Practical Therapies for Diffuse Traumatic Brain Injury in the Mouse: Translational Considerations

by

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Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved November, 2017 by the
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ARIZONA STATE UNIVERSITY

December 2017

ABSTRACT

Approximately 2.8 million Americans seek medical care for traumatic brain injury (TBI) each year. Of this population, the majority are sufferers of diffuse TBI, or concussion. It is unknown how many more individuals decline to seek medical care following mild TBI. This likely sizeable population of un- or self-treated individuals combined with a lack of definitive biomarkers or objective post-injury diagnostics creates a unique need for practical therapies among diffuse TBI sufferers. Practical therapies stand to decrease the burden of TBI among those who would otherwise not seek treatment or do not meet clinical diagnostic criteria upon examination. For this unique treatment niche, practical therapies for TBI are defined as having one or more of the following qualities: common availability, easy administration, excellent safety profile, and cost-effectiveness. This dissertation identifies and critically examines the efficacy of four classes of practical treatments in improving rodent outcome from experimental diffuse traumatic brain injury.

Over-the-counter (OTC) analgesics, omega-3 fatty acids, specialized pro-resolving mediators (SPMs), and remote ischemic conditioning (RIC) were administered before or following midline fluid percussion injury. Behavioral, histological, and molecular analyses were used to assess treatment effects on functional outcome and secondary injury progression. Acute administration of common OTC analgesics had little effect on post-injury outcome in mice. Dietary supplementation with omega-3 fatty acid docosahexaenoic acid (DHA) prior to or following diffuse TBI significantly reduced injury-induced sensory sensitivity and markers of neuroinflammation with no effect on

spatial learning. Intraperitoneal administration of omega-3 fatty acid-derived SPM resolvin E1 significantly increased post-injury sleep and suppressed microglial activation. Aspirin-triggered (AT) resolvin D1 administration improved both motor and cognitive outcome following diffuse TBI. RIC treatment in mice demonstrated little effect on functional outcome from diffuse TBI. Untargeted proteomic analysis of plasma samples from RIC-treated mice was used to identify candidate molecular correlates of RIC. Identification of these candidates represents a vital first step in elucidating the neuroprotective mechanisms underlying RIC. The overall findings suggest that omega-3 fatty acid supplementation, SPM administration, and RIC may serve as effective practical therapies to reduce the somatic, cognitive, and neurological burden of diffuse TBI felt by millions of Americans.

DEDICATION

I must thank my family and friends. My wife Alisha has been incredibly supportive. I cannot fathom moving across the country to pursue this endeavor without her unwavering support and companionship. She has the amazing ability to step back and provide an unbiased and honest opinion about anything. She is my sounding board, the wind that keeps my head in the clouds, and the kite string that keeps me loosely tethered to reality. My parents, Pamela and Darrell, have managed to inspire, fuel, and support me even from 1,871 miles away. My older brother, Casey, and cousin, Kelly, have always served as examples to lead the way and show me there is a light at the end of the tunnel worth pursuing. My aunt Becky remains a needed kindred spirit and open mind in a small town. All of you, along with my grandparents Charles and Eva, have served as the small but tight knit family I needed to know was there, whether I could see you or not. And to my closest friends, Michael and Ethan, I am thankful that physical distance has only brought us closer over the years and hope that I can be as good a friend as you have been to me. I thank all of you for your encouragement, friendship, and love that has brought me here.

AKNOWLEDGMENTS

I didn’t always realize how special my mentor is. Apparently not all mentors move you across the country and let you stay at their home until you find a place. Apparently not all mentors put you on their calendar every week and see you every day. Apparently not all mentors have effective remedies for snow and rain days. Apparently not all mentors are relentlessly optimistic about science and encouraging of constructive failure. Apparently not all mentors are Jonny Lifshitz. It has taken time to realize the small things that make a great mentor, but I’ve gradually learned a lot about being a scientist, mentor, and human being from mine. For that I am thankful.

I would like to acknowledge current and former members of the Lifshitz Translational Neurotrauma Lab for their support, feedback, and friendship over the past few years including Theresa Thomas, Jenna Ziebell, Dan Griffiths, Tim Ellis, Joshua Beitchman, Matt Law, Maha Saber, Bret Tallent, Katie Giordano, and Caitlin Hair. You have each provided small pieces of guidance and camaraderie when needed most. I would especially like to thank Rachel Rowe for serving as my auxiliary mentor and for her shared dedication to so many of the studies contained in this dissertation.

I thank the members of my graduate committee: Janet Neisewander, Sarah Stabenfeldt, Anthony Willyerd, and Patrick Pirrotte. Having experts from such diverse backgrounds has been instrumental to my success and continued growth as a scientist. Thank you for your time and commitment to guiding my research and professional development. This dissertation was supported in part by the National Institutes of Health predoctoral fellowship F31-NS090921 and the Diane and Bruce Halle Foundation.

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CHAPTER 1

INTRODUCTION

TREATING THE UNTREATED: DEFINING PRACTICAL THERAPIES FOR DIFFUSE TRAUMATIC BRAIN INJURY

Approximately 2.8 million Americans seek medical care for traumatic brain injury (TBI) each year (Taylor, Bell et al. 2017). The most common cause of TBI varies by age group with falls most prominent among the very young and elderly. Other common mechanisms of injury include strikes against the head, motor vehicle accidents, and assault (Faul and Coronado 2015). The wide array of long term consequences of TBI can include impaired cognitive or emotional function (Arciniegas, Topkoff et al. 2000; Albensi and Janigro 2003; Masel and DeWitt 2010), sensory sensitivity or dysfunction (Goodrich, Flyg et al. 2013; Goodrich, Martinsen et al. 2014), and loss of neurological function (Arciniegas, Topkoff et al. 2000). These symptoms of TBI, paired with pain from post-concussive headache, can profoundly degrade quality of life from months to years following injury.

Of patients seeking care for TBI, approximately 60-90% are sufferers of mild TBI (Holm, Cassidy et al. 2005). It is unknown how many more individuals decline to seek medical care following mild TBI, but in one survey over 40% of respondents having suffered a TBI reported not seeking medical attention (Setnik and Bazarian 2007). This large population of un- or self-treated individuals combined with a lack of definitive biomarkers or objective post-injury diagnostics creates a unique need for practical therapies among diffuse TBI sufferers. Practical therapies could stand to decrease the

burden of TBI among those who would otherwise not seek treatment for minor symptoms such as headache or do not meet clinical diagnostic criteria upon examination. For this unique treatment niche, we define practical therapies for TBI as having one or more of the following qualities: without risk of harm, readily available, easily administered, and cost-effective. Practical therapies may vary with regard to the window of treatment and could be applied continuously as a prophylactic, immediately after TBI, or in an extended time course following TBI.

In this dissertation, practical therapies were chosen in part for their potential to decrease inflammation following TBI. Following the primary mechanical injury force, secondary injury cascades are activated in the brain which can persist for up to years following injury (Johnson, Stewart et al. 2013). Thus, TBI can be thought of not only as an event but the initiation of a disease process which can be treated. These secondary injury mechanisms include but are not limited to inflammation, oxidative stress, glutamate excitotoxicity, and programmed cell death. Inflammation was chosen as a tractable therapeutic target because of its early activation and potential for chronic persistence. While inflammation is a normal host response to injury which includes necessary repair processes such as clearing of cellular debris, prolonged inflammation in the context of TBI is thought to exacerbate the primary injury and coincide with a worsening of symptoms or impairments (Morganti-Kossmann, Rancan et al. 2002). The goal of this dissertation is to test the efficacy of applying an early practical therapy to prevent an excessive inflammatory state from manifesting along with worsened motor, cognitive, or affective function.

A key element to the translational goal of this dissertation is to evaluate practical therapies using a rodent model of TBI and tests which reflect quality of life in humans. For example, after TBI deficits in balance and dizziness are often reported and investigated with questionnaires and physical exams (Chandrasekhar 2013). In mice, behavioral tests can be used to objectively assess sensorimotor function prior to and following experimental brain injury. One such test, the neurological severity score (NSS), tests mice on eight criteria for ability to balance on and traverse beams of different sizes along with the presence of normal neurological reflexes (Chen, Constantini et al. 1996). Tests such as the NSS allow experimenters to draw conclusions on efficacy that relate to human symptoms. The subsequent chapters of this dissertation use this and similar behavior tests to infer efficacy in domains of sensorimotor, cognitive, and affective function with the long-term translational goal of reducing these burdens among human sufferers of TBI.

Current clinical treatment of diffuse TBI includes a graded return to play or activity approach (May, Marshall et al. 2014). This approach advocates a period of physical and mental rest immediately following TBI that is followed by incremental addition of activity. If new symptoms arise or current symptoms worsen, it is advised to revert back to the previous state of activity until symptoms subside. Practical therapies would ideally be implemented with similar recommendations: if a practical therapy is administered and symptoms worsen, the treatment regimen should be discontinued and additional professional medical care may be warranted. Safety is a paramount consideration in the advancement of practical therapies, but it is not expected that all

individuals will respond uniformly to treatment, especially in the case of a very heterogeneous patient population such as TBI. The current chapter is an overview of promising areas from which practical therapies for mild TBI may arise with particular focus on translational outcome measures—or tests that mirror outcome in the human condition.

1.1 DIET/NUTRITION

Nutritional supplementation and diet modification represent a potential branch of practical therapies for mild TBI. From omega-3 fatty acids, to caloric restriction, to plant-based compounds, nutritional approaches have shown promise of neuroprotection in experimental studies of TBI. This section will provide an overview of research on each of these approaches.

Omega-3 fatty acids are polyunsaturated fatty acids primarily found in cold water fish which chiefly include docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Omega-3 fatty acid have widely studied anti-inflammatory properties (Calder 2017). In the brain, fatty acids are incorporated into cell membranes and subsequently released in response to cellular stress or injury. Western diets are typically high in omega-6 fatty acids, including arachidonic acid (ARA), which displace omega-3 fatty acids, including DHA and EPA, in cell membranes (Bradbury 2011). Omega-6 derived lipid mediators include pro-inflammatory prostaglandin E2 and leukotriene B4 which may prolong inflammation and secondary injury in the absence of omega-3 fatty acids. With omega-3 fatty acid supplementation, the balance of lipid mediator metabolic precursors can be restored (Harrison, Rowe et al. 2016). DHA has demonstrated potent

anti-inflammatory effects in animal models of ischemic stroke (Belayev, Khoutorova et al. 2009) and TBI (Wu, Ying et al. 2011). While the mechanisms of action of DHA in the wake of neurological injury remain widely unknown, its therapeutic potential is compelling (Hasadsri, Wang et al. 2013). Special considerations for use of omega-3 fatty acid as a practical therapy include the relatively long time needed for incorporation into cell membranes. This limits the effective time window to long-term pre-treatment or post-treatment with little effect of acute treatment at time of injury.

A broad category of nutritional supplementation using natural, plant-based compounds has emerged with applications as practical therapies for TBI (for full review, see (Scheff and Ansari 2017)). Among the most studied of these diverse natural compounds are caffeine, curcumin, and resveratrol. Chronic pre-treatment of mice with caffeine before controlled cortical impact TBI was shown to significantly decrease markers of inflammation. This finding was in contrast to that from an acutely-caffeine-treated group in the same study which showed no neuroprotective effects (Li, Dai et al. 2008). Chronic, dietary pre-treatment with curcumin-supplemented food decreased markers of oxidative stress and spatial learning impairment following lateral fluid percussion TBI in rats (Wu, Ying et al. 2006). Acute treatment of rats with resveratrol, a naturally occurring compound found in grapes, for three days following controlled cortical impact injury reduced markers in an array of outcomes including edema, motor impairment, and inflammatory markers (Feng, Cui et al. 2016). Together, these studies demonstrate the breadth of natural compound efficacy with varying beneficial effects in edema, inflammation, and oxidative stress. Unlike omega-3 fatty acid supplementation,

some natural compounds, including resveratrol, showed promise of acute and post-injury treatment instead of relying solely on chronic pre-treatment.

In nearly perfect contrast with nutritional supplementation, caloric restriction is another prophylactic practical therapy which has demonstrated neuroprotection following experimental models of TBI (Rich, Van Landingham et al. 2010; Loncarevic-Vasiljkovic, Pesic et al. 2012). Three months of relatively extreme caloric restriction (50% reduced daily intake) preceding cortical stab injury in rats resulted in significant decreases in secondary injury—particularly neuroinflammatory cascades and microglial activation (Loncarevic-Vasiljkovic, Pesic et al. 2012). Similarly, four months of more modest restriction (30% reduced daily intake) improved spatial memory performance following weight-drop cortical contusion in rats (Rich, Van Landingham et al. 2010). In a more practical approach due to its post-injury application, fasting has been demonstrated to be neuroprotective after controlled cortical impact TBI in mice (Davis, Pauly et al. 2008). This study found that 24 hours of post-injury fasting, but not 48 hours of post-injury fasting, significantly increased tissue sparing following injury. In humans, chronic caloric restriction (25% reduced daily intake) has been demonstrated to be safe and well tolerated (Romashkan, Das et al. 2016). The demonstrated neuroprotective effects and safety of prophylactic caloric restriction and post-injury acute fasting make them ideal candidate practical therapies.

The low cost of supplementation (or negative cost associated with caloric restriction), over-the-counter availability of many fatty acid and plant-based supplements, and often well-studied safety profiles make diet modification a prime avenue from which

to draw practical therapies. The primary limitation to these approaches is in timing: many require chronic pre-treatment prior to an injury which cannot be predicted. While this uncertainty is partially countered by the aforementioned strengths in convenience and availability, chronic diet modification appears particularly appropriate for demographics at high-risk for sustaining TBI such as contact sports participants, the military, and the elderly. Nutritional supplements are regulated by the Food and Drug Administration, but the Dietary Supplement Health and Education Act of 1994 restricted the authority of the FDA concerning supplements. Supplement manufacturers are not required to prove to the FDA that labeling claims are accurate. Because of this often products with similar labeling can contain ingredients of vastly different quality, origin, or processing (Swann 2016). An important part of making dietary modification an effective practical therapy, after basic and clinical research on efficacy, is the establishment of middle ground classification between food and drug for the regulation and assurance of quality among supplements. A dedicated regulatory body for this does not yet exist in the U.S.A.

1.2 EXERCISE

Exercise and self-rehabilitation are examples of practical therapies that are generally health-improving in and out of the context of TBI. Voluntary cardiovascular exercise has been well documented to improve outcome from experimental models of TBI, but timing of exercise in relation to TBI appears to be vital. Consistent cardiovascular exercise pre-treatment has been demonstrated to improve outcome from TBI in mice (Taylor, Montgomery et al. 2015; Zhao, Sabirzhanov et al. 2015). A study in rats, however, demonstrated that post-injury exercise within one week of fluid percussion

brain injury significantly decreased a wide array of potentially beneficial plasticity-related proteins compared to un-exercised controls (Griesbach, Gomez-Pinilla et al. 2004). This finding was supported in mice where late (5 weeks following TBI), but not early (1 week following TBI), exercise improved functional outcome (Piao, Stoica et al. 2013). These studies suggest there may be a critical period acutely following TBI in which exercise is not beneficial or could even worsen long term cognitive and motor symptoms. This potential for harm detracts from the promise of acute post-injury exercise as a practical therapy, but pre-injury exercise and closely regimented post-injury exercise remain viable self-treatment options, particularly when used in a graded approach which can be decreased if symptoms worsen.

Alternatives to cardiovascular exercise which focus on mental and holistic wellbeing have been preliminarily assessed. These approaches understandably rely upon clinical studies due to limitations in adapting treatments to animal models. Both mindfulness-based stress reduction (MBSR) and yoga have shown indications of improvement among TBI patients in domains relevant to their practice. Eight weeks of MBSR for stroke and TBI patients reporting mental fatigue improved self-reported outcome in mental fatigue and tests of neuropsychological function compared to non-treated controls (Johansson, Bjuhr et al. 2012). Two preliminary clinical studies of yoga among TBI patients showed promising results for yoga-related outcomes including breath control, balance, and self-reported psychological wellbeing (Silverthorne, Khalsa et al. 2012; Schmid, Miller et al. 2016). Further research is needed to understand mechanisms behind these alternative exercise regimens, but current research indicates they are very

low-risk practical therapeutic opportunities. Additional consideration of timing of these interventions, similar to those given to cardiovascular exercise, may be warranted as all current clinical studies include patients beyond the acute treatment window.

