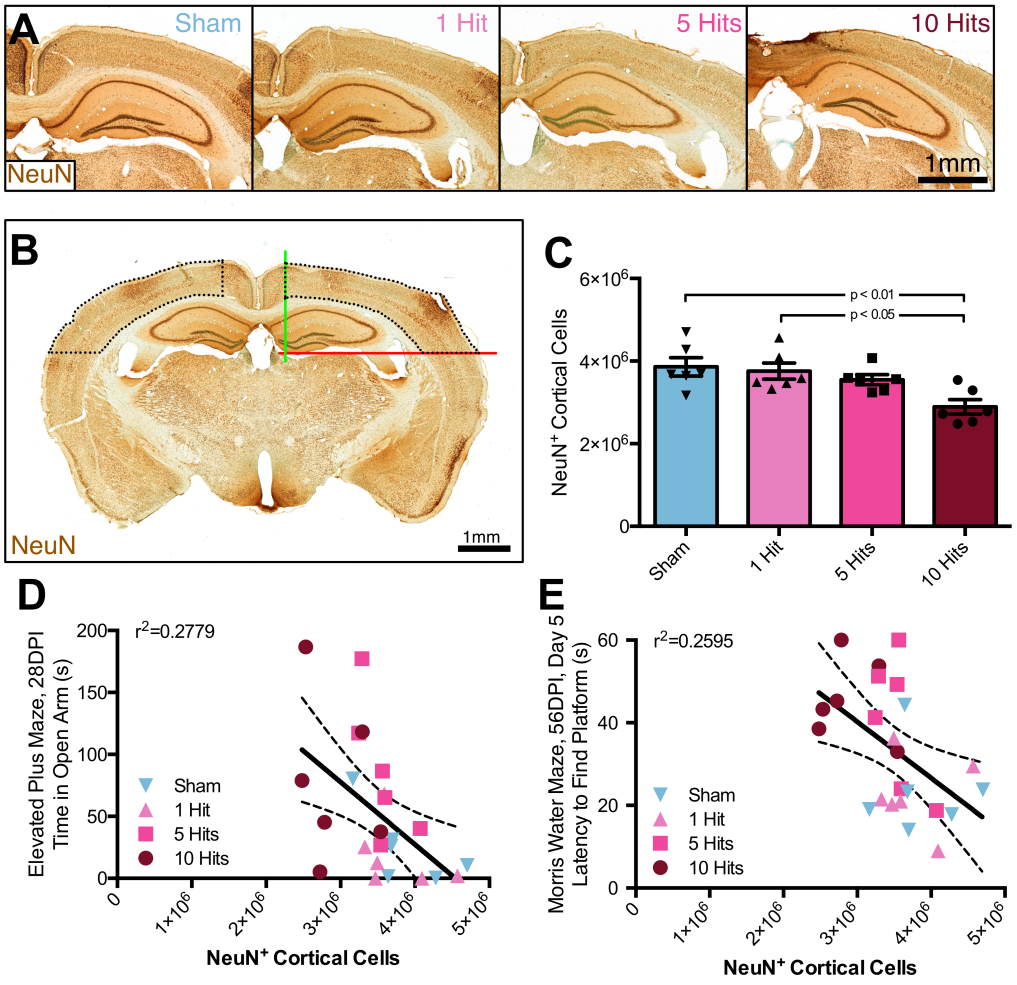

Figure 3.5. rmCHI leads to neuronal loss in the cortex, negatively correlating with behavioral testing. NeuN⁺ neuronal cell populations were estimated using principles of stereology. Representative micrographs of NeuN⁺ staining from mice with 0, 1, 5, or 10 hits at 2MPI (A, scale bar=1mm). NeuN⁺ cells were quantified in cortex as defined by set borders. Within each hemisphere, a medial border was drawn at the apex of the cingulate gyrus of the corpus callosum (B, green line), and a ventral border was drawn at the apex of the thalamus (B, red line, scale bar=1mm). NeuN⁺ neurons were counted in the cortex bound by defined borders, as well as the corpus callosum. At 2MPI, there were less NeuN⁺ neurons in the cortex of 10 hit mice compared to both sham and 1 hit mice (C, 1-way

ANOVA, $F_{(3,20)}$=5.693, p=.0055). NeuN+ cortical neurons negatively correlates with both elevated plus maze time in open arm (D, p=0.0041), as well as latency to reach platform on day 5 of Morris water maze testing (E, p=0.0055). p-values in graphs represent Tukey post-hoc differences.

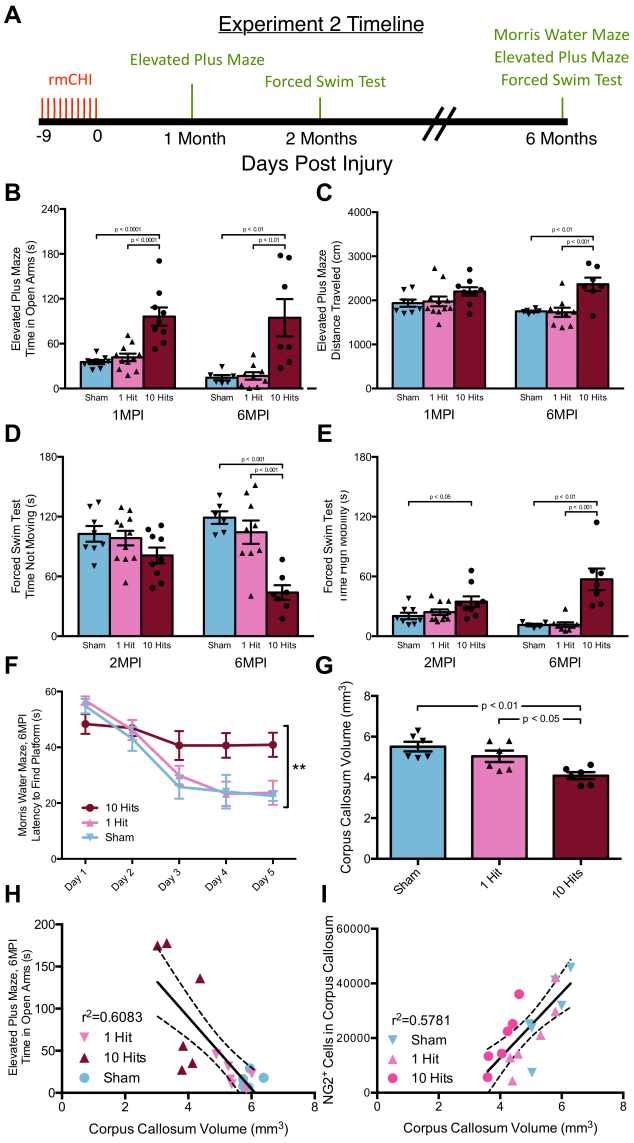

Figure 3.6. Sustained deficits in behavioral tests persist up to 6 months post injury, as well as chronic white matter atrophy within the corpus callosum. In experiment 2, mice with either 0, 1, or 10 hits were tested on the elevated plus maze at 1 and 6MPI, the forced swim test at 2 and 6MPI, and the Morris water maze at 6MPI (A). (B) Mice with rmCHI spent more time in open arms of the elevated plus maze at both 1 (1-way ANOVA, $F_{(2,25)}$=18.31, p<0.0001) and 6 MPI (1-way ANOVA, $F_{(2,19)}$=9.960, p=0.0011). (C) Mice with cmCHI were more active during the elevated plus maze at 6MPI, but not at 1MPI (6MPI 1-way ANOVA, $F_{(2,19)}$=10.26, p=0.0010). (D) At 6MPI, but not at 2MPI, rmCHI mice spent less

time not moving in the forced swim test compared to both 0 and 1 hit groups (1-way ANOVA, $F_{(2,19)}$=15.62, p<0.0001). (E) At both 2MPI and 6MPI, mice with rmCHI spent more time classified as 'High Mobility' (2MPI, 1-way ANOVA, $F_{(2,25)}$ =3.542, p<0.0442; 6MPI, 1-way ANOVA, $F_{(2,17)}$=15.21, p=0.0002). Mice with 10 hits were unable to learn the location of the hidden platform in the Morris water maze at 6MPI (F, 2-way ANOVA, $F_{(8,76)}$=2.858, p<0.0078). Mice with 10 hits had significant white matter atrophy compared to both sham and 1 hit mice (G, 1-way ANOVA, $F_{(2,15)}$=2.532, p<0.01), and corpus callosum volume negatively correlates with latency to time in open arms during the elevated plus maze at 2MPI (H, p=0.0001). Stereological quantification of $NG2^+$ oligodendrocyte precursors reveal a positive correlation with corpus callosum size at 6MPI (I, p=0.0002). p-values in graphs represent Tukey post-hoc differences.