1.3 PHYSIOLOGICAL

Physiological interventions are an emerging field in practical therapies for TBI. Among these, interventions in sleep hygiene have been demonstrated to improve sleep-related symptoms in TBI patients (for review, see (Bogdanov, Naismith et al. 2017)). Sleep disturbances including insomnia, hypersomnia, fatigue and sleep apnea are common among TBI patients (Barshikar and Bell 2017). A promising practical therapy for sleep disturbances after TBI is blue light therapy. Blue light therapy is a self-administered, in-home practical therapy that consists of exposure to high-intensity blue light in the morning. A randomized clinical study demonstrated that four weeks of 45 minute daily light therapy sessions was sufficient to decrease measures of fatigue and daytime sleepiness among TBI patients (Sinclair, Ponsford et al. 2014). With the goal of improving individual sleep quality after TBI, sleep hygiene interventions pose very little risk of harm. Further research is warranted to identify additional sleep routine modifications which improve outcome.

Remote ischemic conditioning (RIC), the process of transiently impeding blood flow to an organ to impart protection to a distant target organ, has demonstrated pre-clinical neuroprotective effects in stroke and now recently in TBI. To date, the majority of clinical and laboratory studies have employed several short cycles (3-5 minutes each) of RIC using a blood pressure cuff (clinically) or femoral artery clamp (pre-clinically)

with equally spaced periods of cuff deflation and limb reperfusion. This non-invasive procedure can provide cellular protection from subsequent or prior injury to the brain and other organs. Though many mechanistic pathways have been proposed including nitric oxide synthase, inflammatory cytokines, opioid receptors, and potassium ATP channels, none have been definitively accepted (for review, see (Tapuria, Kumar et al. 2008)). A study of RIC induced two hours following controlled cortical impact in mice demonstrated significant reduction of TBI-induced motor and cognitive impairments (Sandweiss, Azim et al. 2017). Clinically, TBI patients treated in the emergency department with RIC showed significantly decreased biomarkers of TBI, potentially indicating a less severe injury response (Joseph, Pandit et al. 2015). RIC qualifies as a practical therapy for its ease and speed of administration, cost-effectiveness, and lack of indication of harmful effects. Further studies are needed to identify the most effective RIC administration paradigm with regard to timing relative to TBI and cycle duration.

1.4 OVERVIEW OF DISSERTATION RESEARCH

Practical therapies are a diverse category of approaches as outlined above. The current dissertation is a multi-pronged pre-clinical examination of practical therapies for diffuse TBI with consideration of translationally relevant outcome measures. For this, each study incorporates tests which mirror the long term consequences of TBI in humans including motor, cognitive, and/or affective impairments. A key secondary injury mechanism, inflammation, is identified as a specific therapeutic target of the practical therapies investigated herein for its early activation and potential to perpetuate for years following TBI (Johnson, Stewart et al. 2013). Applying an early therapeutic intervention

such as the currently examined practical therapies has the potential to prevent secondary injury processes from persisting and accordingly improve quality of life after diffuse TBI.

The chapters that follow critically assess four practical therapies for efficacy in a single model of diffuse brain injury. The effects of over-the-counter analgesics, docosahexaenoic acid (DHA), resolvins, and remote ischemic conditioning on outcome from diffuse TBI are measured with numerous overlapping approaches including behavioral analysis, molecular profiling of inflammatory markers, and histological analysis. The order of presentation of these four approaches is not congruent with their chronological progression due to the nature of this multi-pronged assessment in which many of the studies overlapped in their execution or analysis.

In Chapter 2, we hypothesized that common over-the-counter analgesic medications acetaminophen and ibuprofen would improve functional outcome from diffuse TBI by attenuating the injury-induced inflammatory response. Ibuprofen is a non-steroidal anti-inflammatory drug and was hypothesized to have a greater impact on injury-induced inflammation than acetaminophen. Results indicated that acetaminophen-treated mice trended toward better outcome as measured by the rotarod test and neurological severity score, but no robustly significant differences were detected among brain-injured treatment groups. In conclusion, these over-the-counter medications were found to be practical but not efficacious therapies in this application. The finding that these medications did not adversely affect outcome indicated, however, that they may be safe for treating post-concussive symptoms including headache.

In Chapter 3, a dietary supplementation paradigm was tested as a potential practical therapy. We hypothesized that rats fed a DHA-enriched diet either prior to or following diffuse TBI would perform better on tests cognitive and sensory function due to attenuation of injury-induced inflammation. DHA-treated rats were found to behave more similarly to their sham-injured counterparts in a test of sensory sensitivity. As hypothesized, DHA treatment did significantly decrease TBI-induced microglial activation and cytokine production in the brain with greater effects seen from post-injury rather than pre-injury supplementation. These findings suggest that steady-state prophylactic and also post-injury dietary DHA supplementation may serve as effective practical therapies. The primary limitation of DHA as a practical therapy is the relatively long supplementation period required for effective DHA incorporation into cell membranes.

In Chapter 4, a follow-up approach to DHA supplementation was examined in an effort to provide the therapeutic efficacy of DHA without the long supplementation period. Resolvins are lipid mediators of inflammation derived from omega-3 fatty acids. We hypothesized that treatment with resolvins would improve functional outcome from TBI in mice due to the timely resolution of inflammation without the protracted supplementation period required by their omega-3 fatty acid precursors. Results indicated that aspirin-triggered resolving D1 (AT-RvD1), but not resolvin E1 (RvE1) improved both motor and cognitive outcome from TBI. This finding complemented findings from Chapter 3 as AT-RvD1, but not RvE1, is derived specifically from DHA and shared its indications of neuroprotection. While resolvins were demonstrated to be efficacious,

ultimately they are not presently a very practical therapy due to their lack of availability and high cost to the general public. Future studies (of biology, chemistry, and economics) are warranted to determine the plausibility of their development into more practical therapies.

Finally, Chapter 5 examines a physiological approach to practical therapies—RIC. RIC has shown pre-clinical efficacy in improving outcome from neurological injury (see Appendix C for summary table of previous studies). As a pilot study we hypothesized that RIC would similarly improve outcome from diffuse TBI in mice. Simultaneously, a hypothesis-generating approach was undertaken to identify plasma protein correlates of RIC in the context of TBI for further elucidation of the presently unknown mechanistic underpinnings of RIC. While RIC did not improve motor outcome from TBI, cognitive and affective outcome measures were inconclusive due to lack of TBI-induced impairments at the selected time points. Plasma protein correlates of RIC were identified in both the brain-injured condition and sham mice. Proteins selectively responsive to RIC regardless of TBI, including selenium-binding protein 1 and fatty acid binding protein 4 were identified as candidates for future RIC mechanism investigation.

CHAPTER 2

ACUTE OVER-THE-COUNTER PHARMACOLOGICAL INTERVENTION DOES NOT ADVERSELY AFFECT BEHAVIORAL OUTCOME FOLLOWING DIFFUSE TRAUMATIC BRAIN INJURY IN THE MOUSE

Published in Experimental Brain Research (Harrison et al. 2014)

2.1 ABSTRACT

Following mild TBI, patients may self-treat symptoms of concussion, including post-traumatic headache, taking over-the-counter (OTC) analgesics. Administering one dose of OTC analgesics immediately following experimental brain injury mimics the at-home treated population of concussed patients and may accelerate the understanding of the relationship between brain injury and OTC pharmacological intervention. In the current study, we investigate the effect of acute administration of OTC analgesics on neurological function and cortical cytokine levels after experimental diffuse TBI in the mouse.

Adult, male C57BL/6 mice were injured using a midline fluid percussion (mFPI) injury model of concussion (6-10 min righting reflex time for brain-injured mice). Experimental groups included mFPI paired with either ibuprofen (60mg/kg, i.p.; n=16), acetaminophen (40mg/kg, i.p.; n=9), or vehicle (15% ethanol (v/v) in 0.9% saline; n=13) and sham injury paired OTC medicine or vehicle (n=7-10 per group). At 24 hours after injury, functional outcome was assessed using the rotarod task and a modified neurological severity score (NSS). Following behavior assessment, cortical cytokine

levels were measured by multiplex ELISA at 24 hours post-injury. To evaluate efficacy on acute inflammation, cortical cytokine levels were measured also at 6 hours post-injury.

In the diffuse brain-injured mouse, immediate pharmacological intervention did not attenuate or exacerbate TBI-induced functional deficits. Cortical cytokine levels were affected by injury, time, or their interaction. However, levels were not affected by treatment at 6 hours or 24 hours post-injury. These data indicate that acute administration of OTC analgesics did not exacerbate or attenuate brain-injury deficits which may inform clinical recommendations for the at-home treated mildly concussed patient.

2.2 INTRODUCTION

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

The mechanical forces of TBI initiate a cascade of secondary injury processes, including inflammation, which continue for days to weeks following injury (Werner and Engelhard 2007). In conflicting studies, cerebral inflammation has been shown to contribute to either beneficial or deleterious effects after traumatic insult (for review, see (Morganti-Kossmann, Rancan et al. 2002)). TBI triggers a cascade of inflammation-mediating cytokines (Morganti-Kossmann, Rancan et al. 2001; Frugier, Morganti-Kossmann et al. 2010; Semple, Bye et al. 2010; Ziebell and Morganti-Kossmann 2010), which can elicit a range of responses including cell differentiation, immune activation, and cell death (Allan and Rothwell 2001). For the present study, the midline fluid percussion injury (mFPI) experimental model in the mouse induces multifocal neuropathology with translational application to mild diffuse TBI, or concussion. Principally in the first day after mFPI in mice, we have reported significantly increased levels of pro-inflammatory cytokine IL-1β in the cortex (Rowe, Striz et al. 2014) along

with acute neurological impairments manifested within one hour of injury (Rowe, Harrison et al. 2014). In diffuse TBI, the effects of clinically relevant acute pharmacological inhibition of inflammation on functional outcome are not yet understood.

Secondary injury processes initiated by TBI, including inflammation, are tractable therapeutic targets. Inflammation in the wake of TBI is, in part, mediated by the conversion of membrane-released arachidonic acid into pro-inflammatory prostaglandins by cyclooxygenase-2 (COX-2) (Dash, Mach et al. 2000). NSAIDs are widely available over-the-counter drugs used to treat acute pain and inflammation, with mechanisms of action to block COX-1 and/or COX-2, thereby slowing the production of prostaglandins (Vane 1971). Acetaminophen, on the other hand, is presented as an analgesic with actions on cannabinoid receptors (Ottani, Leone et al. 2006; Dani, Guindon et al. 2007), with weaker inflammatory properties. Previous studies suggest anti-inflammatory drugs improve outcome following brain injury as early as 72 hours post-injury (Gopez, Yue et al. 2005; Ng, Semple et al. 2012; Thau-Zuchman, Shohami et al. 2012; Chio, Chang et al. 2013; Gatson, Liu et al. 2013). Treatment with the highly specific COX-2 inhibitor DFU [5,5-dimethyl-3(3-fluorophenyl)-4(4-methylsulfonyl)phenyl-2(^{5}H)-furanone], administered daily for three days following lateral cortical impact in rats attenuated injury-induced prostaglandin production in the brain and improved functional recovery measured by the Morris water maze and neuroscore at 72 hours post-injury (Gopez, Yue et al. 2005). Carprofen, a COX-2 inhibitor, administered daily for seven days following experimental TBI using a closed head injury (CHI) model in mice, also improved

functional recovery (Thau-Zuchman, Shohami et al. 2012). Recovery of function measured by the NSS, however, was not present until 72 hours post-injury (Thau-Zuchman, Shohami et al. 2012). Treatment with anti-inflammatory minocycline for fourteen days following CHI in mice resulted in improved NSS scores starting at 72 hours post-injury, with improvements lasting through day 7 (Ng, Semple et al. 2012). These studies suggest that inhibiting inflammation after mild to severe TBI can improve functional recovery; however, there is evidence to suggest that treatment with ibuprofen over an extended timeframe may worsen cognitive outcome. Rats which were continuously treated with ibuprofen for four months following lateral fluid percussion injury performed significantly worse in the Morris Water Maze than non-treated brain-injured rats (Browne, Iwata et al. 2006). Taken together, previous reports indicate that repeated doses of OTC analgesics, depending on the timeframe, may be beneficial or detrimental to recovery from TBI. The acute nature of neurological impairments induced by the mFPI model necessitates acute behavioral analysis to assess the effects of pharmacological intervention (Rowe, Harrison et al. 2013). The current study delivers ibuprofen and acetaminophen to determine if a single treatment with common over-the-counter (OTC) analgesics after diffuse TBI promotes recovery or worsens behavioral outcome.

The current study investigated the effects of acetaminophen and ibuprofen—two common analgesic drugs with different anti-inflammatory mechanisms—on neurological function and cortical cytokine levels after diffuse TBI in the mouse. We hypothesized

that acute pharmacological inhibition of injury-induced inflammation will lead to a decrease in inflammatory cytokines, possibly altering functional outcome.

2.3 METHODS

2.3.1 Animals

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

2.3.2 Midline Fluid Percussion Injury (mFPI)

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

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

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

has been validated as an overt indicator of injury severity (Hosseini and Lifshitz 2009). The injury hub was removed and the brain was inspected for uniform herniation and integrity of the dura. The dura was intact in all mice; none were excluded as technical failures. The incision was cleaned using saline and closed using sutures. Moderate brain-injured mice had righting reflex recovery times greater than six minutes and a positive fencing response. Sham injured mice recovered a righting reflex within 20 seconds. After spontaneously righting, mice were placed in a heated recovery cage and monitored until ambulatory (approximately 5 to 15 minutes) before being returned to their cage. Adequate measures were taken to minimize pain or discomfort.

2.3.3 Pharmacological Intervention

All mice received either vehicle or drug treatment immediately following induction of injury or sham. Drugs were administered intraperitoneally in 100µl of sterile vehicle solution of normal saline and 15% (v/v) ethanol. Drug-treated mice received either ibuprofen (60 mg/kg; Sigma-Aldrich, St. Louis, MO) or acetaminophen (40 mg/kg; Sigma-Aldrich, St. Louis, MO). These doses were chosen with respect to clinically relevant doses. Dose translations from human to mice were based on body surface area (Reagan-Shaw, Nihal et al. 2008) and were maintained within the maximum daily dose recommended by the United States Federal Drug Administration (www.fda.gov). Both drugs were compared to the same vehicle-treated control group treated with normal saline and 15% (v/v) ethanol.

2.3.4 Behavioral Testing

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

Neurological Severity Score (NSS): Post-traumatic neurological impairments were assessed at 24 hours post-injury using an 8-point NSS paradigm adapted from those previously used in experimental models of TBI (Chen, Constantini et al. 1996; Semple, Bye et al. 2010; Pleasant, Carlson et al. 2011; Ziebell, Bye et al. 2011). One point was given for failure on an individual task, and no points were given if a mouse completed a task successfully. Mice were observed for hind limb flexion, startle reflex, and seeking behavior (presence of these behaviors was considered successful task completion). Mice traversed in sequence, 3, 2, and 1 centimeter beams. The beams were elevated and mice were given 1 minute to travel 30 centimeters on the beams. The task was scored as a

success if the mouse traveled 30 centimeters with normal forelimb and hindlimb position (forelimb/hindlimb did not hang from the beam). Mice were also required to balance on a 0.5 centimeter beam and a 0.5 centimeter round rod for 3 seconds in a stationary position with front paws between hind paws. Non-parametric data are presented as a composite score ranging from 0 to 8 representing performance on all tasks combined. High final NSS scores were indicative of task failure and interpreted as neurological impairment.

2.3.5 Tissue preparation and cytokine quantification

At 6 or 24 hours post-injury mice were given an overdose of sodium pentobarbital and transcardially perfused with ice cold phosphate buffered saline (PBS). Mice were decapitated and the brains were dissected on ice. Cortical biopsies (2mm diameter x 2mm thickness) were taken and snap frozen in methanol cooled over dry ice then stored at -80°C. The protein levels of a panel of inflammation-related cytokines were measured by Quansys Biosciences Mouse Cytokine IR Q-Plex assay (Quansys Biosciences, Logan, UT), according to manufacturer protocol. Cortical biopsies were bead-homogenized using a Precellys 24 in 200 µl of ice-cold Tris-buffered lysis solution supplemented with protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Mini Tablet, Roche Diagnostics, Mannheim, Germany). The cortical homogenate was centrifuged at 3000 RCF for 20 minutes at 4°C in a microcentrifuge. The resulting supernatant (25µl) was loaded per well of the Q-Plex plate, and cytokine levels were determined by Q-Plex assay. Cytokine levels in the cortex were normalized to the total amount of protein in the sample, as determined by BCA Protein Assay (Thermo Scientific, Rockford, IL).

2.3.6 Statistical Analysis

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

2.4 RESULTS

It was not anticipated that drug treatment would change functional outcome in the uninjured sham mice. Statistical analyses confirmed no significant change in rotatrod performance or neurological severity score between any sham treatment groups. Vehicle-treated, ibuprofen-treated, and acetaminophen-treated shams were combined into a single control. As anticipated, anti-inflammatory treatment altered cytokine levels in sham treatment groups; cytokine data were analyzed without combining shams.

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

To assess motor function we used the rotarod task as previously published (Bachstetter, Rowe et al. 2013). Across groups, there was a significant effect on latency to stay on the rotarod ($F(3, 53)=3.688$, $p=0.0174$; Figure 1A; sham n=27, vehicle-treated injury n=10, ibuprofen-treated injury n=12, acetaminophen-treated injury n=8). Rotarod latency was

significantly reduced in vehicle-treated and ibuprofen-treated brain-injured mice compared to sham mice at 24 hours post-injury (Figure 1A). There was no significant latency reduction in acetaminophen-treated brain-injured mice compared to shams (Figure 1A). Further analysis of rotarod performance confirmed the latency data with distance traveled, showing similar significant effects on distance traveled ($F(3, 53)=3.909$, $p=0.0135$; Figure 1B). Distance traveled was significantly reduced in both vehicle and ibuprofen-treated brain-injured mice compared to uninjured sham. There was no difference in distance traveled by acetaminophen-treated brain-injured mice compared to shams. To compensate for trial-based learning, improvement in motor performance was analyzed. Latencies (Figure 1C) and distances (Figure 1D) of each mouse at 24 hours post-injury were compared to their individual baseline scores at training. Brain-injured mice treated with vehicle and ibuprofen showed significantly less improvement in latency to stay on the rod compared to the improvement of uninjured shams ($F(3, 53)=4.553$, $p=0.0065$; Figure 1C). Acetaminophen-treated brain-injured mice did not show a difference in improvement compared to uninjured shams (Figure 1C). All brain-injured mice, regardless of treatment, showed significantly less improvement in distance traveled compared to shams ($F(3, 53)=6.017$, $p=0.0013$; Figure 1D).

Overall, diffuse brain injury reduced motor performance measured on the rotarod task. Acetaminophen-treated brain-injured mice did not show injury-induced impairments measured by latency (Figure 1A, 1C) or distance (Figure 1B), but did have a significantly worse improvement in distance from baseline compared to uninjured shams (Figure 1D). However, the acetaminophen-treated brain-injured mice did not show significant

improvements in motor impairments compared to all other brain-injured groups ($F(2,27)=0.5684$, $p=0.5730$; Figure 1A; ($F(2,27)=1.063$, $p=0.03594$; Figure 1B; ($F(2,27)=0.06751$, $p=0.9349$; Figure 1C). Acute pharmacological intervention, regardless of drug, did not exacerbate or attenuate brain-injury induced motor deficits.

2.4.2 Diffuse TBI resulted in neurological impairments regardless of pharmacological intervention.

All brain-injured mice showed significant neurological impairments measured by the neurological severity score (NSS) compared to uninjured shams, regardless of pharmacological intervention ($KW(4, 57)=27.37$, $p<0.001$; Figure 2; sham n=27, vehicle-treated injury n=10, ibuprofen-treated injury n=12, acetaminophen-treated injury n=8). At 24 hours post-injury all brain-injured groups had significantly higher NSS scores compared to uninjured shams. There was no significant effect of post-injury pharmacological treatment.

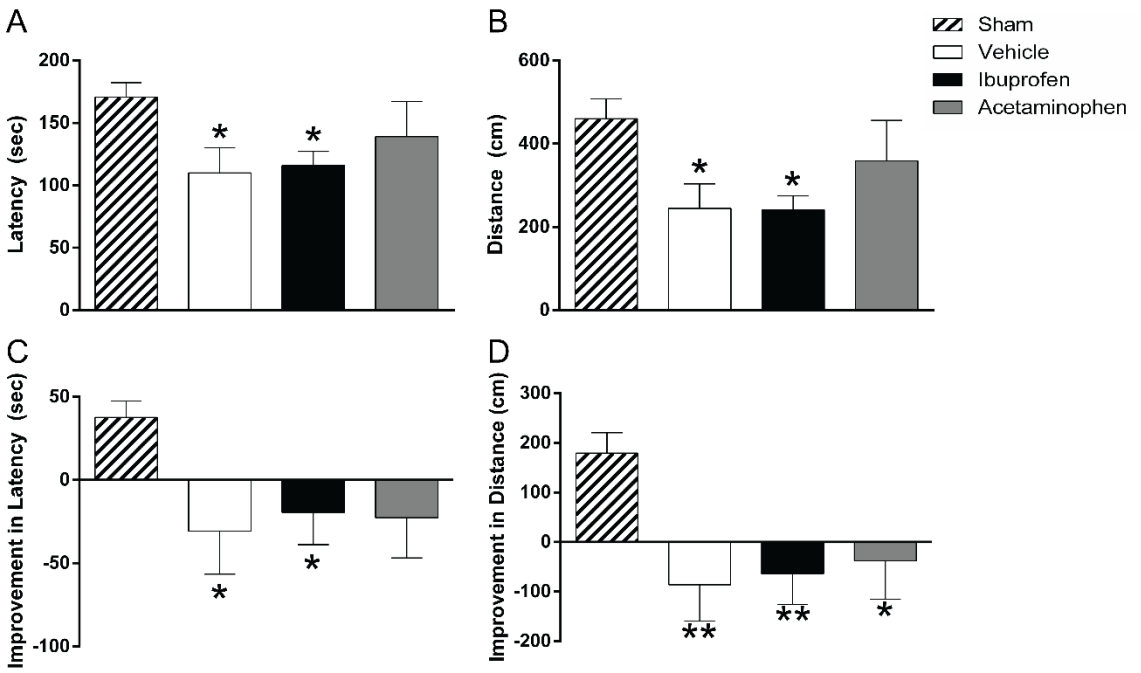

Figure 2.1. No adverse effects of pharmacological intervention on injury-induced motor deficits on the rotarod task.

(A) Injury significantly impaired motor performance as indicated by reduced latency to stay on the rotarod (mean ±SEM; $F(3, 53)=3.688$, $p=0.0174$), with significant differences between vehicle-treated and ibuprofen-treated brain-injured mice compared to uninjured shams at 24 hours post-injury. There was no significant difference between acetaminophen-treated brain-injured mice compared to uninjured shams. **(B)** Reduced distance traveled on the rotarod also indicated a significant injury-induced impairment in motor function (mean ±SEM; $F(3, 53)=3.909$, $p=0.0135$). There was a significant difference between vehicle-treated and ibuprofen-treated brain-injured mice compared to uninjured shams at 24 hours post-injury. There was no difference in distance traveled by acetaminophen-treated brain-injured mice compared to uninjured shams. **(C)** Brain injury significantly impaired the improvement in latency to stay on the rotarod from baseline (mean ±SEM; $F(3, 53)=4.553$, $p=0.0065$) indicated by a difference between vehicle-treated and ibuprofen-treated brain-injured mice compared to uninjured shams at 24 hours post-injury. **(D)** Brain injury also significantly impaired improvement in distance traveled (mean ±SEM; $F(3, 53)=6.017$, $p=0.0013$) between vehicle-treated, ibuprofen-treated, and acetaminophen-treated brain-injured mice compared to uninjured shams at 24 hours post-injury. (sham n=27, vehicle-treated injury n=10, ibuprofen-treated injury n=12, acetaminophen-treated injury n=8; *, $p<0.05$; **, $p<0.01$).

2.4.3 Diffuse TBI resulted in increased cytokine levels at 6 or 24 hours post-injury regardless of pharmacological intervention.

Upon brain dissection, no differences in hemorrhage or gross pathology were noted among treatment groups. To determine the changes in the inflammatory response following diffuse brain injury, we measured inflammatory cytokines in whole cortex at select time points following injury (6 hours, 24 hours). Data from both time points and all measured analytes are presented as measured concentration from cortical homogenate (pg/ml/mg) ± SEM (Table 1). Pro-inflammatory cytokines were found to increase by 6 hours post-injury but did not remain increased at 24 hours post-injury (Figure 3). Anti-inflammatory cytokines were not altered at 6 hours post-injury but increased by 24 hours post-injury (Figure 4). For all analytes, effect of pharmacological treatment was evaluated across brain-injured groups via one-way ANOVA revealing no significant differences in OTC analgesic-treated vs vehicle-treated groups.

Diffuse brain injury resulted in increased cortical levels of pro-inflammatory cytokines IL-6 and TNF-α when compared to uninjured shams at 6 hours post-injury ($F(1, 30)=4.468$, $p=0.0430$, Figure 3B; $F(1, 30)=6.853$, $p=0.0137$, Figure 3C). Another pro-inflammatory cytokine, IL-1α exhibited a modest increase in response to injury which failed to reach statistical significance ($F(1, 30)=1.192$, $p=0.2837$; Figure 3A). Significant injury effects are denoted in Figure 3 by asterisks. No significant injury effects were noted in pro-inflammatory cytokine levels at 24 hours post-injury. By 24 hours post-injury, IL-6 ($F(1, 58)=14.76$, $p=0.0003$, Figure 3B), IL-12 ($F(1,59)=35.61$, $p<0.001$, Table 1), and TNF-α ($F(1, 59)=11.38$, $p=0.0013$; Figure 3C), were each

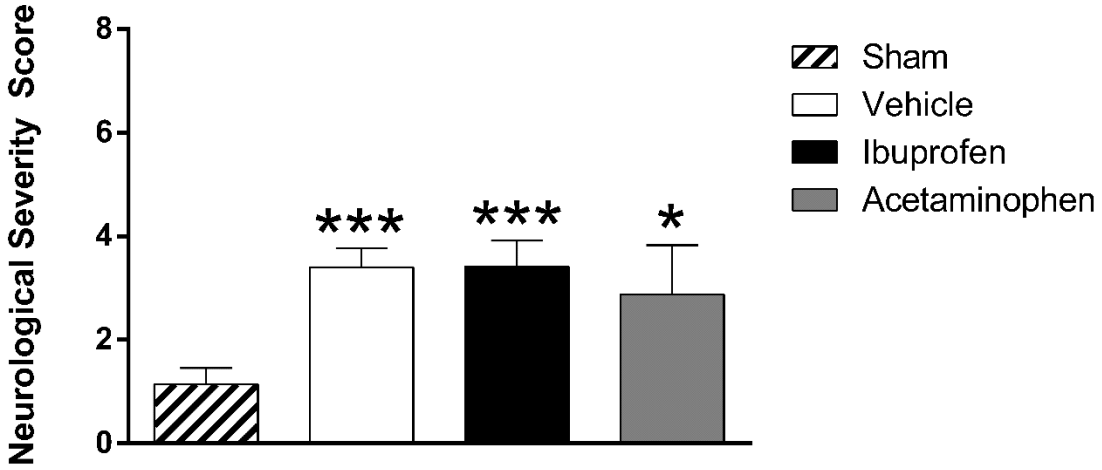

Figure 2.2. No adverse effects of pharmacological intervention on injury-induced neurological impairments.

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

decreased compared to their respective 6 hour measurements. Additionally, pro-inflammatory cytokines IL-1α, IL-1β, and IFNγ were reduced to undetectable levels at 24 hours, precluding statistical comparisons. Significant effects of time between 6 hour and 24 hour measurements are denoted in Figure 3 by crosses.

Diffuse brain injury did not alter cortical levels of anti-inflammatory cytokines IL-4 or IL-10 when compared to uninjured shams at 6 hours post-injury ($F(1, 23)=1.047$, $p=0.3169$; $F(1, 30)=0.2279$, $p=0.6366$, Figure 4A,B) or 24 hours post-injury ($F(1, 23)=0.6964$, $p=0.4126$, Figure 4B). IL-4 levels were increased at 24 hours post-injury compared to 6 hours post-injury; however, 6 hour IL-4 levels were undetectable, precluding statistical comparisons (Figure 4A). Levels of IL-2 and IL-10 were significantly increased at 24 hours post-injury compared to 6 hours post-injury ($F(1, 59)=0.1672$, $p<0.0001$, Figure 4B; ($F(1,59)=25.87$, $p<0.0001$, Table 1). Significant effects of time between 6 hour and 24 hour measurements are denoted in Figure 3 by crosses.

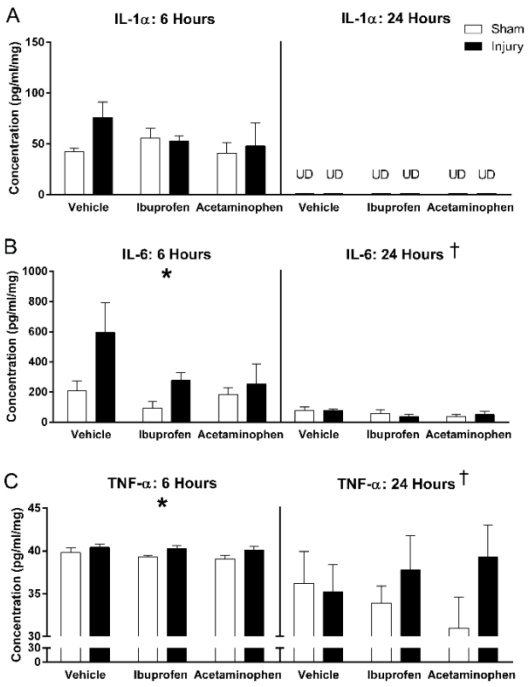

Figure 2.3. Diffuse brain injury increased pro-inflammatory cytokines in the cortex at 6 hours post-injury regardless of pharmacological treatment.

For all analytes, effect of pharmacological treatment was evaluated across brain-injured groups via one-way ANOVA revealing no significant differences in OTC analgesic-treated vs vehicle-treated groups. **(A)** IL-1α was increased in the cortex following brain injury at 6 hours post-injury compared to uninjured shams but failed to reach significance (mean ±SEM; $F(1, 30)=1.192$, $p=0.2837$). Overall, IL-1α decreased 24 hours post-injury to levels that were undetectable (UD) preventing statistical analysis. **(B)** IL-6 was significantly increased in the cortex following brain injury at 6 hours post-injury compared to uninjured shams (mean ±SEM; $F(1, 30)=4.468$, *; $p=0.0430$). Though there was an evident trend of both ibuprofen and acetaminophen toward reduction of injury-induced IL-6 compared to vehicle, these differences did not reach statistical significance ($F(2,18)=1.818$, $p=0.1909$). There were no significant injury-induced changes in IL-6 at 24 hours post-injury (mean ±SEM; $F(1, 22)=0.02819$, $p=0.8682$), however, levels were significantly lower compared to levels at 6 hours post-injury (mean ±SEM; $F(1, 58)=14.76$, †; $p=0.0003$). **(C)** TNF-α was significantly increased in the cortex following brain injury at 6 hours post-injury compared to uninjured shams (mean ±SEM; $F(1, 30)=6.853$, *; $p=0.0137$). There were no significant injury-induced changes in IL-6 at 24 hours post-injury (mean ±SEM; $F(1, 23)=1.756$, $p=0.1981$), however, levels were significantly lower compared to levels at 6 hours post-injury (mean ±SEM; $F(1, 59)=11.38$, †; $p=0.0013$). (6 hrs: sham n=5 per treatment, injury n=7 per treatment; 24 hrs: sham n=5 per treatment, injury n=4-5 per treatment).