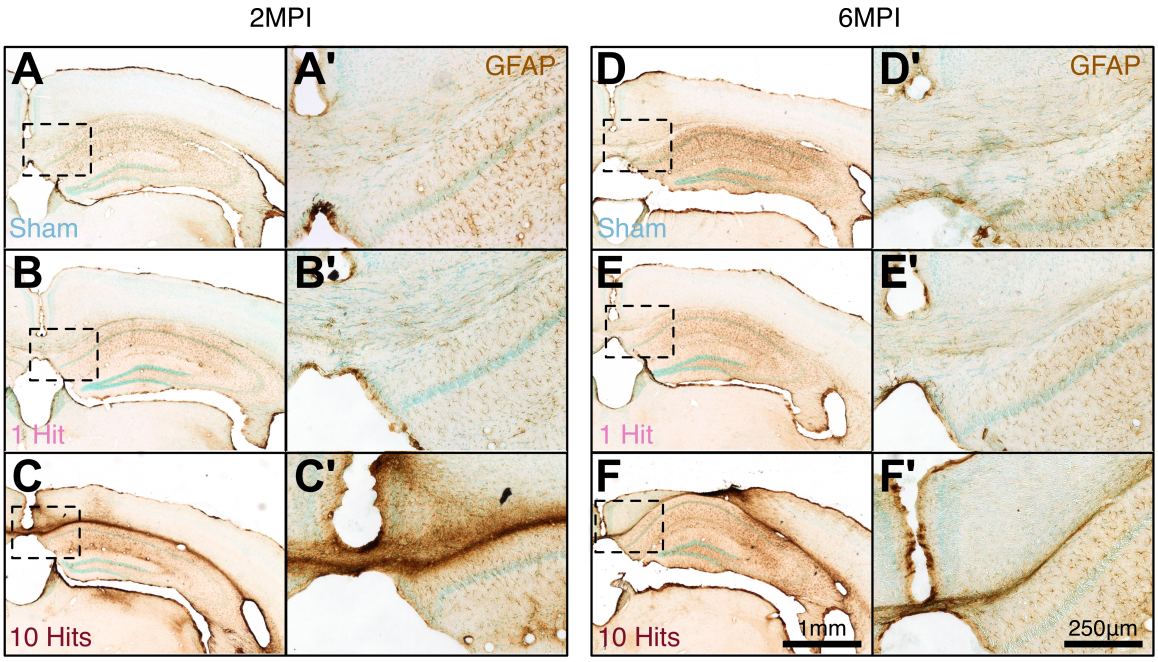

Figure 3.7. rmCHI induces chronic astrogliosis at 2 and 6 months post injury. Mice receiving 10 hits have prominent GFAP immunoreactivity at 2MPI (C, C') and at 6MPI (F, F'), particularly in the corpus callosum, cortex below impact location, and hippocampus. 1 hit mice have similar GFAP immunoreactivity at 2MPI (B, B') and 6MPI (E, E') to sham mice at 2MPI (A, A') and 6MPI (D, D'). Low magnification scale bar = 1mm, high mag scale bar = 250um.

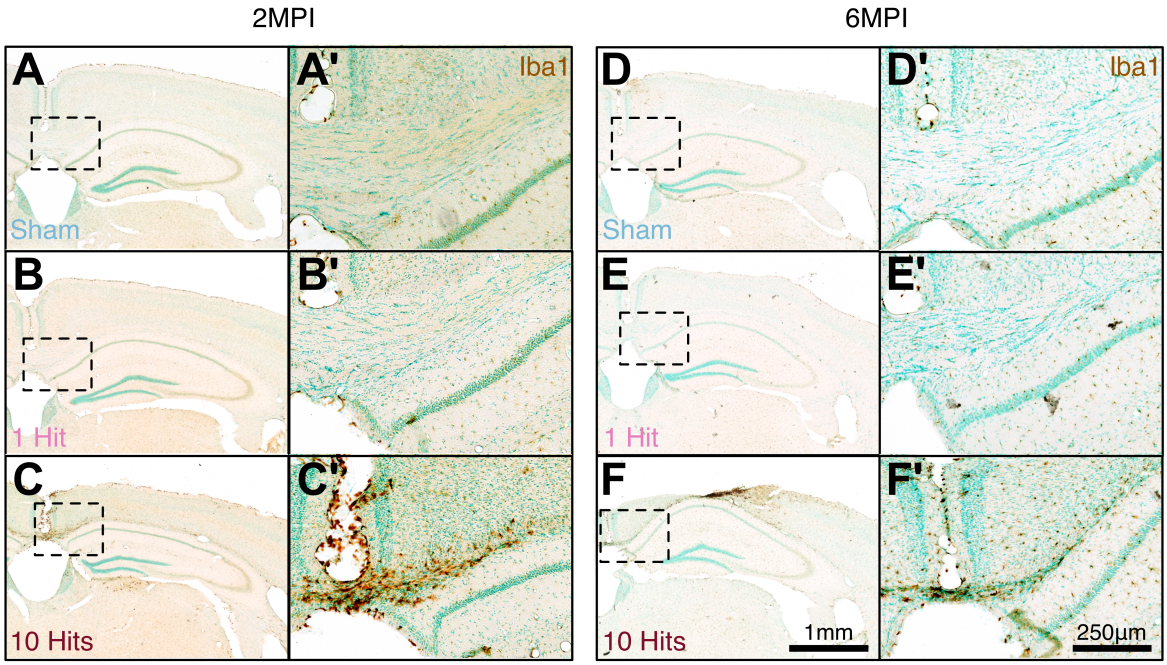

Figure 3.8. rmCHI induces chronic microglial inflammation at 2 and 6 months post injury in white matter tracts. Mice receiving 10 hits have prominent Iba1 immunoreactivity at 2MPI (C, C') and at 6MPI (F, F'), particularly in the corpus callosum. 1 hit mice have similar Iba1 immunoreactivity at 2MPI (B, B') and 6MPI (E, E') to sham mice at 2MPI (A, A') and 6MPI (D, D'). Low magnification scale bar = 1mm, high magnification scale bar = 250um.

CHAPTER 4

In depth rmCHI Model Characterization and Therapeutic Interventions

Introduction

In 1993, the American Congress of Rehabilitation Medicine (ACRM) defined specific criteria for the diagnosis of a mild traumatic brain injury (Mild Traumatic Brain Injury Committee, 1993). To be defined a mild TBI, there must be a traumatically induced physiological disruption of brain function, as manifested by at least one of the following: any loss of consciousness, any loss of memory for events immediately before or after the accident, any alteration in mental state at the time of an accident (e.g., feeling dazed, disoriented, or confused, or focal neurologic deficit(s) that may or may not be transient. To be classified as mild, and not more severe, the injury must be within the following limits: loss of consciousness of 30 min or less, after 30 min a Glasgow Coma Scale (GCS) score of 13-15, and post-traumatic amnesia not greater than 24 hrs. In 2004 this definition was endorsed by the World Health Organization, with minor changes (Carroll et al., 2004). Three assessments on the GCS are scored to get a total score ranging from 1-15. The eye response, verbal response, and motor response have a total of 4, 5, and 6 potential scores respectively. The Glasgow Coma Scale is archaically used to classify TBI for anything over 15, in that there are no further demarcations of anything more *mild*. A score of 15 is relatively normal according to basic eye response, verbal response, and motor response scorings. However, these are under sensitive and better biomarkers are needed for further classification of injury severities.

Clearly, the definition of mild TBI has loose guidelines, with broad possibilities of what an actual *mild* traumatic brain injury is. Diagnosing a TBI as mild is difficult clinically,

as TBI can manifest in a very heterogeneous ways. This is one of the greatest challenges faced in the field: trying to classify mTBI better and create subdefinitions based on a wider range of biomarkers, whether it be through imaging with MRI or CT, metabolomic profiling, proteomic profiling, or other biomarkers.

When developing our rodent model of rmTBI, it was important to us that a singular injury did not have any significant behavioral or pathological consequences. A single mild TBI can result in a wide range of acute symptoms, but in the majority of cases, these symptoms will ultimately subside. This was a priority for us when defining a particular set of injury parameters. The injury itself was to have no long lasting effects. Particular injury parameters of 5.0m/s speed, 1mm depth, and 50ms dwell time cased behavioral and pathological changes for 5 and 10 hit mice, but not 1 hit or sham animals at 1-6MPI. Our initial hypothesis that multiple head injuries result in a unique behavioral and pathological consequence but not a single head injury was supported because we indeed found parameters which only resulted in deficits when 5 or more hits were delivered. However, it became clear that these injury parameters were a bit more severe than we were expecting in the multiple injury groups. While we were content with this initial *mild* definition and validity, we wanted to test a range of additional injury parameters, to find either a *mild* threshold or more mild injury conditions. We still, however, posit that when modeling rmTBI in rodents, it is critical that a behavioral phenotype arises as well as a pathological phenotype, in order for external validity and to be useful for testing therapeutic interventions. To further characterize our model of rmCHI, we reduced the speed of impacts, and reduced the number of injuries to determine a particular threshold where upon behavioral and pathological consequences exist.

A major question surrounding our initial findings in the rmCHI rodent model, was what is happening to commisural fibers crossing between hemispheres in the corpus callosum? We know there was an overall atrophy of volume, partially due to demyelination as assessed by immunohistochemical staining of myelin basic protein. We also know there is cortical neuronal loss. In addition, while there is a decrease in myelin basic protein, we do not see a change in oligodendrocyte progenitor cells within the corpus callosum. To further understand the atrophy and demyelination within corpus callosum, we hypothesized there would be changes to axon myelination in commisural fibers. To investigate this, we performed electron microscopy to assess the quality of myelination. G-ratio is a measure of myelination relative to the axon diameter. Generally, larger diameter axons are myelinated more (more wraps) than smaller axons (Chomiak and Hu, 2009). In addition, G-ratios can change when axons are damaged or when remyelination occurs. For example, when an axon becomes remyelinated, the myelination is more loosely wrapped, altering its g-ratio. Investigating g-ratio will give a more clear view into some of the changes that are occurring in the corpus callosum with regard to axon diameter, myelination, and remyelination.

Another focus of our research is to investigate novel therapeutics that can be used in patients who have no other clinical options. One such therapeutic that is of particular interest to our laboratory is the use of stem cells. Human derived stem cells offer the potential to be transplanted into diseased tissue, to proliferate, differentiate, and integrate into the host (Lindvall and Kokaia, 2010; Zhang et al., 2001). Stem cells are being tested in a wide range of central nervous diseases including Parkinson's disease (Kim et al., 2002; Kriks et al., 2011), Alzheimer's disease (Ager et al., 2015), amyotrophic lateral sclerosis

(Feldman et al., 2014; Glass et al., 2012), stroke (Andres et al., 2011; Chen et al., 2001), multiple sclerosis (Chen et al., 2014; Martino et al., 2010), and spinal cord injury (Mothe and Tator, 2012; Salazar et al., 2010). Specifically, our laboratory has shown that human neural stem cells can be transplanted into the injured rodent spinal cord, promoting functional recovery (Cummings et al., 2005; Hooshmand et al., 2009). We have also shown the efficacy of human derived neural stem cells for the controlled cortical impact model of traumatic brain injury in athymic nude rats (Beretta et al., 2016; Haus et al., 2016).