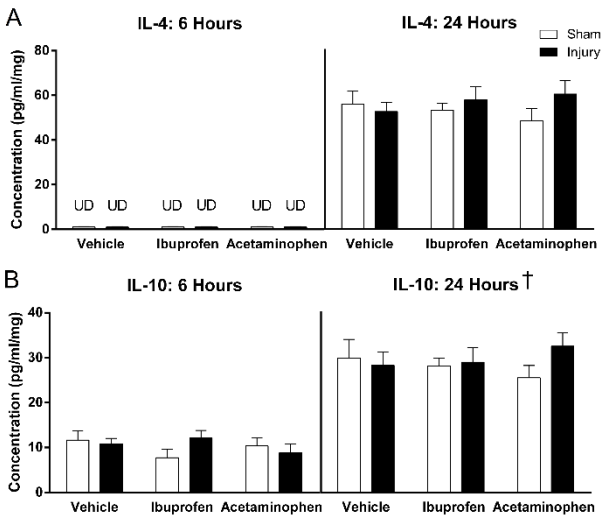

Figure 2.4. Neither brain injury nor pharmacological treatment altered anti-inflammatory cytokine levels in the cortex at 6 hours or 24 hours post-injury.

For all analytes, effect of pharmacological treatment was evaluated across brain-injured groups via one-way ANOVA revealing no significant differences in OTC analgesic-treated vs vehicle-treated groups. **(A)** IL-4 was undetectable in the cortex at 6 hours post-injury, and levels were not statistically analyzed. IL-4 was present in the cortex 24 hours post-injury and there were no significant injury-induced changes compared to uninjured shams (mean ±SEM; $F(1, 23)=1.047$, $p=0.3169$). Overall, IL-4 increased 24 hours post-injury compared to 6 hours post-injury; however, levels were undetectable (UD) preventing statistical analysis. **(B)** There were no injured-induced changes in IL-10 in the cortex at 6 hours (mean ±SEM; $F(1, 30)=0.2279$, $p=0.6366$) or 24 hours (mean ±SEM; $F(1, 23)=0.6964$, $p=0.4126$) post-injury compared to uninjured shams. Levels of IL-10 were significantly higher at 24 hours post-injury compared to levels at 6 hours post-injury (mean ±SEM; $F(1, 59)=0.1672$, †; $p<0.0001$). (6 hrs: sham n=5 per treatment, injury n=7 per treatment; 24 hrs: sham n=5 per treatment, injury n=4-5 per treatment).

Table 2.1. Inflammation-related cytokines in the cortex at 6 and 24 hours following diffuse brain injury.

	Sham Vehicle	Injury Vehicle	Sham Ibuprofen	Injury Ibuprofen	Sham Acetominophen	Injury Acetaminophen
IL-1α 6 Hours	42.4 ± 3.3	76.1 ± 15.1	55.7 ± 9.8	53.2 ± 5.1	40.6 ± 10.8	48.0 ± 22.5
IL-1α 24 Hours	UD	UD	UD	UD	UD	UD
IL-1β 6 Hours	71.2 ± 25.7	62.9 ± 30.1	23.1 ± 16.2	87.9 ± 20.4	60.2 ± 26.9	75.0 ± 29.2
IL-1β 24 Hours	UD	UD	UD	UD	UD	UD
IL-2 6 Hours	61.3 ± 2.8	65.7 ± 5.1	50 ± 4.8	58.4 ± 4.8	57.0 ± 8.5	48.1 ± 3.5
IL-2 24 Hours †	82.9 ± 5.3	71.6 ± 7.1	74.6 ± 1.5	80.6 ± 5.5	68.8 ± 8.0	83.3 ± 8.3
IL-4 6 Hours	UD	UD	UD	UD	UD	UD
IL-4 24 Hours	55.8 ± 3.0	52.4 ± 4.6	53.1 ± 1.0	57.6 ± 2.8	48.3 ± 5.4	60.3 ± 4.9
IL-6 6 Hours *	208.7 ± 65.1	595.7 ± 198.5	95.3 ± 41.6	277.6 ± 52.9	184.3 ± 46.1	255.2 ± 132.7
IL-6 24 Hours †	77.1 ± 24.5	79.1 ± 10.8	59.1 ± 11.1	36.9 ± 34.2	38.8 ± 11.9	51.1 ± 16.4
IL-10 6 Hours	11.6 ± 2.1	10.8 ± 1.1	7.7 ± 1.8	12.1 ± 1.6	10.3 ± 1.8	8.8 ± 1.9
IL-10 24 Hours †	29.7 ± 1.7	28.2 ± 2.3	28.0 ± 0.6	28.8 ± 2.3	25.4 ± 2.7	32.4 ± 2.4
IL-12 6 Hours	296.1 ± 32.0	561.8 ± 221.2	272.6 ± 45.4	309.3 ± 19.5	310.0 ± 41.1	254.8 ± 16.1
IL-12 24 Hours †	231.4 ± 26.1	144.6 ± 16.2	173.2 ± 15.7	172.2 ± 24.5	179.3 ± 25.4	213.6 ± 18.6
TNF-α 6 Hours *	39.8 ± 0.5	40.4 ± 0.3	39.2 ± 0.2	40.3 ± 0.3	39.3 ± 0.4	40.0 ± 0.5
TNF-α 24 Hours	36.1 ± 1.9	35.1 ± 3.2	33.8 ± 0.5	37.7 ± 1.8	30.9 ± 3.5	39.2 ± 3.0
IFNy 6 Hours	21.8 ± 2.3	22.2 ± 3.2	19.0 ± 2.0	23.2 ± 2.6	22.7 ± 5.1	22.0 ± 4.3
IFNy 24 Hours	UD	UD	UD	UD	UD	UD

Data are presented as concentration levels (mean pg/ml/mg ± SEM). IL-6 and TNF-α were both significantly increased in the brain-injured cortex at 6 hours post-injury compared to uninjured shams ($F(1, 30)=4.468$, *; $p=0.0430$; $F(1, 30)=6.853$, *; $p=0.0137$). There were also time dependent decreases in IL-6 and TNF-α at 24 hours post-injury compared to 6 hours post-injury (($F(1, 58)=14.76$, †; $p=0.0003$; $F(1, 59)=11.38$,†; $p=0.0013$). There were time dependent increases in IL-2, IL-10, and IL-12 at 24 hours post-injury compared to 6 hours post-injury ($F(1,59)=25.87$,†; $p<0.0001$; $F(1, 59)=0.1672$, †; $p<0.0001$; $F(1,59)=35.61$, †; $p<0.001$). (UD=undetectable) (6 hrs: sham n=5 per treatment, injury n=7 per treatment; 24 hrs: sham n=5 per treatment, injury n=4-5 per treatment).

2.5 DISCUSSION

In the diffuse brain-injured mouse, immediate pharmacological intervention with over-the-counter analgesics did not adversely affect sensorimotor or neurological outcome. A single, clinically relevant dose of ibuprofen or acetaminophen was hypothesized to reduce early inflammation leading to a worsened functional outcome. In the current study, we show immediate treatment with ibuprofen or acetaminophen did not impact TBI-induced functional deficits measured by the rotarod and neurological severity score (NSS). We also show drug treatment did not alter expression of cortical cytokines at 24 hour post-injury.

There is no approved pharmacological treatment for TBI and current medical care focuses primarily on controlling physiological parameters including intracranial pressure and blood pressure (Wang, Larner et al. 2006), as well as pain. Severity of TBI is categorized based on the Glasgow Coma Scale (GCS) which reliably classifies the severity of TBI based on clinical symptoms with a total GCS score classifying their injury as mild (score: 13-15), moderate (score: 9-12) or severe (score: <9) (Prins, Greco et al. 2013). Given the majority of human TBI encompasses mild to moderate diffuse brain injury for which self-medication may be the primary treatment, the current study sought to replicate the real-life situation in which a survivor of mild TBI self-medicates with a single dose of an OTC analgesic. For this study, we used a moderate severity diffuse brain injury which in our injury model (mFPI) reflects a mild clinical TBI (GCS 13-15). The most frequent symptom after TBI is post-traumatic headache TBI (Theeler, Lucas et al. 2013), making ibuprofen and acetaminophen principal choices for self-

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

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

methylsulfonyl)phenyl-2(^{5}H)-furanone], following lateral cortical impact in rats attenuated injury-induced prostaglandin production in the brain and improved functional recovery measured by the Morris water maze and neuroscore at 72 hours post-injury (Gopez, Yue et al. 2005). Carprofen, a COX-2 inhibitor, administered following closed head injury (CHI) in mice, also improved functional recovery (Thau-Zuchman, Shohami et al. 2012). Recovery of function measured by the NSS, however, was not present until 72 hours post-injury (Thau-Zuchman, Shohami et al. 2012). Treatment with anti-inflammatory minocycline following CHI in mice resulted in improved NSS scores starting at 72 hours post-injury, with improvements lasting through day 7 (Ng, Semple et al. 2012). These studies suggest that inhibiting inflammation can improve functional recovery. While the administration of analgesics has been primarily shown to positively influence functional outcome, these studies have incorporated multiple dosing strategies either before or after TBI. While the results are experimentally valid, they do not address the situation faced by a mildly concussed individual not seeking medical attention. In this scenario, an individual would likely self-treat prominent symptoms, including headache, with OTC analgesics immediately post-injury. Experimentally, it would be expected that a single dose of OTC analgesics would have less profound effects upon outcome than a more aggressive dosing strategy.

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

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

Our experimental model of concussion has shown elevated levels of pro-inflammatory cytokines peaking between three and nine hours post-injury (Bachstetter, Rowe et al. 2013; Rowe, Striz et al. 2014). In the current study, we measured a panel of inflammation related interleukins at 6 and 24 hours post-injury to investigate the presence or absence of inflammation following injury and at the time behavioral testing was completed. Interestingly, the pattern of cytokine levels over time reflected their

functionality and immune properties. Immune mediators that are secreted following brain injury can be divided into subgroups: archetypal pro-inflammatory cytokines (IL-1, TNF, IL-6), anti-inflammatory cytokines (IL-2, IL-4, IL-10, transforming growth factor-beta), and the chemotactic cytokines or chemokines (Banchereau, Pascual et al. 2012; Woodcock and Morganti-Kossmann 2013). Our data showed, regardless of pharmacological treatment, pro-inflammatory cytokines were increased in the cortex at 6 hours but not 24 hours post-injury. In contrast, we found anti-inflammatory cytokines were increased in the cortex at 24 hours post-injury compared to 6 hours post-injury. Our experimental model of injury has shown injury-induced elevations in cortical chemokines with a similar timecourse as the pro-inflammatory cytokines measured in this study, reaching significant increases at 6 hours post-injury (Bachstetter, Rowe et al. 2013). While chemokines were not measured in the current study, based on previously reported data, we predict similar elevations occurred.

IL-1α and IL-1β are key mediators of the inflammatory response both peripherally and centrally (Woodcock and Morganti-Kossmann 2013). The IL-1 family of cytokines are regulators of inflammation in relation to acute TBI (Woodcock and Morganti-Kossmann 2013) and previous temporal associations of injury-induced cytokine levels in our injury model have shown increased IL-1β peaking between 3 and 9 hours post-injury (Bachstetter, Rowe et al. 2013; Rowe, Striz et al. 2014). In this study, there were increases in production of IL-1 but the injury effect did not reach significance. By 24 hours post-injury, the IL-1 cytokines had become undetectable supporting the role of IL-1 in acute inflammation following TBI. IL-6 and TNF-α are also associated with the acute

immune response following TBI. Our study measured cytokines identified as key regulators of the acute phase response including , IL-6, and TNFα (Gabay and Kushner 1999). We found both IL-6 and TNF-α were significantly increased in brain-injured cortex compared to uninjured sham at 6 hours supporting their role as key regulators of the acute phase response. When measured at 24 hours post-injury there was a time dependent reduction in pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12, IFNγ) suggesting that acute inflammation following experimental diffuse brain injury has resolved, which may or may not emerge at later time points.

In contrast, we found a time dependent increase in anti-inflammatory cytokines (IL-2, IL-4, and IL-10) at 24 hours post-injury compared to 6 hours post-injury. IL-10 has been shown to have inhibitory effects on the production of pro-inflammatory cytokines (IL-1, IL-6, TNF-α) which supports different patterns of anti-inflammatory versus pro-inflammatory cytokines after diffuse brain injury. IL-10 is significantly increased at 24 hours post-injury which could have inhibitory effects on the pro-inflammatory cytokines, which further validates IL-10 inhibition of IL-1β and TNF to dampen the inflammatory response (Woodcock and Morganti-Kossmann 2013). Overall, there were both injury-induced and time dependent changes in cortical cytokine levels at both 6 and 24 hours post-injury, however there were no alterations dependent upon pharmacological intervention.

Regardless of treatment there were no significant reductions in cytokine levels following the administration of OTC analgesics with varying anti-inflammatory properties. Overall, immediate pharmacological intervention following brain injury did

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

2.6 CONCLUSIONS

In the diffuse brain-injured mouse, immediate pharmacological intervention did not attenuate or exacerbate TBI-induced functional deficits. Pro-inflammatory cortical cytokine levels were elevated at 6 hours post-injury and anti-inflammatory cytokines were elevated at 24 hours post-injury. We conclude that while a single dose of OTC analgesics does not significantly inhibit the immediate injury-induced inflammation, it does not adversely affect functional outcome. Further investigation is needed to examine time of drug treatment and multiple dosing.

2.7 ACKNOWLEDGMENTS

I would like to thank all collaborators on this project: Rachel Rowe for her contributions to study design, experiment execution, data interpretation, and manuscript preparation; Jonathan Lifshitz for his contributions to study design, data interpretation, and manuscript preparation; Bruce O'Hara for his contributions to data interpretation and

manuscript preparation; P. David Adelson for his contributions to data interpretation and manuscript preparation.

CHAPTER 3

DIETARY DOCOSAHEXAENOIC ACID (DHA) ALLEVIATES LONG-TERM SENSORY HYPERSENSITIVITY AND INFLAMMATION INDUCED BY EXPERIMENTAL DIFFUSE BRAIN INJURY IN RATS

Published in Current Research: Concussion (In Press)

3.1 ABSTRACT

Docosahexaenoic acid (DHA) is an omega-3 fatty acid that has importance for brain development and function. In the current study, we investigated the effect of dietary supplementation with DHA either before or after experimental brain injury. After 28 days on predetermined diets, rats were subjected to moderate midline fluid percussion injury or sham surgery and the effects of DHA-enriched diets and traumatic brain injury (TBI) on spatial learning, sensory hypersensitivity, neuroinflammation and fatty acid profiles were evaluated. The results provide novel evidence that sufficient dietary intake of DHA, fed either before or after TBI, decreased injury-induced sensory sensitivity and that post-injury DHA supplementation most effectively attenuated injury-induced neuroinflammation. This divergence suggests that the pre-injury protective mechanisms of DHA may differ from post-injury mechanisms. Overall, the results indicate sufficient DHA intake may be an effective nutritional approach to minimizing pathology and maximizing recovery after TBI.

3.2 INTRODUCTION

In the United States alone, over 1.7 million people per year sustain traumatic brain injuries (TBI) (Faul, Xu et al. 2010), which may grossly underestimate the true incidence due to underreporting, particularly of diffuse TBI. The long term symptoms of TBI can include impaired cognitive or emotional ability (Arciniegas, Topkoff et al. 2000; Albensi and Janigro 2003; Masel and DeWitt 2010), sensory sensitivity or dysfunction (Goodrich, Flyg et al. 2013; Goodrich, Martinsen et al. 2014), and loss of neurological function (Arciniegas, Topkoff et al. 2000). TBI is induced by mechanical forces which initiate a protracted cascade of secondary injury processes, including inflammation and oxidative stress (Werner and Engelhard 2007). To date, many pharmacological therapies have been selected to target specific cellular and molecular cascades which contribute to secondary injury in the hours to days following TBI. In attenuating secondary signaling, the approach is to alleviate long term symptoms of TBI.

The shortcomings of recent clinical trials for TBI therapeutics (Margulies and Hicks 2009) suggest that a drug that targets a single secondary injury process may not be sufficient to alleviate the multifaceted pathophysiology of TBI. Pleiotropic agents that target multiple components of the secondary injury cascade may prove more effective. One potential such approach, investigated in the current study, may be the maintenance of sufficient levels of docosahexaenoic acid (DHA). DHA is the most concentrated omega-3 polyunsaturated fatty acid (PUFA) in the nervous system, and dietary sources of this nutrient include mothers' milk, cold water fish, marine algae, and supplements containing DHA. Randomized, controlled trials in humans indicate that DHA has an

excellent safety profile (Hughbanks-Wheaton, Birch et al. 2014) and that elevation of its tissue concentration throughout the body allows for better cognitive function in healthy, aging populations (Yurko-Mauro, McCarthy et al. 2010; Salem, Vandal et al. 2014) and improved neurodevelopment (Mulder, King et al. 2014). The positive findings in these clinical trials are likely due to the multiple actions of DHA, which include regulation of apoptotic signaling (Sinha, Khare et al. 2009), decreased inflammatory signaling (Chang, Kuan et al. 2013) and reduced oxidative stress (Chaung, Chang et al. 2013).

While DHA administration has already shown favorable effects in animal models of TBI (Bailes and Mills 2010; Wu, Ying et al. 2011; Wu, Ying et al. 2013; Begum, Yan et al. 2014), the present study was designed with the novel goal of assessing DHA administration before and after diffuse TBI within the same study using a defined DHA-enriched diet, rather than direct administration of DHA-containing oil. Dietary administrations of DHA before or after TBI represent two equally valid scenarios of prophylaxis and therapy, respectively, which may differ in efficacy and mechanism. Behavioral outcome measures were chosen to model the clinical conditions of cognitive impairment and sensory sensitivity. We evaluated each administration schedule by testing post-injury learning with the Morris water maze (MWM), sensory hypersensitivity with the whisker nuisance task (WNT), and neuroinflammation with [^{3}H]PK11195 binding density—a ligand of the translocator protein (TSPO). TSPO has previously been shown to predominantly label activated microglia and exhibited regional co-localization with microglial activation following CNS injury, along with some macrophages and to a lesser extent astrocytes (Weissman and Raveh 2003; Cao, Thomas et al. 2012). To further

understand the effect of DHA supplementation and TBI on central and peripheral fatty acid profiles, fatty acid analysis was conducted on brain, plasma, and red blood cells. We hypothesized that both pre- and post-injury DHA supplementation would increase brain DHA content, improve functional outcome from TBI and reduce post-traumatic neuroinflammation.

3.3 METHODS

3.3.1 Animals

Adult male Sprague-Dawley rats were obtained from Harlan, Inc. (Indianapolis, IN), housed in pairs on a 12h:12h light schedule with *ad libitum* access to rat chow and water. The ordering, receipt and dietary supplementation of the animals were timed to accommodate the study design calling for 28 days of supplementation and a standard injury weight of 350 grams. All animals were between 84-96 days of age at the time of injury. All animal procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

3.3.2 Diets

The animal diets were designed to provide either 0% (control) or ~1 wt% DHA, expressed as a percentage of the total fatty acids. The diets were based on the AIN-93G standard, but their fat contents consisted of controlled blends of coconut oil, high-oleic safflower oil, TBHQ-free soy oil (Dyets, Inc.), and DHASCO® oil (DSM Nutritional Products). The oils were analyzed as fatty acid methyl esters (FAME) prior to diet formulation by Dyets, Inc., and the diets were similarly analyzed prior to their use in the study. Thus, the oil blends allowed for a balancing of all fatty acids between diets except

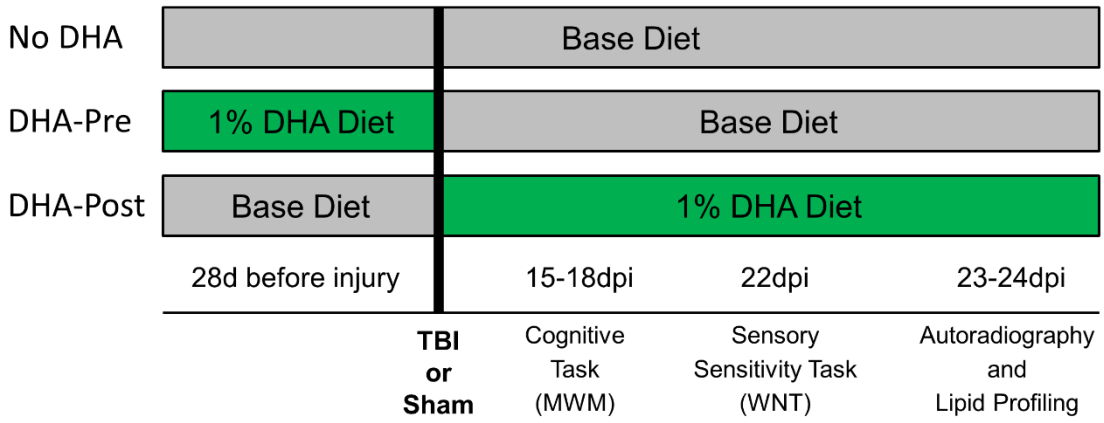

Figure 3.1. Experimental Design

All rats received a base diet containing No DHA (gray) or an equivalent diet containing DHA as ~1% of the total fatty acids (green). One group received a base diet for the entire study. The second group was given a DHA diet for 28 days prior to surgery/injury, followed by a base diet. The third group was given a base diet prior to surgery/injury followed by a post-injury treatment of the DHA diet for 23-24 days post-injury. Each experimental arm included both traumatic brain injury (TBI) and uninjured control (Sham) animals. For analysis of Morris Water Maze (MWM), Whisker Nuisance Task (WNT), and fatty acid profiles, a combined sham group was formed from random selection among diet groups. Following injury, rats were evaluated for spatial learning in the MWM task (15-18 days post-injury; dpi). Sensory sensitivity was assessed using the WNT at 22 days post-injury. Following behavior tasks, tissue was collected for autoradiography and lipid profiling (23-24 dpi).

Table 3.1. Diet Compositions

Ingredient		No DHA Diet	DHA Diet
Casein		20.00	20.00
L-cystine		0.30	0.30
Sucrose		10.00	10.00
Cornstarch		39.75	39.75
Dyetrose[a]		13.20	13.20
Cellulose		5.00	5.00
Mineral Mix 210025[b]		3.50	3.50
Vitamin Mix 310025[c]		1.00	1.00
Choline		0.25	0.25
Fat Composition:		7.00	7.00
	Coconut Oil	2.34	2.30
	Safflower Oil	3.82	3.68
	Soybean Oil[d]	0.84	0.84
	DHASCO Oil[e]	0.00	0.18
Fatty Acid Composition:		(wt% Total Fatty Acids)	
	ΣSat	41.06	41.14
	ΣMono	41.71	41.14
	18:2n6	15.88	15.45
	18:3n3	1.29	1.28
	20:4n6	0.02	0.01
	22:6n3	0.00	0.92
	ΣPUFA	17.30	17.78
	Σn3	1.37	2.29
	Σn6	15.92	15.49
	n6:n3	11.59	6.76

[a] Carbohydrate composition (%): monosaccharides, 1; disaccharides, 4; trisaccharides, 5; tetrasaccharides and higher, 90.

[b] AIN-93G mineral mix (mg/100g diet): calcium, 500; phosphorus, 156.1; potassium, 360; sodium, 101.9; chloride, 157.1; sulphur, 30; magnesium, 50.7; iron, 3.5; copper, 0.6; manganese, 1; chromium, 0.1; iodine, 0.02; selenium, 0.02; fluoride, 0.1; boron, 0.05; molybdenum, 0.02; silicon, 0.5; nickel, 0.05; lithium, 0.01; vanadium, 0.01

[c] AIN-93VX vitamin mix (units/100g diet): thiamin, 0.6 mg; riboflavin, 0.6 mg; pyridoxine, 0.7 mg; niacin, 3 mg; pantothenate, 1.6 mg; folate, 0.2 mg; biotin, 0.02 mg; cyanocobalamin, 2.5 mg; vitamin A, 400 IU; Vitamin E, 7.5 IU; Vitamin D3, 100 IU; Vitamin K1, 0.08 mg.

[d] TBHQ-free

[e] Provided by DSM Nutritional Products

for DHA content and total omega-3 content (see Table 1 for details). The diets were fed for 28 days prior to sham or brain injury. Directly after injury or sham surgeries, the animals were either maintained on a DHA-deficient diet (controls), switched to DHA-deficient diets (pre-injury DHA), or switched to DHA-sufficient diets (post-injury DHA; see Figure 1). The animals were randomized to the diets, and the researchers were blinded from the treatments throughout all experimental procedures.

3.3.3 Midline Fluid Percussion Injury

A total of 100 rats were subjected to midline fluid percussion injury (mFPI) consistent with methods previously described (Lifshitz 2007; Lifshitz 2008; McNamara, Lisembee et al. 2010; Cao, Thomas et al. 2012; Ziebell, Taylor et al. 2012). Rats were anesthetized using 5% isoflurane in 100% oxygen for five min and the head of the rat was placed in a stereotaxic frame with continuously delivered isoflurane at 2.5% via nosecone. While anesthetized, body temperature was maintained using a Deltaphase® isothermal heating pad (Braintree Scientific Inc., Braintree, MA). A midline incision was made exposing bregma and lambda, and fascia was removed from the surface of the skull. A trephine (4.8 mm outer diameter) was used for the craniotomy, centered on the sagittal suture between bregma and lambda without disruption of the dura. An injury cap prepared from the female portion of a Luer-Loc needle hub was fixed over the craniotomy using cyanoacrylate gel and methyl-methacrylate (Hygenic Corp., Akron, OH). The incision was sutured at the anterior and posterior edges and topical Lidocaine ointment was applied. Rats were placed in a heated recovery cage and monitored until ambulatory.

For injury induction, rats were re-anesthetized (60-90 min after surgery) with 5% isoflurane delivered for five min. The dura was visually inspected through the hub to make sure it was intact with no debris. The hub was then filled with normal saline and attached to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). An injury of moderate severity for our injury model (2.0 atm) was administered by releasing the pendulum onto the fluid-filled cylinder. Sham-injured rats underwent the same procedure except the pendulum was not released. Rats were monitored for the presence of a forearm fencing response and righting reflex times were recorded for the injured rats as indicators of injury severity (Hosseini and Lifshitz 2009). The injury hub was removed and the brain was inspected for uniform herniation and integrity of the dura through the craniotomy. The incision was cleaned using saline and closed using sutures. Moderate brain-injured rats had righting reflex recovery times greater than five min and a positive fencing response; injury severities were comparable between brain-injured diet groups. Sham-injured rats recovered a righting reflex within 20 sec. After spontaneously righting, rats were placed in a heated recovery cage and monitored until ambulatory (approximately 5 to 15 min) before being returned to their cage. Adequate measures were taken to minimize pain or discomfort. Mortality was not encountered as a result of surgery or TBI.

3.3.4 Morris Water Maze (MWM)

Spatial learning ability was assessed in 100 rats using a MWM testing paradigm similar to those used in other experimental models of TBI (Smith, Soares et al. 1995; Murai, Pierce et al. 1998; Smith, Nakamura et al. 1998; Prins and Hovda 2001; Guseva,

Hopkins et al. 2008; Pleasant, Carlson et al. 2011; Rowe, Harrison et al. 2014). Briefly, animals were tested in a 127 cm diameter × 56 cm tall circular pool with a 13.5 cm diameter circular platform submerged 1 cm below the waterline. Black, non-toxic powdered paint was added to obscure the platform. The lighting of the room and various spatial cues were constant throughout the acquisition period. At selected time points post-injury (15, 16, 17, 18 days), rats were tested in sets of four trials per day. Rats started from one of four starting points (North, South, East, West). If the animal did not find the platform in 60 sec, it was placed there by the handler and allowed to rest for 15 sec. The animals were allowed a 5 min rest between each trial. During all phases of the MWM, the animals' performance was recorded and analyzed with Accuscan Instruments EzVideoDV Automated Tracking System (Columbus, OH) that allowed quantification of escape latency and swim speed for the tests. Data are presented as average daily latency to find the hidden platform in seconds and average swim speed (cm/sec) for all trials.

3.3.5 Whisker Nuisance Task (WNT)

Protocols were conducted as described previously to evaluate sensory sensitivity (McNamara, Lisembee et al. 2010; Learoyd and Lifshitz 2012). A darkened plastic test cage (16.5 × 38.1 × 55.9 cm) lined with an absorbent pad was used. Rats were acclimated to the test cage for 5 min prior to testing. Testing involved manually stimulating the whiskers of both mystacial pads with a wooden applicator stick for three periods of 5 min with periods (≤1 min) of non-stimulation between periods. Animals were tested individually and the absorbent pad and wooden applicator stick were replaced for each animal after cleaning the test cage. For each five min period, observations were made

regarding the predominant observed behavior. These observations were recorded as discontinuous, categorical data for (1) movement, (2) stance and body position, (3) breathing quality, (4) whisker position, (5) whisking response, (6) evading stimulation, (7) response to stick presentation, and (8) grooming response. Normal behavior for each category consisted of those typically seen in uninjured animals during stimulation and was recorded as a score of zero. For example, an animal that was relaxed and looking upward during stimulation was given a score of 0 for stance and body position. Meaningful abnormal behaviors expressed in response to whisker stimulation were assigned scores of 1–2, depending on degree of expression. For example, an animal that cowered and showed a guarded position was given a score of 2 for stance and body position. The maximum whisker nuisance score was 16 (two points for each of eight categories), where each two point increase indicated the meaningful expression of 1-2 behaviors. Higher scores indicate abnormal responses to the stimulation overall, in which the rat freezes, becomes agitated or is aggressive. Lower scores indicate normal responses, in which the rat is either soothed or indifferent to the stimulation. A five min period of non-stimulation was scored immediately following the final stimulatory trial as a control for spontaneously occurring behaviors (McNamara, Lisembee et al. 2010; Learoyd and Lifshitz 2012).

3.3.6 Tissue Extraction

Following rapid decapitation on day 23 or 24 post-TBI approximately 3 ml of whole, trunk blood was collected from all animals. Each whole blood sample was split equally and centrifuged at 100*g* for 15 min at 4ºC. Plasma was transferred to a fresh tube. Prior

to closure, the tube's headspace was purged with nitrogen gas. The remaining red blood cell pellet was resuspended in 2 volumes of isotonic saline solution and centrifuged again. The supernatant was removed, and the pellet was resuspended again in 2 volumes of isotonic saline. The pelleted red blood cells and the isotonic saline supernatant were then stored together at -80ºC. After blood collection, the brain was extracted and flash frozen in isopentane chilled on dry ice. Brains were then stored at -80ºC until use.

3.3.7 [^{3}H]PK11195 autoradiography

Radioligand binding was carried out using protocols previously described (Cao, Thomas et al. 2012; van Bregt, Thomas et al. 2012). Briefly, rats were euthanized by decapitation and brains were removed and flash frozen in an isopentane-dry ice slurry. Brains were cryosectioned at 16 µm. Translocator protein 18 kDa (TSPO) receptor densities were measured using [^{3}H]PK-11195 autoradiography, as previously described (Little, McLaughlin et al. 1998; van Bregt, Thomas et al. 2012). A ligand concentration of 1 nM ([^{3}H]PK-11195 specific activity 85.5 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA, USA) was used for incubation. RayMax Beta High Performance Autoradiography Film was used to visualize ligand binding. All films were processed using Kodak GBX developer. Binding data were analyzed using NIH image v1.59 with a Sony XC-77 CCD camera via a Scion LG-3 frame-grabber. Areas of interest were outlined manually to include cortex (18-24 sections per rat), hippocampus (18-24 sections per rat), thalamus (9-13 sections per rat), and substantia nigra (6 sections per rat). For each brain region, sham rat sections were measured to acquire a background intensity level. A threshold intensity value was set at the highest gray scale value measured for any sham operated

rat. Regions of interest were drawn by their anatomical boundaries (Paxinos and Watson 2007) and the percentage of pixels over threshold was calculated for each region.