As we have shown that our model of rmCHI leads to cortical neuronal death and white matter atrophy, we believe that human neural stem cell therapy offers an attractive approach for treatment of individuals suffering from chronic deficits associated with rmTBI and/or CTE. White matter atrophy in the corpus callosum was partially due to demyelination, as well as axonal loss, based on both loss of myelin basic protein as well as loss of projecting cortical neurons. Neural stem cells transplanted into either the cortex or the corpus callosum could differentiate into different cell types based on their specific need within the niche. We have also shown that with injury that there is no overall drop in NG2+ oligodendrocyte progenitor cells in the corpus callosum, or a change in density relative to an atrophied corpus callosum. This suggests that the appropriate number of oligodendrocyte progenitors are populating the damaged corpus callosum. However, this does not tell us about the mature oligodendrocyte population, or the number of commisural fibers crossing through the corpus callosum. To this end, we hypothesize that transplanting human derived neural stem cells into an injured mouse brain would promote functional recovery by differentiation into newborn neurons in the cortex, as well as promoting more oligodendrocyte myelination of axons in the corpus callosum.

All previous investigations utilizing our new model of rmCHI have been in C57BL/6J male mice. In order to have sufficient cell survival and minimal host rejection, a form of immunosuppression must be used in any future human stem cell transplant experiments. As opposed to transplanting into an immunocompetent mouse with immunosuppressants, it is better to transplant human cells into an immunodeficient mouse (Anderson et al., 2011). We have chosen to test NOD-*scid* Gamma mice as a suitable rodent strain for receipt of human cells (Ishikawa et al., 2005; Shultz et al., 2005). NSG mice are similar to the classic immune-compromised mouse line, NOD-*scids*, in that they both lack T and B cells. However, NGS mice also have carry a null allele of the IL2 receptor gamma, resulting in a deficiency of natural killer cells. NSGs also have a longer lifespan than NOD-*scids*, making them even more attractive for long-term survival studies following human cell transplantation. Different strains, especially on a different strain background, may have different baseline behavioral phenotypes, as well as different responses to injury. Therefore, it is imperative that before testing the efficacy of human neural stem cells for rmCHI, it is critical to test the injury parameters on this new strain to characterize their behavioral profile, as well as their pathological response to the injuries.

In addition to stem cell based therapies, we are also interested in novel small molecules that can be administered following rmTBI to alleviate functional symptoms and/or pathologies. Several approaches can be taken when thinking about delivering a therapeutic for rmTBI. One approach is preventative. For example, taking a drug prior to an activity in which you might suffer a head injury, a football game. This is certainly one option, although this type of preventative care would require a lot of dosing and does not account for injuries that occur in situations that are unavoidable, ie car crashes or falls. The

alternative approach is to treat either immediately after injury or in the weeks and months after injuries as symptoms have or are developing. One major pathological consequence of rmCHI in our model is prolonged microglial activity, particularly in white matter tracts. With our collaborators at Vivreon Biosciences, we are interested in testing an inhibitor of calcium release activated channels (CRAC). CRAC channels are involved in activating microglia and have been shown to decrease the activation of microglia in vitro (unpublished data). We hypothesize that by decreasing microglial activation, white matter pathology will be less severe, as well as behavioral deficits. We will also be testing another compound with collaborators who have developed an allosteric modulator of nicotinic acetylcholine receptors (Ng et al., 2007). Our goal is to identify new molecules that have potential to be administered following rmTBI to alleviate behavioral deficits and neuropathology.

Decreased Injury Severity Study

We have previously characterized a closed head traumatic brain injury as *mild*. However, brain injuries occur on a spectrum, and to categorize them as *mild* may be too general. Particular characteristics, symptoms, and pathologies ought to be designated and particularly defined in rodent models of TBI to more precisely tier injury severity. To this end, we sought to investigate thresholds, or various changes in injury parameters, that would alter and grade neuropathology and behavioral deficits associated with multiple head injuries.

We tested the two main variables of the model, the number of hits and the velocity of hits. Previously, we have shown that two sets of injury parameters had very similar

pathology and behavior consequences: 10 hits over 10 days at 5 m/s & 5 hits over 10 days at 5 m/s. In order to identify injury parameter thresholds that produce or do not produce similar results, we tested either half speed (2.5 m/s) or less hits (3 hits). 10 week old male C57Bl/6J mice (n=12/group) were divided into 4 groups: A) Sham, B) 3 hits 5 m/s, C) 5 hits 2.5 m/s, D) 5 hits 5 m/s (as a positive control). Mice hit 3 times were hit every other day, and mice hit 5 times were hit every 24 hours. Mice were tested in elevated plus maze at 1 month post injury (MPI) and the Morris water maze at 2MPI.

Mice were tested at 1MPI on the elevated plus maze, as previously reported. Briefly, mice were placed into a 4-armed raised maze 29" off the ground, with two open arms, and two arms closed in with walls 7.5" high. Mice were given 5 minutes to explore the arms in a dark room, and recorded using an infrared camera and Ethovision software (Noldus Information Technology, Leesburg, VA). There was a significant effect of group on time spent in open arms (Figure 4.1A, $F_{(3,44)} = 16.36$, $p<0.0001$). Sham mice spent less time in open arms compared to both 3 hit 5m/s (Tukey post-hoc, $p<0.01$) and 5 hit 5.0m/s (Tukey post-hoc, $p<0.0001$) mice, but not compared to 5 hit 2.5m/s mice. In addition, 5 hit 5.0m/s mice spent more time in open arms than both 5 hit 2.5m/s (Tukey post-hoc, $p<0.0001$) and 3hit 5.0m/s (Tukey post-hoc, $p<0.05$). There was a significant effect of group on total distance traveled (Figure 4.1B, $F_{(3, 44)}=3.681$, $p=0.0189$), although Tukey post-hoc testing revealed no differences between any groups. There was also a significant effect of group on open arm entries (Figure 4.1C, $F_{(3, 44)}=7.887$, $p=0.0003$). Sham mice had less open arm entries compared to both 3 hit 5m/s (Tukey post-hoc, $p<0.05$) and 5 hit 5.0m/s (Tukey post-hoc, $p<0.01$) mice, but not compared to 5 hit 2.5m/s mice. In addition, 5 hit 2.5m/s

mice had less open arm entries compared to both 3 hit 5.0m/s (Tukey post-hoc $p<0.05$) and 5 hit 5.0m/s mice (Tukey post-hoc $p<0.01$).

At 2mpi, these same mice were tested for spatial learning using the Morris water maze test. There was a significant interaction of group vs time (Figure 4.1D, $F_{(12, 176)} = 3.278$, $p=0.0003$), where a graded behavioral profile became apparent. On day 5 of testing, sham mice were able to locate the platform quicker than both 3 hit 5.0m/s (Tukey post-hoc, $p<0.0001$) and 5 hit 5.0m/s mice (Tukey post-hoc, $p<0.0001$). In addition, 5 hit 2.5m/s mice were able to locate the hidden platform faster than both 3 hit 5.0m/s (Tukey post-hoc, $p<0.01$) and 5 hit 5.0m/s mice (Tukey post-hoc, $p<0.01$).

To determine if these drops in severity had an effect on neuropathology, we assessed corpus callosum volume blind to experimental condition. Using unbiased stereology we quantified changes in corpus callosum volume throughout the entire brain. 30μm sections were stained with Cresyl violet for volume quantification. Contours of the corpus callosum were drawn using StereoInvestigator software v11.08.01 (MicroBrightField, Inc., Williston, VT) on every 12th section to evaluate estimated volume in a systematic, non-biased, design-based approach. Anterior and posterior limits of corpus callosum were bound by sections that contained intact corpus callosum connecting the two hemispheres in the same coronal plane. A 100um grid was laid down to assess corpus callosum volume and coefficient of error for each animal was less than 0.10.

Indeed, there was a significant effect of group on corpus callosum size (Figure 4.1E, $F_{(3,26)=}9.336$, $p=0.0002$). Similar to behavioral deficits, sham mice had smaller corpus callosum than both 3 hit 5.0m/s (Tukey post-hoc, $p<0.01$) and 5 hit 5.0m/s (Tukey post-hoc, $p<0.01$) mice. In addition, 5 hit 2.5m/s mice had smaller corpus callosum volumes

compared to both 3 hit 5.0m/s (Tukey post-hoc, $p<0.01$) and 5 hit 5.0m/s mice (Tukey post-hoc, $p<0.01$).

To summarize, two major injury parameters were altered to identify phenotypes associated with reduced injury. We have previously found that both 5 hits and 10 hits at 5.0m/s result in corpus callosum atrophy, neuronal loss, and behavioral changes relating to anxiety and learning and memory. Using our control injury parameters of 5 hits 5.0m/s, we lowered two variables, number of hits or velocity of hits. In doing so we tested uninjured shams, compared to historic 5 hits normal speed, same hit number but half velocity, or less hits at same velocity. Dropping the number of hits at *full* velocity (5.0m/s) does not have an effect on neuropathology or behavioral changes. We know from previous studies that a singular hit at 5.0m/s produces no changes in behavior or pathology compared to 5 hits. Here, we observe that 3 hits at 5.0m/s produces similar results to 5 hits. However, 5 hits at half speed, 2.5m/s, does not produce significant behavioral or neuropathological changes. Thus the threshold for additive effects of a 5m/s injury is greater than 1 and may be as high as 3 hits. Conversely, the threshold for velocity sufficient to injury a repetitive injury is greater than 2.5m/s.

Electron Microscopy Analysis of Corpus Callosum

At 2 months post injury, C57Bl/6J mice were sacrificed for electron microscopy and assessment of g-ratio of myelinated axons. Mice were first perfused with room temperature PBS, 10mL/min for 5 minutes. Next, mice were perfused with 4% paraformaldehyde/0.5% gluteraldehyde (pH 7.4) at 37°C in 0.1M phosphate buffer for 10 minutes. To dissect the corpus callosum, a sagittal incision was made through the excised brain along the midline.

A section was recovered after a 2nd blade was inserted 1mm lateral on either side of midline. Corpus callosum was isolated from these 1mm sections, and subsequently divided into three regions, anterior, medial, and posterior, each roughly 2mm long. Posterior regions were used for analysis.