3.3.8 Fatty Acid Profiling

All brain, plasma and red blood cell samples were freeze-dried prior to homogenization and Folch extraction (Folch, Lees et al. 1957). Lipids from red blood cell samples were extracted using the methods of Bligh and Dyer (Bligh and Dyer 1959). Briefly, tricosanoic free fatty acid was added to each sample as an internal standard. Butylated hydroxytoluene was added to all samples before saponification with 0.5 N methanolic NaOH, and all samples were purged with N_2 throughout the process to minimize oxidation. Fatty acids were converted to methyl esters with 14% BF3/methanol at 100ºC for 30 min (Morrison and Smith 1964). Fatty acid methyl esters were analyzed by GLC using a Hewlett Packard 6890 equipped with a flame ionization detector. The fatty acid methyl esters were separated on a 30-meter FAMEWAX capillary column (Restek; 0.25 mm diameter, 0.25 µm coating thickness) using hydrogen at a flow rate of 40 mL/min with a split ratio of 48:1. Chromatographic run parameters included an oven starting temperature of 130ºC that was increased at 6ºC/min to 225ºC, where it was held for 20 min before increasing to 250ºC at 15ºC/min, with a final hold of 5 min. The injector and detector temperatures were constant at 220ºC and 230ºC, respectively. Peaks were identified by comparison of retention times with external fatty acid methyl ester standard mixtures from NuCheck Prep. The fatty acid profiles were expressed as weight percent of the total fatty acids in each sample.

3.3.9 Statistical Analysis

Data are shown as mean ± SEM and analyzed using statistical software (GraphPad-Prism 6). Differences in escape latencies for the MWM were analyzed using a repeated measure two-way ANOVA across injury group and testing day. Differences in overall swim speed for the MWM between groups were analyzed using a one-way ANOVA. Non-parametric whisker nuisance task data were analyzed using a Kruskal-Wallis test with a Dunn's multiple comparison test since the data obtained from the WNT were not continuous. Differences in autoradiography data were analyzed by one-way ANOVA with a Tukey's post-hoc comparison. Fatty acid profiles were compared using a two-way ANOVA between brain injury and diet groups with a Tukey's post-hoc comparison. Statistical significance was assigned when $p<0.05$. Initial group sizes were determined for each outcome measure by power analysis (GPower; Dusseldorf University) and final group sizes are indicated in the figure legends.

3.4 RESULTS

It was not anticipated that dietary supplementation with DHA would change functional outcome or inflammation in uninjured sham rats. Statistical analyses confirmed no significant differences in Morris water maze performance ($F(3,34)=1.2$, $p=0.3$) or whisker nuisance task scores ($KW(3,34)=4.7$, $p=0.1$) between any sham groups. There were no statistical differences in ligand binding density between sham groups (Cortex, $F(2,19)=0.9673$, $p=0.4$). Uninjured shams treated with No DHA supplementation, pre-DHA supplementation, and post-DHA supplementation were combined into a single sham control group with a group size comparable to injured groups. Sham animals were

selected using a random number generator (Microsoft Excel). In contrast to the above, it was anticipated that DHA administration would affect DHA tissue content regardless of injury status. Therefore, sham animals were not combined for lipid profiling analyses that investigated the potential effect of brain injury on the fatty acid profiles.

3.4.1 Spatial Learning

To test spatial learning following TBI, 12-14 rats per experimental arm were tested in the MWM for four consecutive days starting at day 15 post-injury. Latency to find the submerged platform was measured to assess learning and swim speed was evaluated as a control for gross motor dysfunction. A two-way ANOVA indicated all groups had a significant time-dependent improvement in latency to find the hidden platform ($F(3,147)=62.35$, $p<0.0001$; Figure 2A). There was no significant treatment effect on performance measured by latency to find the platform at the selected post-injury time points ($F(3,49)=0.9860$, $p=0.4071$; Figure 2A). A probe trial for recall was not pursued as all groups improved over time. Further analysis indicated no significant difference in average swim speed across groups ($F(3,49)=0.4616$, $p=0.6553$; Figure 2B).

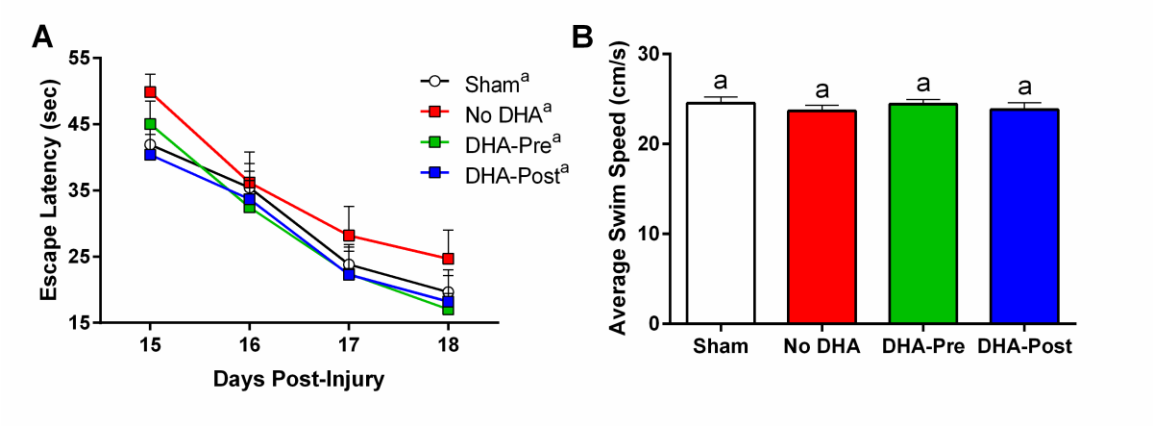

Figure 3.2. Spatial learning in the Morris Water Maze was not affected by brain injury or dietary administration of DHA.

(A) A repeated measure two-way ANOVA showed a significant time-dependent improvement in latency to the platform, indicating that animals in all groups learned the task. There was no significant treatment effect. **(B)** A one-way ANOVA showed no significant treatment effect on average swim speed. Data presented as mean ± SEM (Non-matching letters indicate Tukey's post-hoc specific effects $p<0.05$; combined sham n=14, TBI No DHA n=14, TBI DHA-Pre n=13, TBI DHA-Post n=12).

3.4.2 Sensory Sensitivity

We have previously reported increased sensory sensitivity in rats following diffuse TBI, as a late-onset injury-induced morbidity using the whisker nuisance task (McNamara, Lisembee et al. 2010). All animals that underwent MWM testing were also subjected to the WNT (N=12-14 per experimental arm). A Kruskal-Wallis test indicated an overall significant effect on sensory sensitivity ($KW(4,53)=16.02$, $p=0.0011$; Figure 3A). Further analysis using Dunn's multiple comparison test indicated brain-injured rats receiving No DHA dietary supplementation had significantly higher whisker nuisance task scores compared to the uninjured sham control group. However, brain-injured rats receiving DHA-supplemented diets either before or after injury were not significantly different from uninjured shams. There was no significant difference in sensory sensitivity when animals were scored on the WNT with no whisker stimulation ($KW(4,53)=4.38$, $p=0.2232$; Figure 3B).

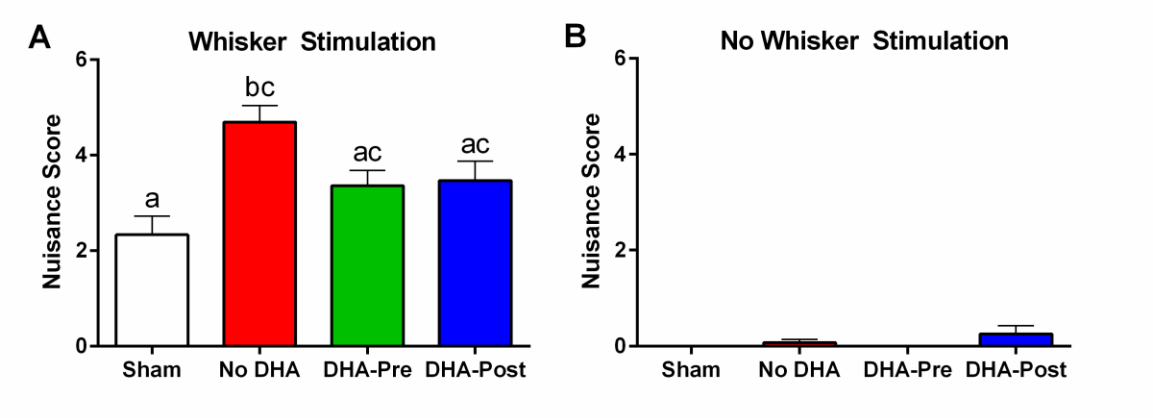

Figure 3.3. Injury-induced increase in sensory sensitivity observed during the Whisker Nuisance Task was mitigated by dietary administration of DHA.

(A) Sensory sensitivity assessed by the whisker nuisance task (WNT) at 22 days post-injury was significantly increased compared to uninjured sham without dietary supplementation. There was no difference in sensory sensitivity in brain-injured rats receiving dietary DHA supplementation compared to the combined uninjured sham group. **(B)** There was no significant difference in sensory sensitivity between groups when animals were scored on the WNT after cessation of whisker stimulation. Data presented as mean ± SEM (Non-matching letters indicate Dunn's post-hoc specific effects $p<0.05$; combined sham n=14, TBI No DHA n=14, TBI DHA-Pre n=13, TBI DHA-Post n=12).

3.4.3 Inflammation

The effect of DHA on brain injury-induced inflammation has not been previously evaluated after mFPI. Here, [H^3]PK11195 was used to label activated glia as an index of neuroinflammation at 23-24 days post-injury. Approximately 75% of the available brain tissue was used for this analysis (N=8-9 for each experimental group). Uninjured sham rats displayed minimal glial activation, with [H^3]PK11195 binding limited to areas normally without an intact blood-brain barrier (Figure 4A), while all brain-injured groups indicated at least some regions of increased microglial activation (Figure 4B-D). Binding density quantification was conducted in four regions of interest: cortex, hippocampus, thalamus and substantia nigra (Figure 4E-H). A one-way ANOVA indicated an overall main treatment effect in the cortex ($F(3,33)=13.40$, $p<0.0001$; Figure 4E) and hippocampus ($F(3,33)=13.93$, $p<0.0001$; Figure 4F). Tukey's multiple comparison test indicated brain-injured rats treated with No DHA or DHA-Pre had significantly greater binding density in the cortex and hippocampus compared to uninjured sham rats. DHA-Post treated rats showed binding density in the cortex and hippocampus equivalent to sham rats and significantly lower than both other injured groups. A one-way ANOVA indicated an overall main treatment effect in the thalamus ($F(3,33)=4.157$, $p=0.0133$; Figure 4G) and the substantia nigra ($F(3,33)=7.147$), $p=0.0008$; Figure 4H). Tukey's multiple comparison test indicated brain-injured rats treated with No DHA had significantly higher binding density in the hippocampus and substantia nigra compared to uninjured sham rats. In the substantia nigra, the binding density of brain-injured rats treated with No DHA was also significantly higher compared to brain-injured rats treated

with DHA-Post. Overall, TBI resulted in increased [H^3]PK11195 binding density, indicative of microglial activation.

Following the behavioral studies and autoradiography, the remaining brains from each group (N=5-6) were used for lipid profile analysis by GC/FID. A two-way ANOVA indicated diffuse brain injury significantly reduced DHA fatty acid levels in brain ($F(1,27)=4.860$, $p=0.0362$; Figure 5A) but not plasma ($F(1, 19)=0.7678$, $p=0.3918$; Figure 5C) or red blood cells ($F(1,19)=0.1805$, $p=0.6757$; Figure 5E). A two-way ANOVA indicated a significant overall effect of diet in the brain ($F(2,27)=6.073$, $p=0.0066$; Figure 5A). Tukey's multiple comparison test indicated significantly increased brain levels of DHA fatty acid in rats receiving DHA supplementation (pre or post) compared to rats receiving No DHA. Similarly, diet exerted a significant effect in the plasma ($F(2,19)=62.80$, $p<0.0001$; Figure 5C). Tukey's multiple comparison test indicated significantly increased DHA fatty acid levels in the plasma of rats receiving Pre-DHA compared to rats receiving No DHA, and significantly increased levels in the rats receiving Post-DHA compared to rats receiving Pre-DHA or No DHA. Lastly, a two-way ANOVA indicated a significant dietary effect in red blood cells ($F(2,19)=8.928$, $p=0.0018$; Figure 5E). Tukey's multiple comparison test indicated significantly increased DHA fatty acid levels in the brain of rats receiving Post-DHA supplementation compared to rats receiving Pre-DHA or No DHA. To distinguish between DHA and EPA mediated effects, comparisons of EPA levels were made. Post-DHA increased levels of EPA in the

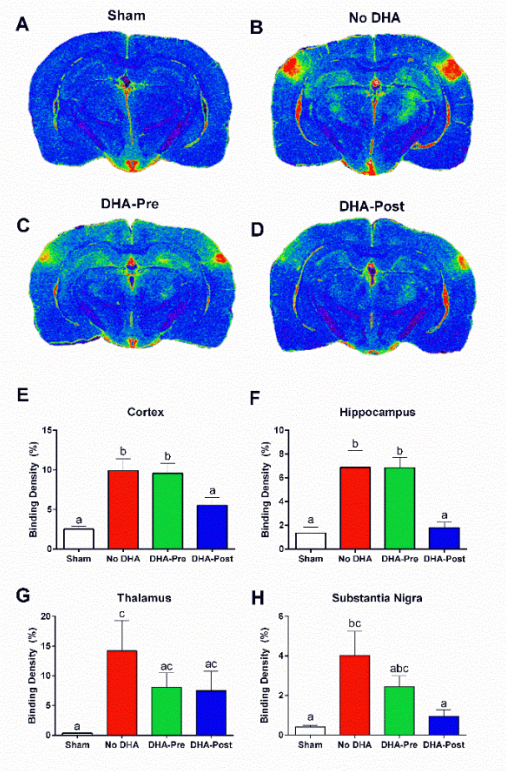

Figure 3.4. Dietary DHA reduced microglial activation following diffuse brain injury.

[H^3]PK11195 autoradiography was used to label activated microglia 23-24 days post-injury. **(A-D)** Representative coronal sections from each treatment group are labeled by [H^3]PK11195 ligand-binding, with binding densities ranging from maximal to minimal indicated by colors red, yellow, green, blue. Uninjured sham rats displayed minimal microglial activation, with [H^3]PK11195 binding limited to areas normally without an intact blood-brain barrier, while all brain-injured groups indicated focal regions of increased microglial activation. Binding density quantification was conducted in four regions of interest. **(E)** In the cortex, brain-injured rats treated with No DHA or DHA-Pre had significantly higher binding densities than sham rats. Binging density in DHA-Post treated rats were equivalent to sham rats and significantly lower than both other injured groups. **(F)** The same pattern of activation among groups was observed in the hippocampus. **(G)** In the thalamus, No DHA treated rats had significantly higher binding density than sham rats. **(H)** In the substantia nigra, No DHA treated rats had significantly higher binding density than sham and DHA-Post treated rats. Overall, TBI resulted in increased [H^3]PK11195 binding density, indicative of greater microglial activation. DHA administration before or after TBI reduced microglial activation, but this effect was most evident in DHA-Post treated rats (Non-matching letters indicate Tukey's post-hoc specific effects $p<0.05$; combined sham n=12, TBI No DHA n=8, TBI DHA-Pre n=9, TBI DHA-Post n=8). DHA administration before or after TBI reduced microglial activation, but this effect was most evident among DHA-post treated rats.

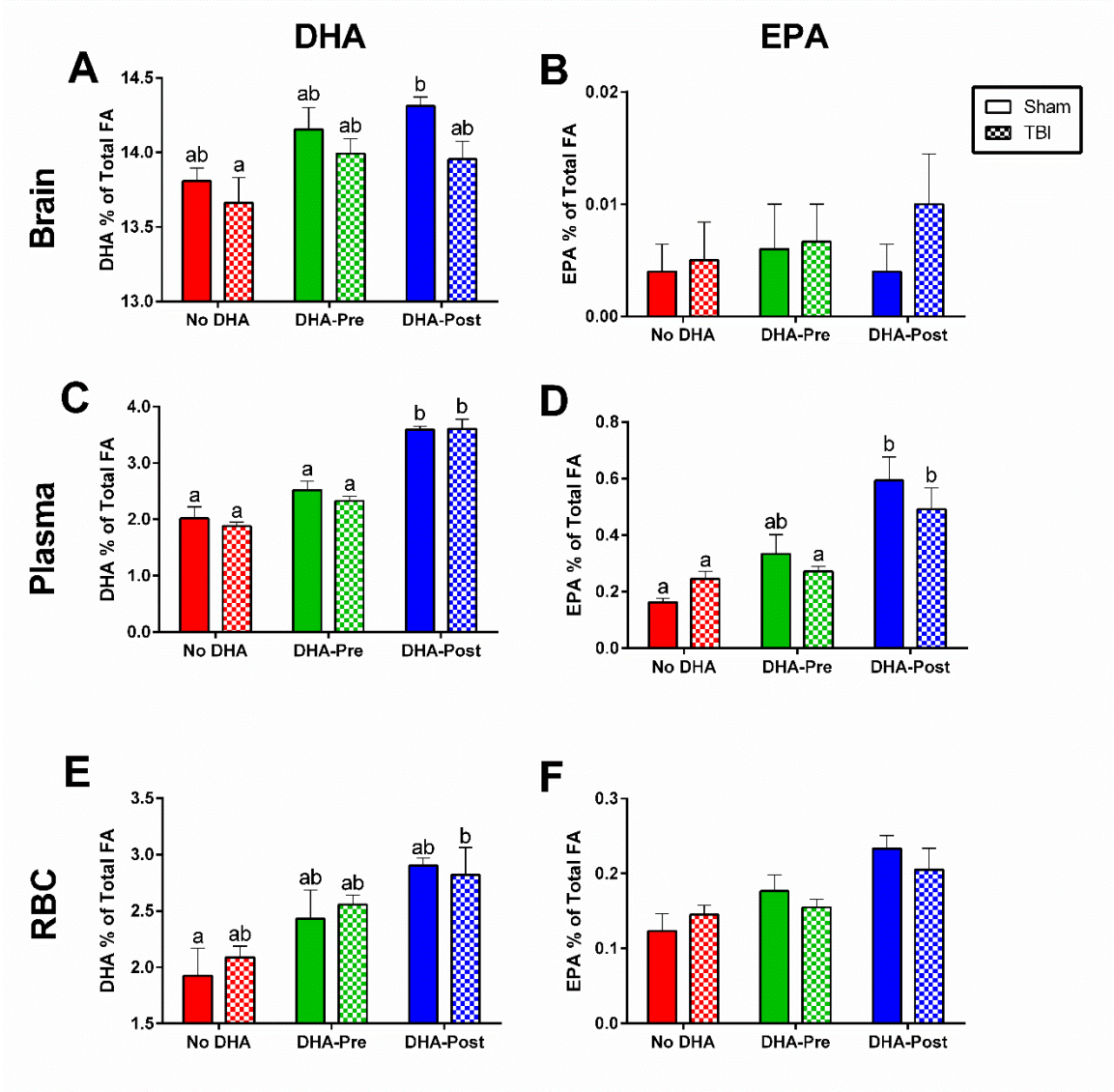

Figure 3.5. Dietary DHA supplementation and diffuse TBI altered brain levels of DHA but not EPA.

Diffuse brain injury significantly reduced DHA fatty acid levels (main effect of TBI) in the brain **(A)**, but not in plasma **(C)** or red blood cells **(E**; RBC**)**. DHA supplementation significantly increased the level of DHA fatty acids (main effect of diet) in the brain, plasma, and red blood cells. DHA dietary supplementation increased EPA levels in plasma **(D)** and RBC **(F)**, but not the brain **(B).** Data presented as mean percentage DHA of total fatty acids ± SEM (Non-matching letters indicate Tukey's post-hoc specific effects $p<0.05$; Brain: sham n=5 per treatment, TBI n=6 per treatment; Plasma and RBC: sham n=3 per treatment, TBI n=4-6 per treatment).

plasma (Figure 5D; $F(2,19)=16.63$, $p<0.0001$) and red blood cells (Figure 5F; $F(2,19)=6.785$, $p=0.006$), but did not affect brain levels of EPA (Figure 5B; $F(2,26)=0.2810$, $p=0.281$).

In addition to TBI-induced decreases in DHA, fatty acid profiling indicated a significant injury-induced increase in brain arachidonic acid (20:4ω6), a precursor of pro-inflammatory prostaglandins (Table 2). Overall, two-way ANOVAs indicated dietary DHA supplementation resulted in significantly altered fatty acid profiles in brain, plasma and red blood cells. See Table 3.2 for full statistical results.

Table 3.2. Dietary DHA and TBI affected levels fatty acids in brain, plasma, and red blood cells.

Brain	No DHA Sham	No DHA TBI	DHA-Pre Sham	DHA-Pre TBI	DHA-Post Sham	DHA-Post TBI	Effect	F	p
18:2	0.57 ± 0.02	0.57 ± 0.02	0.62 ± 0.01	0.63 ± 0.03	0.64 ± 0.02	0.63 ± 0.02	Diet	F(2,27)= 5.481	0.0100
20:4ω6	11.04 ± 0.07	11.25 ± 0.07	10.90 ± 0.10	11.09 ± 0.07	11.02 ± 0.12	11.00 ± 0.09	Injury	F(2,27)= 2.584	0.0386
22:0	0.32 ± 0.01	0.32 ± 0.01	0.28 ± 0.02	0.28 ± 0.01	0.30 ± 0.01	0.31 ± 0.01	Diet	F(2,27)= 4.159	0.0266
22:2	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.01	Diet	F(2,27)= 16.02	<0.0001
22:6ω3 (DHA)	13.81 ± 0.09	13.66 ± 0.17	14.06 ± 0.13	14.15 ± 0.08	14.23 ± 0.09	13.95 ± 0.12	Diet Injury	F(2,27)= 6.073 F(1,27)= 4.860	0.0066 0.0362
24:0	0.38 ± 0.02	0.41 ± 0.02	0.33 ± 0.02	0.34 ± 0.01	0.38 ± 0.02	0.39 ± 0.01	Diet	F(2,27)= 6.770	0.0041
24:1	0.44 ± 0.04	0.49 ± 0.02	0.37 ± 0.05	0.40 ± 0.02	0.48 ± 0.04	0.43 ± 0.04	Diet	F(2,27)= 4.642	0.0185
Plasma	**No DHA Sham**	**No DHA TBI**	**DHA-Pre Sham**	**DHA-Pre TBI**	**DHA-Post Sham**	**DHA-Post TBI**	**Effect**	**F**	**p**
16:1	3.45 ± 0.35	3.68 ± 0.24	2.80 ± 0.51	2.62 ± 0.46	2.80 ± 0.53	3.54 ± 0.33	Injury	F(1,19)= 5.541	0.0295
18:0	7.42 ± 0.30	6.97 ± 0.21	8.29 ± 0.33	8.24 ± 0.32	8.01 ± 0.37	7.49 ± 0.28	Interaction	F(2,19)= 7.470	0.0040
22:6ω3 (DHA)	2.05 ± 0.10	1.88 ± 0.07	2.71 ± 0.54	2.53 ± 0.53	2.00 ± 0.15	1.99 ± 0.15	Diet	F(2,19)= 62.80	<0.0001
RBC	**No DHA Sham**	**No DHA TBI**	**DHA-Pre Sham**	**DHA-Pre TBI**	**DHA-Post Sham**	**DHA-Post TBI**	**Effect**	**F**	**p**
16:1	1.08 ± 0.14	1.06 ± 0.24	1.50 ± 0.21	1.34 ± 0.02	0.85 ± 0.00	1.03 ± 0.12	Diet	F(2,19)= 5.675	0.0117
18:0	11.14 ± 0.46	11.88 ± 0.54	9.93 ± 0.88	10.55 ± 0.18	11.72 ± 0.21	11.08 ± 0.41	Diet	F(2,19)= 4.661	0.0225
18:1ω7	3.82 ± 0.11	3.87 ± 0.30	4.16 ± 0.12	4.30 ± 0.09	3.90 ± 0.07	3.94 ± 0.09	Diet	F(2,19)= 3.862	0.0391
22:4ω6	1.83 ± 0.14	2.00 ± 0.10	1.41 ± 0.07	1.52 ± 0.08	1.73 ± 0.07	1.61 ± 0.15	Diet	F(2,19)= 8.207	0.0027
22:5ω6	0.51 ± 0.05	0.57 ± 0.01	0.35 ± 0.03	0.45 ± 0.04	0.39 ± 0.05	0.34 ± 0.03	Diet	F(2,19)= 10.77	0.0007
22:6ω3 (DHA)	2.13 ± 0.27	2.09 ± 0.10	2.28 ± 0.11	2.46 ± 0.08	2.90 ± 0.07	2.82 ± 0.24	Diet	F(2,19)= 10.22	0.0010

Fatty acid methyl ester (FAME) analysis was conducted on brain, plasma, and red blood cells (RBC) from each group. All fatty acids significantly altered by diet or TBI in comparison to untreated, sham animals are shown. Data are presented as mean percentage of fatty acid content ± SEM. 16:1-palmitoleic acid; 18:1ω7-vaccenic acid; 18:2-linoleic acid; 20:4-arichidonic acid; 22:0-behenic acid; 22:2-docosadienoic acid; 22:4ω6-docosatetraenoic acid; 22:5ω3-docosapentaenoic acid; 22:5ω6-docosapentaenoic acid; **22:6ω3-docosahexaenoic acid**; 24:0-nervonic acid; 24:0-lignoceric acid; 24:1-nervonic acid. (Brain: sham n=5 per treatment, TBI n=6 per treatment. Plasma and RBC: sham n=3 per treatment, TBI n=4-6 per treatment)

3.5 DISCUSSION

The current study was designed with the goals of: (1) directly comparing the therapeutic efficacy of dietary DHA supplementation before vs after diffuse brain injury on translational outcomes; (2) contributing to the understanding of the role of DHA supplementation in trauma-induced neuroinflammation; and (3) establishing a comprehensive fatty acid profile detailing the influences and interactions of TBI and DHA supplementation. Dietary DHA supplementation both before and after diffuse brain injury attenuated late-onset sensory hypersensitivity as detected by the whisker nuisance task. DHA supplementation also reduced [^{3}H]PK-11195 binding density, an index of neuroinflammation, at 28 days post-injury. This effect, however, was somewhat asymmetrical with regard to timing, as post-TBI DHA supplementation showed greater efficacy than pre-TBI supplementation. TBI resulted in a significant loss of brain DHA content and a significant increase in brain arachidonic acid, a precursor of pro-inflammatory prostaglandins. These data add to the body of evidence supporting the beneficial effects of omega-3 fatty acid supplementation on outcome from brain injury.

Several studies have shown that DHA (Bailes and Mills 2010) and fish oil (containing both EPA and DHA) supplementation can attenuate TBI-induced functional deficits (Wu, Ying et al. 2013; Desai, Kevala et al. 2014; Russell, Berman et al. 2014). Based on these findings, it was expected that brain-injured rats require more time to locate the submerged platform compared to uninjured animals; DHA supplementation was hypothesized to shorten the search durations. During days 15 to 17 post-injury, all groups had equivalent latencies to locate the submerged platform, indicating no learning

impairment, which precluded an evaluation of recall. No motor impairment was indicated by the equivalence of swim speeds. In two previous reports, supplementation with DHA following lateral fluid percussion brain injury in rats served to preserve cognitive function as measured in the Morris water maze (Wu, Ying et al. 2011; Wu, Ying et al. 2013). Literature in the currently used midline fluid percussion injury model has indicated a spatial learning deficit using the MWM at earlier post-injury time points (Hamm, Lyeth et al. 1993), but rats in the current study did not exhibit a significant injury-induced cognitive deficit when tested in the MWM at later post-injury time points (15-18 days). Thus, the effect of DHA supplementation on spatial learning was inconclusive. It is possible that these impairments could have resolved by the time of testing. Alternatively, lateral FPI, CCI, and the impact-acceleration weight-drop models are known to cause lesions and swelling of the brain parenchyma not typically resulting from midline FPI (Zhang, Cai et al. 2014). It may be that greater injury forces are required to elicit a sustained cognitive impairment. Such circumstances would provide some explanation for the differences in this study from earlier studies (Mills, Hadley et al. 2011; Wu, Ying et al. 2011) for the effects of DHA on spatial learning after TBI.

We have previously reported increased sensory sensitivity in rats following diffuse TBI as a late-onset morbidity (McNamara, Lisembee et al. 2010; Learoyd and Lifshitz 2012) that reflects clinical symptomology of agitation (Goodrich, Flyg et al. 2013; Goodrich, Martinsen et al. 2014). The whisker nuisance task was used to test for sensory hypersensitivity 22 days post-injury. Brain-injured rats not supplemented with DHA exhibited significantly increased nuisance scores compared to uninjured shams;

both DHA supplemented groups exhibited sensory sensitivity levels comparable to sham. DHA supplementation before or after TBI attenuated whisker nuisance scores by approximately two points from non-supplemented injured rats; the magnitude of this effect is meaningful as it indicates that treatment completely ameliorated one adverse reaction to sensory stimulation or a reduced the intensity of two different reactions to sensory stimulation. The timing of DHA supplementation cannot delineate the critical period to affect sensory sensitivity, however histopathology and inflammation data suggest a window of 7-10 days post-injury may be optimal (Cao, Thomas et al. 2012; Lifshitz and Lisembee 2012; Ziebell, Taylor et al. 2012)

Following TBI, the role of inflammation remains somewhat unclear, with evidence for its contribution to both beneficial and detrimental outcomes (for review, see Morganti-Kossmann et al. 2002(Morganti-Kossmann, Rancan et al. 2002)). TBI results in the acute expression of inflammation-mediating cytokines (Morganti-Kossmann, Rancan et al. 2001; Frugier, Morganti-Kossmann et al. 2010; Semple, Bye et al. 2010), which elicit a range of responses including immune activation and programmed cell death (Allan and Rothwell 2001). Some cytokine signaling can persist into sub-acute and chronic stages of injury. Omega-3 fatty acids can inhibit the production of pro-inflammatory cytokines in response to insults both *in vitro* (De Caterina, Cybulsky et al. 1994; Khalfoun, Thibault et al. 1997) and *in vivo* (Yaqoob and Calder 1995). In the current study, [^{3}H]PK11195 autoradiography was used to measure the efficacy of DHA supplementation on suppressing the microglial response to TBI. Two regions of interest, the cortex (Cao, Thomas et al. 2012) and substantia nigra (van Bregt, Thomas et al.

2012), were chosen for their vulnerability to mFPI. The remaining two areas of interest, the thalamus (Lifshitz, Kelley et al. 2007; McNamara, Lisembee et al. 2010) and hippocampus (Hamm, Lyeth et al. 1993), were chosen for their roles in the whisker nuisance task and MWM, respectively. Although [^{3}H]PK11195 binding has some limitations in that it can partially label macrophages and astroglia, it predominately labels activated microglia (Venneti, Lopresti et al. 2009). Despite these limitations, post-injury DHA supplementation significantly reduced injury-induced inflammation as measured by [^{3}H]PK11195 autoradiography in three of four regions of interest. Furthermore, pre-injury DHA supplementation resulted in binding densities not significantly higher than that of uninjured shams nor significantly lower than that of non-supplemented rats; a result interpreted as a modest reduction in this index of inflammation.

The discrepancy in efficacy between the two supplementation regimens may indicate that while both pre- and post-injury DHA impart functional benefit in the WNT, they may act through separate mechanisms. One set of possibilities is that pre-injury DHA may attenuate the initiation of the beneficial immune response acutely post-injury, while post-injury DHA may help resolve the ongoing immune response. While this supposition has not been tested directly in the context of TBI *in vivo*, *in vitro* pretreatment with DHA increases the phagocytic activity of microglia and promotes the M2, alternatively activated microglial phenotype (Chen, Zhang et al. 2014) that is more closely associated with tissue repair than with the inflammatory cascades of the M1 phenotype (Cherry, Olschowka et al. 2014). It therefore seems plausible that the intermediate [^{3}H]PK11195 findings we observed for the DHA pretreatment group could

have been due to increased phagocytosis being part of an activated microglial phenotype and DHA being withdrawn from the pretreatment group directly after injury. In contrast, the post-injury DHA group likely benefited from the suppression of M1-related pro-inflammatory cytokines (*e.g.* IL1β, TNFα, etc.; (Cao, Thomas et al. 2012; Cherry, Olschowka et al. 2014) by DHA or its docosanoid metabolites (Michael-Titus and Priestley 2014).