Tissue was then processed to for electron microscopy resin embedding. Tissue was first briefly washed in 0.2M sodium cacodylate. Next, tissue was placed in 1% osmium tetroxide/1.5% potassium ferrocyanide at 4°C for 90 minutes. Tissue was then washed in 0.2M sodium cacodylate twice for 15 minutes, each at 4°C. Tissue was then post-fixed in 2% gluteraldehyde at 4°C for 1.5 hours. Tissue was then washed 3 times in phosphate buffer for 10 minutes, followed by a 10 minute wash in diH_2O. Next, the tissue was dehydrated through a series of alcohols: 50% ethanol (3x5minutes), 75% ethanol (3x5minutes), 85% ethanol (3x5minutes), 95% ethanol (3x5minutes), 100% ethanol (3x10minutes). Next, tissue was rinsed 3 times for 20 minutes each in propylene oxide. Tissue was then placed into a 1:1 mixture of propylene oxide:resin and placed into a vacuum overnight. Resin consisted of Epon (24mL), DDSA (dodecenylsuccinic anhydride, 6mL), NMA (nadic methyl anhydride, 20mL), and DMP-30 (dimethylaminomethyl phenol, 1.1mL). The next day tissue was placed in fresh 100% resin for 8 hours in a vacuum. Finally, tissue was then placed into BEEM caps filled with 100% resin, and cured in an oven overnight at 55°C .

Resin blocks were trimmed using a specimen trimmer (Leica EM Trim2) to shape the block. Blocks were then cut on an ultramicrotome (Leica EM UC6) to collect 1um sections, and allowed to dry on a slide. Sections were stained with Toluidine Blue to visualize axons and confirm correct orientation. Ultrathin sections of 80-100um were then collected on 150 mesh grids and let to dry overnight. The next day, grids were stained with

uranyl acetate for 1 minute, followed by 5 rinses in ddH_2O. Next, sections were stained in lead citrate for 3 minutes, followed by 5 rinses in ddH_2O.

Specimens were imaged using a transmission electron microscope (Joel JEM-1400, JOEL USA, Peabody, MA) at 4000x. Approximately 100 axons were quantified in 2-4 fields of view using ImageJ (NIH, USA, v1.47). G-ratio was calculated from traced and quantified axons, as (axon area)/(axon + myelin area). Due to limitations on imaging and sample preparation, final n/group were 2 sham, 2 1-hit, and 1 10-hit mice. Example image captures of sham and 10 hit mice can be seen in Figure 2A and 2B respectively.

To get a better understanding of the myelination integrity of commisural fibers within the corpus callosum, we calculated g-ratios from axons, defined as axon area divided by myelin+axon area, in the cross-sectional 2-dimensional plane captured by electron microscopy. Upon analysis, we found a significant effect of group on g-ratio (Figure 4.2C, 1way ANOVA, $F_{(2, 369)}=10.02$, $p<0.0001$). 10 hit mice had significantly lower g-ratios than both sham (Figure 4.2C, Tukey post-hoc, $p<0.0001$) and 1 hit mice ($p<0.05$). A frequency distribution (Figure 4.2D) reveals a shift for lower g-ratios following rmCHI.

The drop in g-ratio is most likely due to increased axon and myelin total width. Our analysis shows that 10 hit mice have lowered g-ratio values than sham and 1 hit mice. If axon diameters remained the same, and myelin thickness increases, the g-ratio would go down. Likely, the myelin thickness is increased, due to remyelination or dysmyelination. Injured axons can have looser myelin wraps, but also remyelinated axons have been shown to have less loosely packed wraps of myelin around axons. Another possibility, although less probable, is that the axons could be actively demyelinating. At 2MPI, most injury cascades have terminated, although there is chronic neuroinflammation present. Chronic

active microglial could be playing a role in demyelinating axons or inhibiting remyelination. Further analysis of axon myelination, the time course of demyelination and remyelination, and native oligodendrocyte progenitor proliferation and differentiation in response to injury will help us get a better understanding of mechanisms underlying corpus callosum pathology associated with rmCHI.

NOD-*scid* Gamma mice show no deficits following rmCHI

Stem cell transplants are an attractive option for treating the effects of repeated mild traumatic brain injury. We have previously shown that rmCHI has long-standing consequences in rodents, out to 6 months following injury. One strategy to alleviate these symptoms is to introduce multi-potent neural stem cells that are capable of differentiating into neurons, oligodendrocytes, or astrocytes. We have identified two separate areas were cellular changes are occurring, within the cortex, as well as the corpus callosum. One strategy is to introduce neural stem cells to repopulate neuronal loss in cortex, increase oligodendrocyte progenitor pools within corpus callosum, and/or increase oligodendrocytes and myelination for commisural fibers within the corpus callosum. Previous studies have shown efficacy of neural stem cell transplantation in other models of traumatic brain injury, but this approach has not been investigated in a rodent model of repeated mild closed head injury.

To transplant neural stem cells into a rodent model, a few grafting options need to be carefully evaluated. Our ultimate goal is to transplant human neural stem cells into humans in the clinic. Therefore, it is imperative to test a clinically relevant cell population, ie human as opposed to murine. In order for a human cell to graft successfully with

minimal immune rejection, one needs to either immunosuppress a fully immune competent mouse, or utilize mice with compromised immune systems. It is our preference to use immunocompromised rodents, as they have the highest cell graft efficiency (Anderson et al., 2011). We chose a newly developed mouse strain from Jackson laboratories, NOD-*scid* gamma (NSG, catalog # 005557). NSG mice are similar to the classic immunocompromised mouse line, NOD-*scids*, in that they both are absent of T and B cells. However, these mice also have carry a null allele of the IL2 receptor gamma, resulting in a deficiency of natural killer cells. NSGs also have a longer lifespan than NOD-*scids*, making them even more attractive for long-term survival studies following cell transplantation.

Before a cell transplantation study in NSGs is to be conducted, it is important to verify their behavioral profile, both in a naïve uninjured state, as well as an injured state. To this end, we conducted an experiment to behaviorally profile uninjured and rmCHI injured NSG mice at chronic timepoints. 10 week old male NSG mice were divided into 4 groups (n=12/gp): A) Sham, B) 1 Hit 5.0m/s, C) 5 Hits 2.5m/s, D) 5 Hits 5.0m/s. Following rmCHI, mice were tested on a battery of behavioral tasks including elevated plus maze at 1 and 6MPI, novel place and novel object recognition at 2MPI, and Morris water maze at 2 and 6MPI. Following behavioral testing at 2MPI, half of the animals in each group were sacrificed and transcardially perfused with PBS followed by 4% paraformaldehyde for histological analysis. For behavioral testing at 6MPI, n=6/group.

Mice were tested on the elevated plus maze at 1 and 6MPI as previously described. There were no differences between groups in outcome measures at either time point. Specifically, there was no effect of group on distance traveled (Figure 4.3A, 1-way ANOVA, $F_{(3,43)}$=0.5475, p=0.6525), time spent in open arms (Figure 4.3B, 1-way ANOVA,

$F_{(3,43)}$=1.302, p=0.2865), or open arm entries (Figure 4.3C, 1-way ANOVA, $F_{(3,43)}$=0.9388, p=0.4302) at 1MPI. Similarly, at 6MPI, there was no effect of group on distance traveled (Figure 4.3A', 1-way ANOVA, $F_{(3,20)}$=0.4665, p=0.7089), time in open arm (Figure 3B', 1-way ANOVA, $F_{(3,20)}$=0.9844, p=0.4200), or open arm entries (Figure 4.3C', 1-way ANOVA, $F_{(3,20)}$=0.8486, p=0.4836).

Mice were tested for learning performance at both 2 and 6MPI using the Morris water maze. Mice were given 4 trials per day over 5 days to locate the location of a hidden platform in a static quadrant. Each trial began at a different dropoff location, and this location was randomized throughout the 5 days of testing. At 2mpi, there were no differences detected in water maze performance between groups (Figure 4.3D, 2way repeated measures ANOVA, $F_{(12, 172)}$=0.4904, p=0.9184), in that no group had faster latency to reach platform by day 5. Similar results were observed at 6MPI, with no detectable difference in learning (Figure 4.3D', 2way repeated measures ANOVA, $F_{(12, 80)}$=1.352, p=0.2071).

Having not observed any differences in water maze performance, we decided to test a pair of memory tasks, the novel object recognition (NOR) and novel place recognition (NPR) tasks. Following several days of habituation in open field boxes, two objects were placed in separate quadrants. On day 1 of testing, mice are exposed to these 2 objects. On day 2 of testing, 1 of the objects is repositioned to a different quadrant, as part of the novel place recognition test. On day 3 of testing, the moved item is then swapped with a novel object, to test novel object recognition. Time spent exploring each of these items is quantified by a blind observer from taped recordings. The discrimination index is calculated as (time spent exploring novel object - time spent exploring familiar object)/

time spent exploring either object). If a novel object were explored more relative to the familiar object, the index would approach 1, but if objects were explored equally, the discrimination index would equal 0. For both novel place recognition and novel object recognition, no group showed preferences for the novel place or object, and there were no differences between injury groups (Figure 4.4, 1-way ANOVAs, NPR: $F_{(3, 41)}$=0.8012, p=0.5004, NOR: $F_{(3, 41)}$=1.050, p=0.3803).

Surprisingly, NSG mice exhibited no significant changes as a result of any injury parameter tested on any behavioral or histological measure assessed. Injury had no measurable effect on behavioral measures of anxiety, learning, and memory, at both early and chronic time points. Moreover, previously observed white matter atrophy was not observed in NSG mice, at either at 2MPI or 6MPI, relative to shams. There was, however, a slight significance of corpus callosum atrophy at 1MPI between 1 hit 5.0m/s and 5 hit 5.0 m/s mice (Figure 4.3E, 1-way ANOVA, $F_{(3, 16)}$=4.139, p=0.0238, Tukey post-hoc, p<0.05). There were two outliers, one from the 1hit 5.0m/s group and another from 5 hit 5.0m/s group that were excluded via Grubb's test. Increasing n would likely bring these groups closer together. There was no significant effect of white matter atrophy at 6MPI between any groups (Figure 4.3E', 1-way ANOVA, $F_{(3, 19)}$=0.5767, p=0.6573).

It was quite surprising to have observed no changes either behaviorally or neuropathologically in the Nod-*scid* Gamma mice at either 1MPI or 6MPI. Further experimental avenues need to be explored to understand why there were no deficits. Strain differences certainly affect behavioral performance. Baseline behaviors can be different than C57BL/6J mice, as well as the response to injury. A more likely explanation however, lies in the inflammatory response to injury. With no T cells, B cells, and a severe deficiency

in NK cells, some sort of compensatory mechanism could be in place that is helping mediate damage. Ongoing studies investigating the time course of other inflammatory cells are underway to further understand the dynamic injury.

Experimental Therapeutics to Protect the Effects of rmCHI on Mice

AVL-3288 is a type 1 positive allosteric modulator of α7 nicotinic acetylcholine receptors (Ng et al., 2007), developed and provided as a gift from collaborators at UC Irvine, Dr Kelvin Gee and Dr Tim Johnson. Deficits in nAChR expression and function are associated with cognitive decline in Alzheimer's disease and schizophrenia (Freedman et al., 1995; Guan et al., 2000). AVL-3288 locally targets cholinergic activation of neurons, as opposed to other therapeutic strategies elevating acetylcholine systematically. By targeting cholinergic neurons with α7 nAChRs in the limbic system and hippocampus, we hypothesize that allosteric modulation of these receptors will reverse the cognitive deficits associated with repeated mild traumatic brain injury. To test this hypothesis, we administered AVL-3288 or vehicle to mice after rmCHI model of rmTBI, followed by a battery of behavioral tests.

Vivreon 2.0 is a proprietary small molecule inhibitor of calcium release activated channels (CRAC). CRAC channels play a critical role in microglial activation, and our collaborators at Vivreon Biosciences have demonstrated their proprietary molecules can inhibit microglial activation *in* vitro (data unpublished). Inhibiting microglial activation *in vivo* can alter the injury response to repeated mild traumatic brain injury, potentially minimizing pathology associated with increased microglial presence. We have seen increased Iba1+ microglia activity at chronic timepoints (2 and 6MPI), particularly in the

atrophied corpus callosum of mice following rmCHI. Vivreon 2.0 offers a potential strategy to inhibit this microglial activation, potentially leading to decreased atrophy within the corpus callosum.

Two experiments were run in parallel, testing both AVL-3288 and Vivreon 2.0 for their therapeutic potential. 10 week old, male C57Bl/6J mice (n=11-12) were given rmCHI, via 5 hits over 5 days as previously described. As these studies were unfunded, no uninjured sham controls were used in this study; rather all animals were injured, and given either drug or vehicle and compared to historical controls (shams) of C57BL/6J mice. AVL-3288 was diluted and dosed intraperitoneally to animals at 0.3mg/kg in 5%DMSO, 5% solutol, and 90% saline. AVL-3288 vehicle control was 5% DMSO, 5% solutol, and 90% saline. Mice were given AVL-3288 drug or AVL-3288 vehicle control at 8 timepoints: 1 hour following first hit, 1 hour following final hit, 2 days pre 1MPI behavioral testing, 1 day pre-1MPI behavior testing, 2 days prior to 2MPI behavior testing, and 1 day prior to 2MPI behavior testing. Vivreon 2.0 and Vivreon vehicle control solutions were provided by Vivreon Biosciences. Mice received subcutaneous injections of Vivreon 2.0 or vehicle once daily for 4 weeks. The first injection was 1 hr prior to their first hit. Daily injections lasted for 1 month, with the final injection coming 1 day before behavioral testing of elevated plus maze at 1mpi. Vivreon 2.0 was injected at 25mg/kg, in a solution containing propylene glycol, PEG400, and Tween 80. Vehicle control omitted Vivreon 2.0 but contained propylene glycol, PEG400 and Tween 80. Cognitive behavior was assessed at 1MPI using the elevated plus maze test and the forced swim test, and at 2mpi using the Morris water maze test. All testing was conducted blind to treatment group.

At 1MPI, mice were first tested on the elevated plus maze. Briefly, mice were placed into a 4-armed raised maze, with two open arms, and 2 arms closed in with walls. Mice were given 5 minutes to explore the arms in a dark room, and recorded using a camera and analyzed using Ethovision software. Total distance traveled, time in open arms, and open arm entries were analyzed by Ethovision tracking software. The following day, mice were tested in the forced swim test. Mice were placed into a 1L beaker filled 70% with room temperature water for a 6 minute trial. Mice were recorded by videocamera and analyzed by Ethovision software. Only the last 3 minutes of the trial were analyzed for two outcome measures. Moving was determined by calculating the duration for which the center point of the animal was changing location with a start velocity set at 1.25cm/s and a stop velocity set at 1.0cm/s. Mobility was quantified using changes in pixel area of the subject between samples collected. Standard software thresholds were used to segment mobility into 'immobile', 'mobile', and high mobility' levels, where the immobile threshold was set to 6% and the high mobility threshold was set to 18%. At 2MPI mice, were tested in the Morris water maze over 5 days. Mice were given 4 trials per day, with a 20 minute intertrial test period. A hidden platform remained stationary in the northwest quadrant of the tank. Mice were released at 4 different entry points to the tank each day, requiring them to use spatial cues in the room to locate the submerged, hidden platform.

Mice tested at 1MPI on the elevated plus maze exhibited no effects of AVL-3288 administration. There was no significant effect of drug on either total distance traveled (Figure 4.5A, unpaired t test, p=0.8281) or time spent in open arms (Figure 4.5B, unpaired t test, p=0.7980). In addition, mice tested at 1MPI exhibited no changes due to AVL-3288 administration on multiple outcome measures during the forced swim test. There was no

effect of drug on time immobile (Figure 4.5D, unpaired t test, p=0.2939), time highly mobile (Figure 4.5E, unpaired t test, p=0.7866), or time not moving (Figure 4.5F, unpaired t test, p=0.3148). Moreover, drug administration had no effect on learning performance as assessed by the Morris water maze. AVL-3288 did not lead to decreased time to reach the hidden platform over 5 training days (Figure 4.5C, 2way ANOVA, $F_{(4,84)}$=1.942, p=0.1109). There was a trend that AVL-3288 administration actually had a worse effect on learning behavior in comparison to vehicle injected rmTBI mice, although this effect did not reach significance.

Mice tested at 1MPI on the elevated plus maze exhibited no effects of Vivreon 2.0 administration. There was no significant effect of drug on either total distance traveled (Figure 4.6A, unpaired t test, p=0.2553) or time spent in open arms (Figure 4.6B, unpaired t test, p=0.2758) in comparison to vehicle. In addition, mice tested at 1MPI exhibited no changes due to Vivreon 2.0 administration on multiple measures during the forced swim test. There was no effect of drug on time immobile (Figure 4.6D, unpaired t test, p=0.8538), time highly mobile (Figure 4.6E, unpaired t test, p=0.0723), or time not moving (Figure 4.6F, unpaired t test, p=0.7741) in comparison to vehicle. Moreover, drug administration had no effect on learning performance as assessed by the Morris water maze. Vivreon 2.0 did not lead to decreased time to reach the hidden platform over 5 training days (Figure 4.6C, 2way ANOVA, $F_{(4,72)}$=0.7147, p=0.5846) in comparison to vehicle.

Neither compound had any significant effect on any behavioral measure assessed. This came as a surprise, and even in the case of the AVL-3288 compound, the drug treated group performed worse on the water maze than the injured control group, although not statistically significant. There were a few issues and complications associated with this

experiment. First, there was no uninjured control group to verify the injury actually caused behavioral deficits. However, we have compared sham to rmTBI in C57Bl/6J mice in over 10 separate cohorts, and have always detected an injury effect. Second, the individual drugs' pharmacokinetics in relation to injury sequelae may not have been optimized. Vivreon's compound had a very quick half-life (approximately 20 minutes), and is being further developed with an oral delivery mechanism that could have longer lasting effects in vivo. In addition, dosing daily may have caused increased stress throughout both vehicle and treated groups. Daily subcutaneous injections for an entire month is not ideal, and could have lead to a wide range of neuropathological changes and responses to the injury. As no behavioral deficits were observed, no histological assessments were undertaken. However, a closer examination of the drugs effect on microglial activation and cholinergic neurons in the hippocampus would help us get a better understanding of the drugs impact following rmCHI. These samples are in storage for future histological analysis.

References

Ager, R.R., Davis, J.L., Agazaryan, A., Benavente, F., Poon, W.W., LaFerla, F.M., Blurton-Jones, M., 2015. Human neural stem cells improve cognition and promote synaptic growth in two complementary transgenic models of Alzheimer's disease and neuronal loss. Hippocampus 25, 813-826.

Anderson, A.J., Haus, D.L., Hooshmand, M.J., Perez, H., Sontag, C.J., Cummings, B.J., 2011. Achieving stable human stem cell engraftment and survival in the CNS: is the future of regenerative medicine immunodeficient? Regen Med 6, 367-406.

Andres, R.H., Horie, N., Slikker, W., Keren-Gill, H., Zhan, K., Sun, G., Manley, N.C., Pereira, M.P., Sheikh, L.A., McMillan, E.L., Schaar, B.T., Svendsen, C.N., Bliss, T.M., Steinberg, G.K., 2011. Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. Brain 134, 1777-1789.

Beretta, S., Cunningham, K.M., Haus, D.L., Gold, E.M., Perez, H., Lopez-Velazquez, L., Cummings, B.J., 2016. Effects of Human ES-Derived Neural Stem Cell Transplantation and Kindling in a Rat Model of Traumatic Brain Injury. Cell Transplant.

Carroll, L.J., Cassidy, J.D., Holm, L., Kraus, J., Coronado, V.G., Injury, W.H.O.C.C.T.F.o.M.T.B., 2004. Methodological issues and research recommendations for mild traumatic brain injury: the WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury. J Rehabil Med, 113-125.

Chen, J., Li, Y., Wang, L., Zhang, Z., Lu, D., Lu, M., Chopp, M., 2001. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. Stroke; a journal of cerebral circulation 32, 1005-1011.

Chen, L., Coleman, R., Leang, R., Tran, H., Kopf, A., Walsh, C.M., Sears-Kraxberger, I., Steward, O., Macklin, W.B., Loring, J.F., Lane, T.E., 2014. Human neural precursor cells promote neurologic recovery in a viral model of multiple sclerosis. Stem Cell Reports 2, 825-837.

Chomiak, T., Hu, B., 2009. What is the optimal value of the g-ratio for myelinated fibers in the rat CNS? A theoretical approach. PLoS One 4, e7754.

Cummings, B.J., Uchida, N., Tamaki, S.J., Salazar, D.L., Hooshmand, M., Summers, R., Gage, F.H., Anderson, A.J., 2005. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc Natl Acad Sci U S A 102, 14069-14074.

Feldman, E.L., Boulis, N.M., Hur, J., Johe, K., Rutkove, S.B., Federici, T., Polak, M., Bordeau, J., Sakowski, S.A., Glass, J.D., 2014. Intraspinal neural stem cell transplantation in amyotrophic lateral sclerosis: phase 1 trial outcomes. Ann Neurol 75, 363-373.

Freedman, R., Hall, M., Adler, L.E., Leonard, S., 1995. Evidence in postmortem brain tissue for decreased numbers of hippocampal nicotinic receptors in schizophrenia. Biological psychiatry 38, 22-33.

Glass, J.D., Boulis, N.M., Johe, K., Rutkove, S.B., Federici, T., Polak, M., Kelly, C., Feldman, E.L., 2012. Lumbar intraspinal injection of neural stem cells in patients with amyotrophic lateral sclerosis: results of a phase I trial in 12 patients. Stem Cells 30, 1144-1151.

Guan, Z.Z., Zhang, X., Ravid, R., Nordberg, A., 2000. Decreased protein levels of nicotinic receptor subunits in the hippocampus and temporal cortex of patients with Alzheimer's disease. J Neurochem 74, 237-243.

Haus, D.L., Lopez-Velazquez, L., Gold, E.M., Cunningham, K.M., Perez, H., Anderson, A.J., Cummings, B.J., 2016. Transplantation of human neural stem cells restores cognition in an immunodeficient rodent model of traumatic brain injury. Experimental neurology 281, 1-16.

Hooshmand, M.J., Sontag, C.J., Uchida, N., Tamaki, S., Anderson, A.J., Cummings, B.J., 2009. Analysis of host-mediated repair mechanisms after human CNS-stem cell transplantation for spinal cord injury: correlation of engraftment with recovery. PLoS One 4, e5871.

Ishikawa, F., Yasukawa, M., Lyons, B., Yoshida, S., Miyamoto, T., Yoshimoto, G., Watanabe, T., Akashi, K., Shultz, L.D., Harada, M., 2005. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. Blood 106, 1565-1573.

Kim, J.H., Auerbach, J.M., Rodriguez-Gomez, J.A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S.H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K., McKay, R., 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 418, 50-56.

Kriks, S., Shim, J.W., Piao, J., Ganat, Y.M., Wakeman, D.R., Xie, Z., Carrillo-Reid, L., Auyeung, G., Antonacci, C., Buch, A., Yang, L., Beal, M.F., Surmeier, D.J., Kordower, J.H., Tabar, V., Studer, L., 2011. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. Nature 480, 547-551.

Lindvall, O., Kokaia, Z., 2010. Stem cells in human neurodegenerative disorders--time for clinical translation? The Journal of clinical investigation 120, 29-40.

Martino, G., Franklin, R.J., Baron Van Evercooren, A., Kerr, D.A., Stem Cells in Multiple Sclerosis Consensus, G., 2010. Stem cell transplantation in multiple sclerosis: current status and future prospects. Nat Rev Neurol 6, 247-255.

Mild Traumatic Brain Injury Committee, A.C.o.R.M., Head of Interdisciplinary Special Interest Group, 1993. Definition of mild traumatic brain injury. Journal of Head Trauma Rehabilitation 8, 86-87.

Mothe, A.J., Tator, C.H., 2012. Advances in stem cell therapy for spinal cord injury. The Journal of clinical investigation 122, 3824-3834.

Ng, H.J., Whittemore, E.R., Tran, M.B., Hogenkamp, D.J., Broide, R.S., Johnstone, T.B., Zheng, L., Stevens, K.E., Gee, K.W., 2007. Nootropic alpha7 nicotinic receptor allosteric modulator derived from GABAA receptor modulators. Proc Natl Acad Sci U S A 104, 8059-8064.

Salazar, D.L., Uchida, N., Hamers, F.P., Cummings, B.J., Anderson, A.J., 2010. Human neural stem cells differentiate and promote locomotor recovery in an early chronic spinal cord injury NOD-scid mouse model. PLoS One 5, e12272.

Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., Mangada, J., Greiner, D.L., Handgretinger, R., 2005. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol 174, 6477-6489.

Zhang, S.C., Wernig, M., Duncan, I.D., Brustle, O., Thomson, J.A., 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 19, 1129-1133.

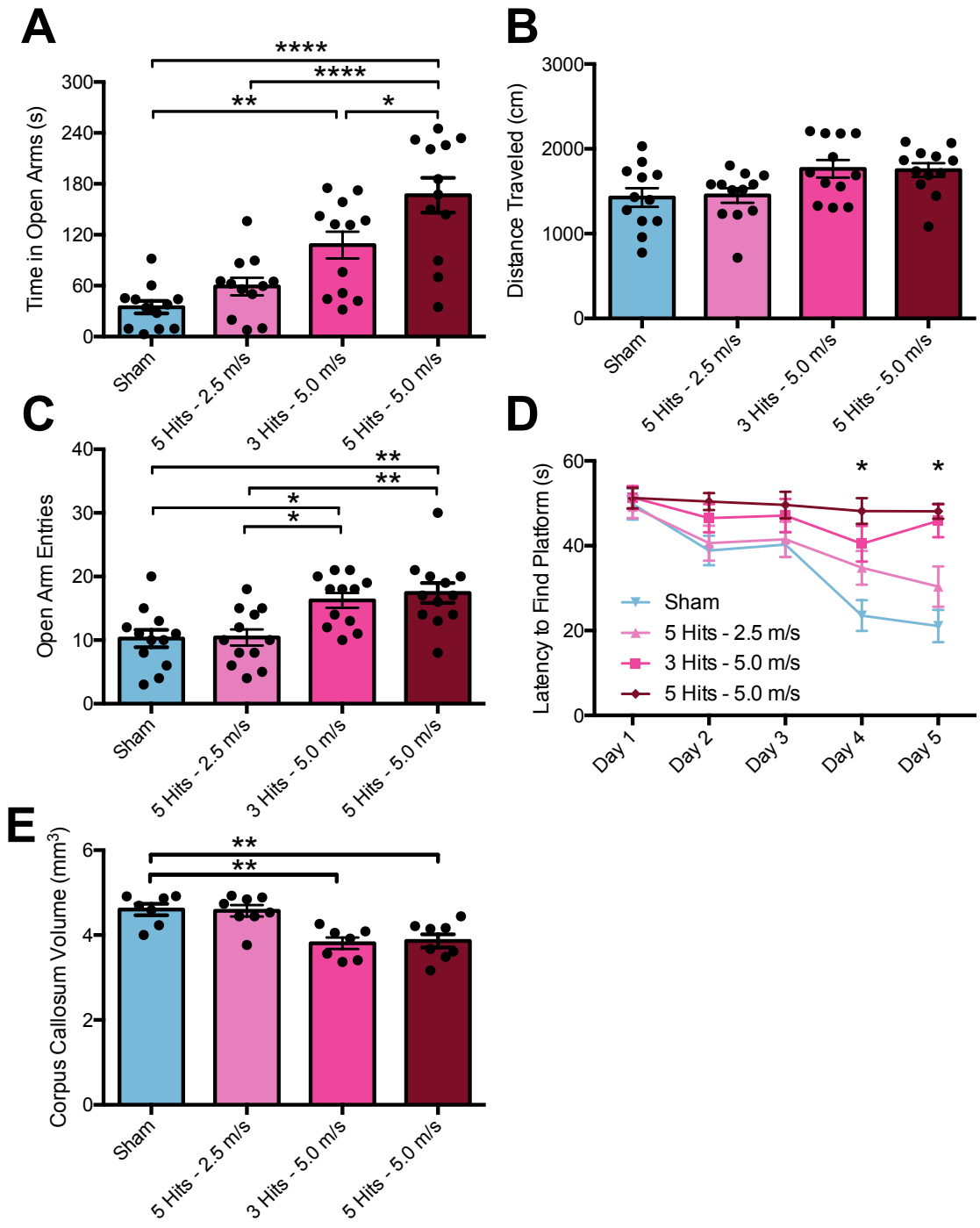

Figure 4.1. Decreasing the speed parameter of rmCHI injuries does not induce behavioral changes, while decreasing the number of hits at full velocity still induces effects. In the elevated plus maze test at 1mpi, mice with 5 hits or 3 hits at 5.0m/s showed increased time in open arms (A) and increased open arm entries (C) compared to sham

controls and mice with 5 hits at 2.5m/s. No changes in distance traveled during the elevated plus maze were observed across groups (B). Learning deficits in the elevated plus maze were observed in mice hit either 5 or 3 times at 5.0m/s but not at 5hits 2.5m/s (D). Dropping the velocity to 2.5m/s over 5 hits did not cause corpus callosum atrophy, however 3 hits at 5.0m/s had similar atrophy to 5 hits at 5.0m/s (17%) compared to sham controls (E).

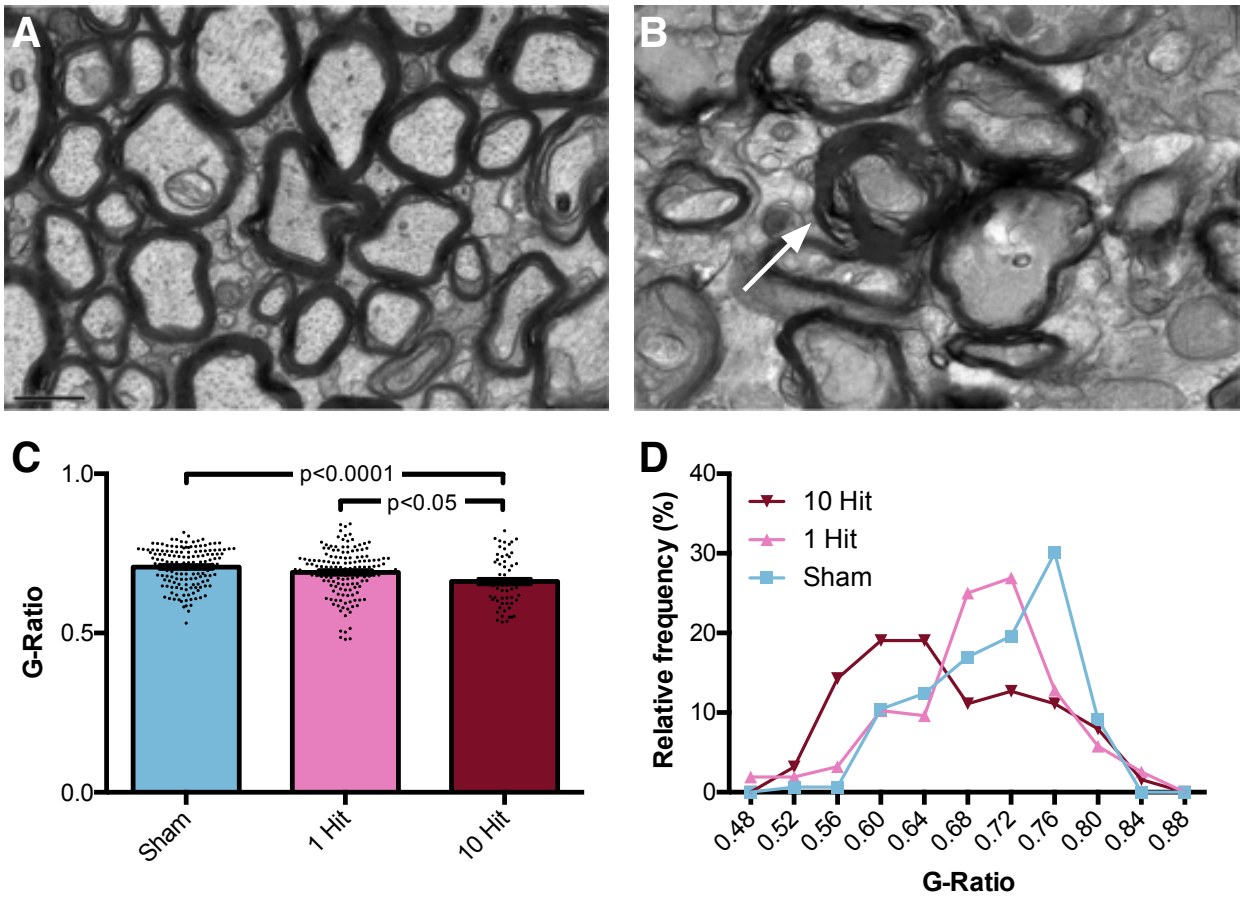

Figure 4.2. Electron microscopy analysis reveals decrease in G-ratio following rmCHI. Cross-section example of healthy corpus callosum axons, tightly myelinated (A, 6000x, scale bar = 0.5um) compared to unhealthy axons with loosely wrapped myelin sheaths (B, arrow). 10 Hit rmCHI mice had lower G-ratios than both sham and 1 hit mice (C, 1way ANOVA, $p<0.0001$). D, Histogram analysis reveals a shift to lower G-ratios for mice suffering rmCHI at 2mpi.

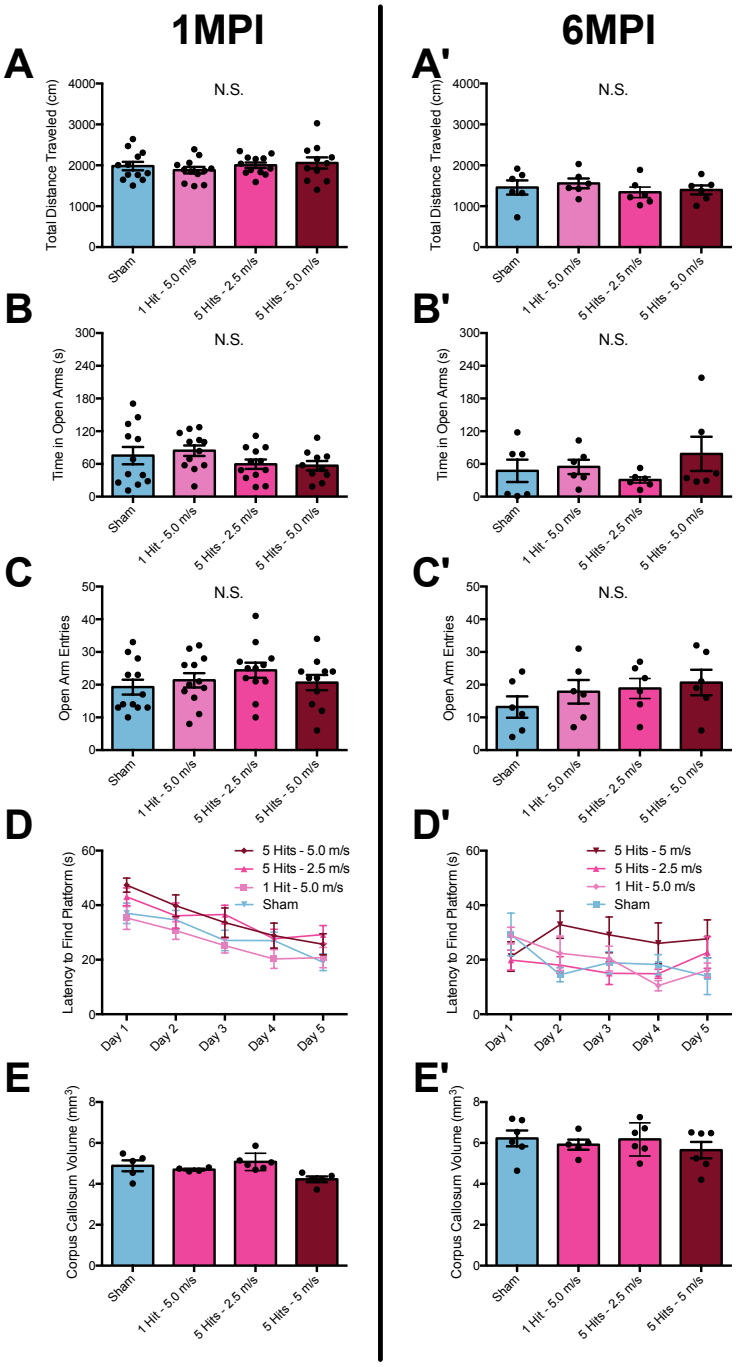

Figure 4.3. rmCHI in Nod-*scid* Gamma mice does not induce changes in behavioral performance at both 1MPI or 6MPI, nor is there a decrease in corpus callosum volume. No differences were observed at 1MPI on total distance traveled (A), time in open arms (B), or open arm entries (C). No differences were observed at 6MPI on total distance

traveled (A'), time in open arms (B'), or open arm entries (C'). No differences were observed in latency to find platform during the Morris water maze testing at 1MPI (D) or 6MPI (D'). Corpus callosum atrophy was not observed at either 1MPI (E) or 6MPI (E').

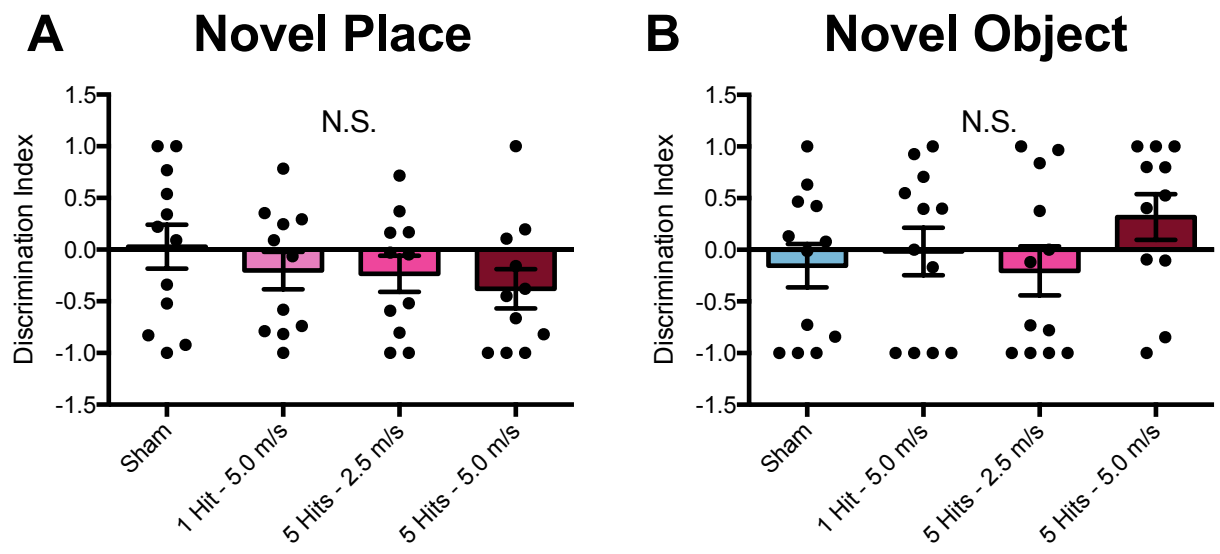

Figure 4.4. rmCHI in Nod-*scid* Gamma mice does not induce changes in Novel Object Recognition or Novel Place Recognition task at 2MPI. No differences were observed at 2MPI as assessed by discrimination index at 1MPI (A) or 6MPI (B). Discrimination index is equal to the time spent exploring novel object minus the time spent exploring familiar object, over time spent exploring either object.

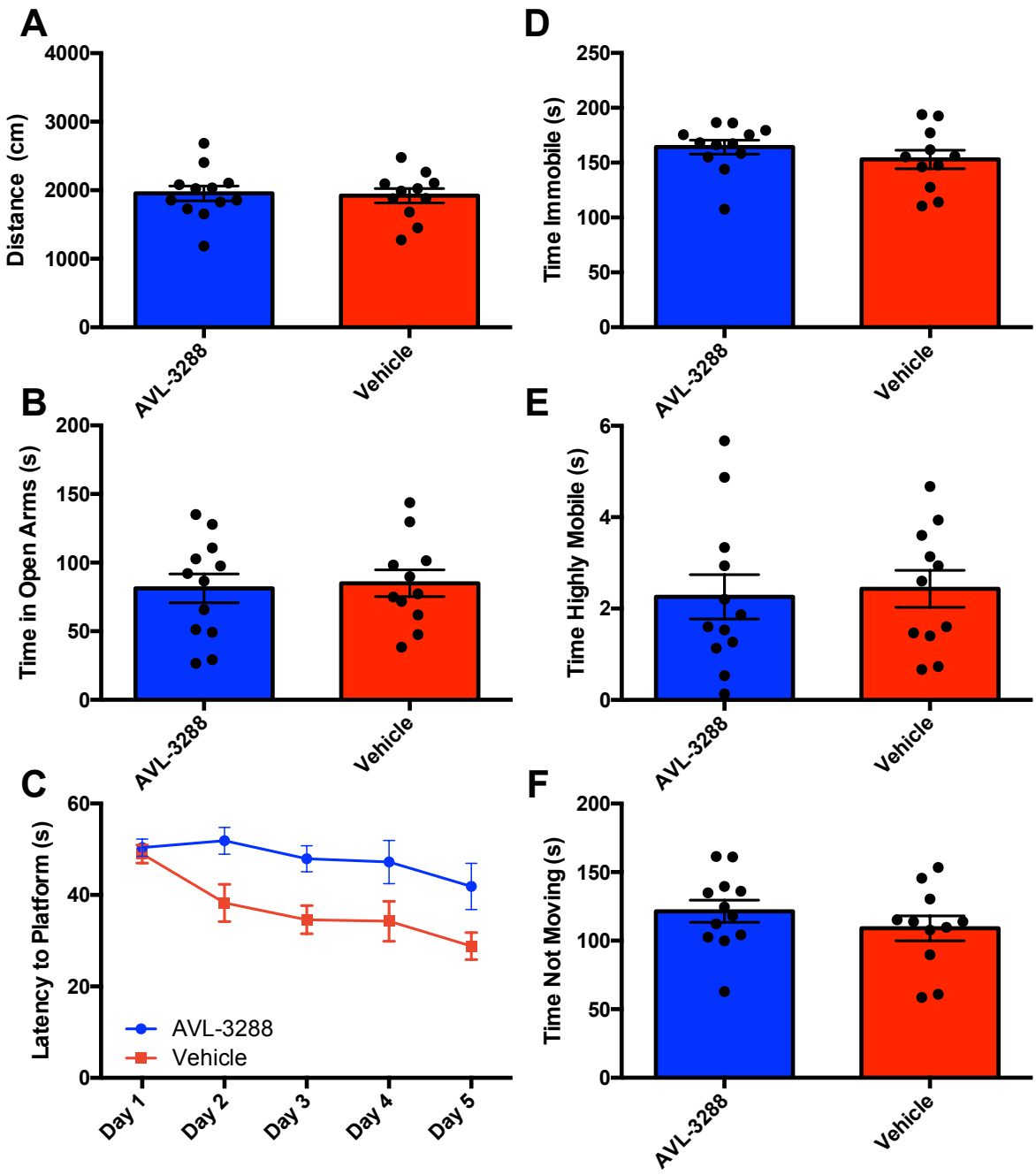

Figure 4.5. AVL-3288 does not improve behavior performance at 1mpi or 2mpi. 1mpi following rmCHI, mice showed no differences with treatment of AVL-3288 in total distance traveled (A) or time in open arm (B) in the elevated plus maze task. There was no significant improvement, or worsening, of latency to reach platform during Morris water maze testing at 2mpi (C, 2 way anova, p=0.1109) between drug and vehicle groups. There

was no change in behavior during the forced swim test at 1mpi between drug and vehicle groups for time immobile (D), time highly mobile (E), or time not moving (F).

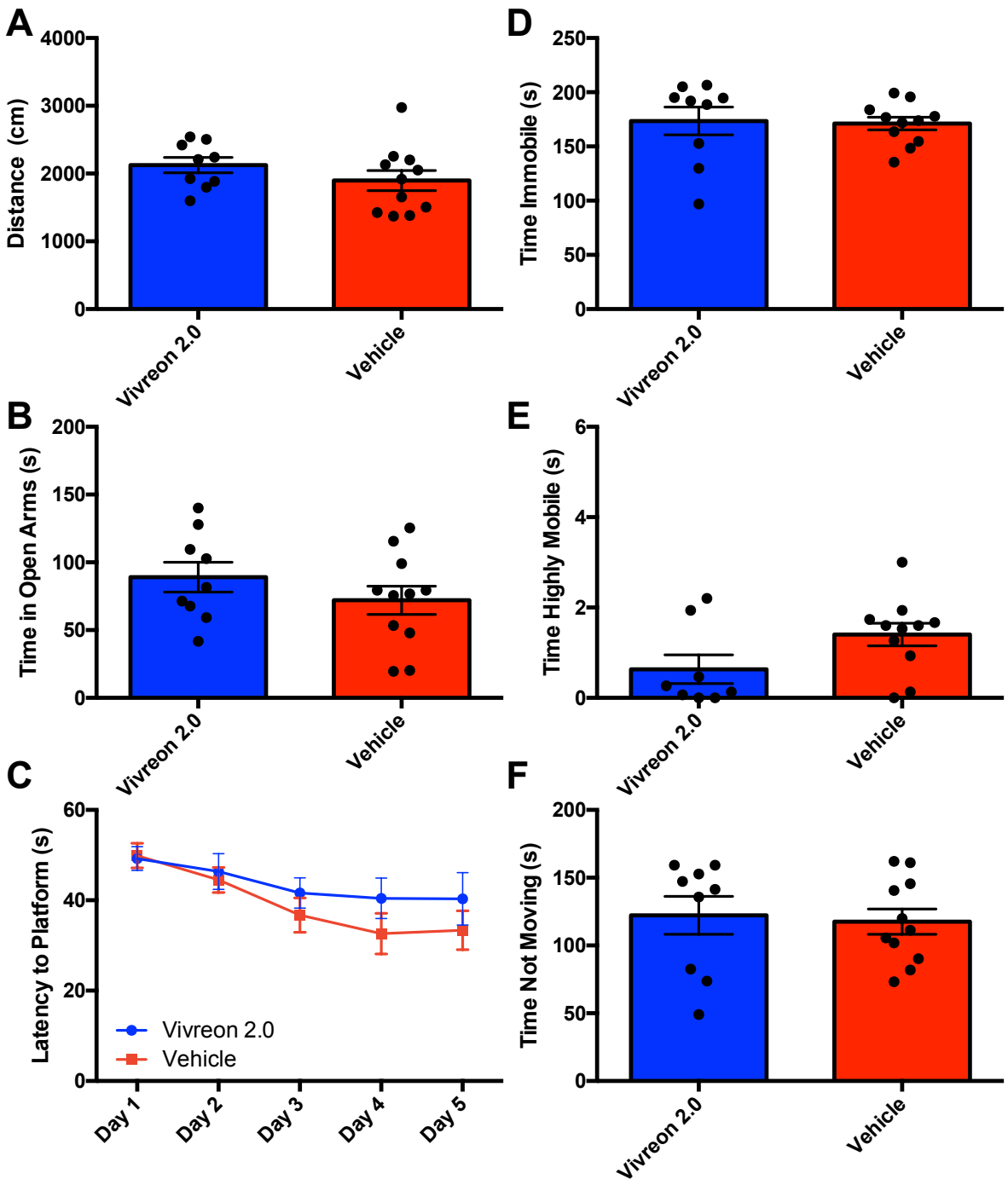

Figure 4.6. Vivreon 2.0 does not improve behavior performance at 1mpi or 2mpi.

1mpi following rmCHI, mice showed no differences with treatment of AVL-3288 in total distance traveled (A) or time in open arm (B) in the elevated plus maze task. There was no significant improvement, or worsening, of latency to reach platform during Morris water

maze testing at 2mpi (C, 2 way anova, p=0.5846) between drug and vehicle groups. There was no change in behavior during the forced swim test at 1mpi between drug and vehicle groups for time immobile (D), time highly mobile (E), or time not moving (F).

CHAPTER 5

Summary

Repeated mild traumatic brain injury is an underreported disease that is a result of heterogeneous injuries leading to a wide range of cognitive and behavioral changes. Development of rodent models of rmTBI are critical to further understanding mechanisms underlying pathology and functional deficits. A strong biomarker of rmTBI is changes to white matter tracts, often assessed by diffusion tensor imaging, although the mechanisms underlying these changes are not well understood. We sought to develop a model of rmTBI that exhibits changes in white matter tracts to further understand disease progression and its relationship to behavioral changes.

The work presented in this dissertation has shown the following:

- In a review of traumatic brain injury literature, 68% of papers did not evaluate a functional outcome past 1-month post TBI; 90% of papers reviewed did not make a functional assessment 2 or more months following injury; of these papers that investigated a 2 month timepoint, 84% demonstrated a functional deficit in a behavioral measure
- A novel model of mouse rmCHI was developed that is easily reproducible, has high throughput, and is clinically relevant
- rmCHI leads to ~30% corpus callosum atrophy that is associated with chronic microglial and astroglial reactivity and decreased myelin basic protein at chronic timepoints

- White matter atrophy is closely correlated with behavioral outcome tasks including measures of anxiety and learning
- White matter atrophy is not due to a decrease in oligodendrocyte precursor cells, but rather likely due to loss of axons from projecting cortical neurons as well as white matter myelin
- Nod-*scid* gamma mice exposed to the same rmCHI injury parameters do not exhibit any of the neuropathologies or functional changes as wildtype C57Bl/6J mice
- Experimental compounds tested, including AVL-3288 and Vivreon 2.0, did not have an effect on behavioral outcomes following rmCHI