To better understand the lipidomic landscape following DHA administration and TBI, fatty acid analysis was conducted on brain, plasma, and red blood cells. Our findings confirm a recent report (Wu, Ying et al. 2014) that TBI significantly decreased brain DHA content. As such, the loss of brain DHA after brain injury is likely, regardless of the nature of the impact of TBI model. TBI was also shown to increase brain arachidonic acid content, a precursor of pro-inflammatory prostaglandins (Korbecki, Baranowska-Bosiacka et al. 2013). Dietary supplementation of DHA significantly influenced brain content of six other fatty acids. The finding that brain EPA content was not increased provides evidence to support the independent role of DHA in the current study. In the periphery, plasma and red blood cell fatty acid profiles were also significantly modified by dietary manipulation of DHA, but not TBI.

Potential reasons for the decrease of brain DHA content could include post-injury loss of DHA into the cerebrospinal fluid (Farias, Heidenreich et al. 2011), decreased brain DHA uptake as seen in models of Alzheimer's disease (Vandal, Alata et al. 2014), or higher rates of brain DHA utilization after TBI since damaged brain cells require more energy (Giza and Hovda 2001). Less conversion of its precursor, α-linolenic acid, to

DHA in the brain is also possible but unlikely given that the healthy brain only has a conversion efficiency of ≤1%, most α-linolenic acid is converted in the liver, and the brain rates are unchanged by omega-3 deficiency (Igarashi, DeMar et al. 2007). More data are needed to support these possible explanations, but they are consistent with the decreased expression of sirtuins, which are metabolic regulators, after TBI (Wu, Ying et al. 2011). More refined time courses would aid in understanding the interaction between brain injury and DHA availability.

Recent reports suggest that the neuroprotective effects of omega-3 fatty acids may be in part due to interactions of specialized pro-resolving mediator (SPM) derivatives (Buckley, Gilroy et al. 2014), including the resolvins (Harrison, Rowe et al. 2015), lipoxins (Luo, Li et al. 2013), and protectins (Bazan, Eady et al. 2012), with specific cellular mechanisms to be identified. To this effect, our group tested the therapeutic efficacy of two omega-3 fatty acid-derived SPMs for experimental diffuse brain injury in the mouse. The DHA-derived SPM tested, aspirin-triggered resolvin D1, but not the EPA-derived SPM, resolvin E1, significantly preserved motor and cognitive function compared to vehicle (Harrison, Rowe et al. 2015). Similarly, another DHA-derived SPM, aspirin-triggered neuroprotectin D1, was sufficient to reduce neuroinflammation (Orr, Palumbo et al. 2013) and improve performance on a battery of tests of neurological function following experimental stroke in rats (Bazan, Eady et al. 2012). These data are consistent with our lipid profile findings that only brain DHA content increased with DHA supplementation even though both DHA and EPA increased in the plasma. Our results are also consistent with the rapid metabolism and oxidation of EPA by the brain

(Chen and Bazinet 2015). Overall, these data add further support to the notion that DHA may play an important role as a precursor for bioactive lipid mediators in the context of acute neurological injury.

Findings of the current study could be taken into consideration for the design of future investigations. While both pre- and post-injury DHA supplementation resulted in attenuation of sensory hypersensitivity, only post-injury DHA supplementation effectively reduced inflammation measured by [^{3}H]PK11195 binding density. Further studies are needed to examine other potential mechanisms through which pre-injury DHA supplementation contributes to and sustains therapeutic efficacy for weeks. Measurement of cytokine and chemokine levels could provide additional insight into specific pathways of action. The current study was focused on post-acute outcomes which coincide with late-onset morbidities. Extensions of this study could investigate the role of DHA supplementation in the acute window following TBI, however the current experimental model focuses on enduring neurological symptoms.

3.6 CONCLUSIONS

In conclusion, we found that dietary DHA supplementation showed efficacy at attenuating long-term sensory hypersensitivity and reducing inflammation, measured by [^{3}H]PK-11195 binding density. Additionally, TBI resulted in significant changes across the fatty acid profile, notably including loss of brain DHA content. These results indicate that pre-injury supplementation of DHA may offer therapeutic efficacy against trauma-induced deficits and post-injury supplementation may serve as an intervention to

replenish brain DHA content, both of which could lessen the permanent injury burden resulting from TBI.

3.7 ACKNOWLEDGMENTS

I would like to thank all collaborators on this project: Christopher M. Butt, James Pauly, Jonathan Lifshitz, and Norman Salem Jr. for their contributions to study design, data interpretation, and manuscript preparation; Jacob Jones and Kelly Wynalda for their contributions to experiment execution and data interpretation; Rachel Rowe for her contribution to data interpretation and manuscript preparation.

CHAPTER 4

RESOLVINS AT-D1 AND E1 DIFFERENTIALLY IMPACT FUNCTIONAL OUTCOME, POST-TRAUMATIC SLEEP, AND MICROGLIAL ACTIVATION FOLLOWING DIFFUSE BRAIN INJURY IN THE MOUSE

Published in Brain, Behavior, and Immunity (Harrison et al. 2015)

4.1 ABSTRACT

Traumatic brain injury (TBI) is induced by mechanical forces which initiate a cascade of secondary injury processes, including inflammation. Therapies which resolve the inflammatory response may promote neural repair without exacerbating the primary injury. Specific derivatives of omega-3 fatty acids loosely grouped as specialized pro-resolving lipid mediators (SPMs) and termed resolvins promote the active resolution of inflammation. In the current study, we investigate the effect of two resolvin molecules, RvE1 and AT-RvD1, on post-traumatic sleep and functional outcome following diffuse TBI through modulation of the inflammatory response.

Adult, male C57BL/6 mice were injured using a midline fluid percussion injury (mFPI) model (6-10 min righting reflex time for brain-injured mice). Experimental groups included mFPI administered RvE1 (100ng daily), AT-RvD1 (100ng daily), or vehicle (sterile saline) and counterbalanced with uninjured sham mice. Resolvins or saline were administered daily for seven consecutive days beginning 3 days prior to TBI to evaluate proof-of-principle to improve outcome. Immediately following diffuse TBI, post-traumatic sleep was recorded for 24 hours post-injury. For days 1-7 post-injury, motor outcome was assessed by Rotarod. Cognitive function was measured at 6 days

post-injury using Novel Object Recognition (NOR). At 7 days post-injury, microglial activation was quantified using immunohistochemistry for Iba-1.

In the diffuse brain-injured mouse, AT-RvD1 treatment, but not RvE1, mitigated motor and cognitive deficits. RvE1 treatment significantly increased post-traumatic sleep in brain-injured mice compared to all other groups. RvE1 treated mice displayed a higher proportion of ramified microglia and lower proportion of activated rod microglia in the cortex compared to saline or AT-RvD1 treated brain-injured mice. Thus, RvE1 treatment modulated post-traumatic sleep and the inflammatory response to TBI, albeit independently of improvement in motor and cognitive outcome as seen in AT-RvD1-treated mice. This suggests AT-RvD1 may impart functional benefit through mechanisms other than resolution of inflammation alone.

4.2 INTRODUCTION

Each year over 1.7 million people sustain traumatic brain injuries (TBI) in the United States alone (Faul, Xu et al. 2010). The consequences of TBI for the individual can include diminished quality of life manifested through a range of symptoms including acute and chronic pain (Tham, Palermo et al. 2013), loss of neurological function (Arciniegas, Topkoff et al. 2000), and impaired cognitive or emotional ability (Arciniegas, Topkoff et al. 2000; Albensi and Janigro 2003; Masel and DeWitt 2010). Few therapies are available to treat these debilitating morbidities and many promising therapeutic agents have failed to achieve efficacy in clinical trials (Margulies and Hicks 2009). Animal models have been established to reproduce the complex pathophysiology of TBI and offer valuable means through which therapies can be tested on the resulting physiological, sensorimotor, and cognitive deficits. Pharmacological attempts to lessen the burden of TBI include a wide swath of treatment approaches, routinely using experimental compounds largely designed to target the cellular and molecular cascades which contribute to secondary injury in the hours to days following TBI (McIntosh, Juhler et al. 1998; Margulies and Hicks 2009).

TBI is induced by mechanical forces which initiate a cascade of secondary injury processes, including inflammation (Werner and Engelhard 2007). To complicate our understanding of underlying processes, cerebral inflammation has been shown to contribute to both beneficial and detrimental effects on outcome (for review, see (Morganti-Kossmann, Rancan et al. 2002)). Following TBI, the brain is inundated with inflammation-mediating cytokines (Morganti-Kossmann, Rancan et al. 2001; Frugier,

Morganti-Kossmann et al. 2010; Semple, Bye et al. 2010) which prompt a spectrum of responses including cell differentiation, immune activation, and cell death (Allan and Rothwell 2001). While the role of early-onset inflammation in the pathophysiology of TBI is debatable, clinical and experimental data suggest that chronic over-production of inflammatory cytokines aggravate the primary injury (Schmidt, Heyde et al. 2005; Lloyd, Somera-Molina et al. 2008; Cao, Thomas et al. 2012), and thereby impact outcome. Our lab has previously reported that the cortical primary somatosensory barrel fields (S1BF) are a primary site of neuropathology following experimental diffuse traumatic brain injury (Hall and Lifshitz 2010; Lifshitz and Lisembee 2012), with pronounced microglial activation and the induction of a previously understudied rod morphology of activated microglia (Cao, Thomas et al. 2012; Ziebell, Taylor et al. 2012; Taylor, Morganti-Kossmann et al. 2014). Rod microglia appear to constitute a phenotypically distinct class of activated microglia which present following acute neurological injury including diffuse TBI, infection and seizure (Wirenfeldt, Clare et al. 2009; Ziebell, Taylor et al. 2012; Mukherjee, Zeitouni et al. 2013). The relative distribution of microglial morphologies can thereby serve as an indicator neuroinflammation and underlying pathological and reparative processes. The current study further assesses neuroinflammation after diffuse TBI using a semi-quantification method of microglial activation to determine proportions of ramified (unactivated) microglia in relation to morphological phenotypes activated in response to injury—activated and rod microglia.

Cytokines are elevated and regulate inflammation following TBI (Semple, Bye et al. 2010; Ziebell and Morganti-Kossmann 2010). These cytokines, including pro-

inflammatory interleukin-1 beta (IL-1β), can also serve dual roles as sleep regulatory substances (SRSs) which influence sleep-wake behavior through action on the sleep circuits within the brain (Krueger, Takahashi et al. 1995; Krueger, Obal et al. 2001; Krueger, Rector et al. 2007). The elevation of IL-1β following midline fluid percussion brain injury in the mouse has previously been documented to correspond temporally with an acute increase in post-traumatic sleep during the first six hours post-injury (Rowe, Striz et al. 2014). These data suggest a relationship between inflammation and SRSs such that post-traumatic sleep may serve as an indicator of SRS action.

While inflammation is an essential component of the repair process, excessive or prolonged inflammation can aggravate injury-related damage (Bachstetter, Rowe et al. 2013). Therapies which resolve the inflammatory response may promote neural recovery without exacerbating the primary injury. Certain derivatives of omega-3 fatty acids loosely grouped as specialized pro-resolving lipid mediators (SPMs) have been shown to promote the resolution of inflammation resulting from multiple induction pathways (for review, see (Recchiuti and Serhan 2012)). These lipid mediators of inflammation are described as eicosanoids or docsanoids depending on their derivation from either eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) respectively (Serhan, Hong et al. 2002) and encompass subclasses including resolvins, protectins, lipoxins, and maresins (Recchiuti and Serhan 2012). Dietary supplementation with precursors of SPMs, particularly DHA, has shown therapeutic potential by reducing lesion size and improving neurological function following a model of ischemic stroke (Belayev, Khoutorova et al. 2009) and TBI (Mills, Hadley et al. 2011; Wu, Ying et al. 2011) in rats

by decreasing axonal injury and preserving cognitive function. Further, DHA supplementation after experimental stroke led to increased brain levels of another docosanoid, neuroprotectin D1 (NPD1) (Belayev, Khoutorova et al. 2011), a lipid mediator of inflammation which was then shown to ameliorate the functional and histological consequences of experimental stroke on its own (Bazan, Eady et al. 2012). Therefore, pharmacotherapy targeting SPMs provides a pleiotropic approach to regulating inflammation resulting from TBI, rather than genetically or chemically targeting a single signaling pathway.

Considering the similar inflammatory pathophysiologies of stroke and TBI, these data suggest that SPMs may regulate inflammation resulting from TBI. In the current study, two lipid mediators of inflammation, resolvin E1 (RvE1) and aspirin-triggered resolvin D1 (AT-RvD1), are tested for their impact upon post-traumatic sleep, sensorimotor and cognitive outcome, and microglial activation after diffuse experimental brain injury in the mouse. These endogenous SPMs are proposed to bring the injury-induced inflammatory response to conclusion as evidenced by a reduction in post-traumatic sleep, shift in the microglial morphology profile, and thereby improvement in functional outcome. We hypothesize that RvE1 and AT-RvD1 will improve functional outcome from diffuse TBI through modulation of the inflammatory response.

4.3 METHODS

4.3.1 Animals

Male C57BL/6 mice (Harlan Laboratories, Inc., Indianapolis, IN) were used for all experiments (n=73). Mice were housed in a 12 h light/12h dark cycle at a constant

temperature (23°C ± 2° C) with food and water available *ad libitum* according to the Association for Assessment and Accreditation of Laboratory Animal Care International. All mice used in this study were singly housed. Mice were acclimated to their environment following shipment for at least three days prior to any experiments. After surgery, mice were evaluated daily during post-operative care by a physical examination and documentation of each animal's condition. Animal care was approved by the Institutional Animal Care and Use Committees at St. Joseph's Hospital and Medical Center (Phoenix, AZ).

4.3.2 Midline Fluid Percussion Injury (mFPI)

Mice (20-24g) were subjected to midline fluid percussion injury (mFPI) consistent with methods previously described (Lifshitz 2008; Harrison, Rowe et al. 2014; Rowe, Harrison et al. 2014; Rowe, Harrison et al. 2014; Rowe, Striz et al. 2014). Group sizes are indicated in the results section and figure legends for individual studies. Mice were anesthetized using 5% isoflurane in 100% oxygen for five minutes and the head of the mouse was placed in a stereotaxic frame with continuously delivered isoflurane at 2.5% via nosecone. While anesthetized, body temperature was maintained using a Deltaphase® isothermal heating pad (Braintree Scientific Inc., Braintree, MA). A midline incision was made exposing bregma and lambda, and fascia was removed from the surface of the skull. A trephine (3 mm outer diameter) was used for the craniotomy, centered on the sagittal suture between bregma and lambda without disruption of the dura. An injury cap prepared from the female portion of a Luer-Loc needle hub was fixed over the craniotomy using cyanoacrylate gel and methyl-methacrylate (Hygenic Corp.,

Akron, OH). The incision was sutured at the anterior and posterior edges and topical Lidocaine ointment was applied. The injury hub was closed using a Luer-Loc cap and mice were placed in a heated recovery cage and monitored until ambulatory before being returned to their sleep cage.

For injury induction 24 hours post-surgery, mice were re-anesthetized with 5% isoflurane delivered for five minutes. The cap was removed from the injury-hub assembly and the dura was visually inspected through the hub to make sure it was intact with no debris. The hub was then filled with normal saline and attached to an extension tube connected to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). An injury of moderate severity for our injury model (1.4 atm) was administered by releasing the pendulum onto the fluid-filled cylinder. Sham-injured mice underwent the same procedure except the pendulum was not released. Mice were monitored for the presence of a forearm fencing response and righting reflex times were recorded for the injured mice as indicators of injury severity (Hosseini and Lifshitz 2009). The righting reflex time is the total time from the initial impact until the mouse spontaneously rights itself from a supine position. The fencing response is a tonic posturing characterized by extension and flexion of opposite arms that has been validated as an overt indicator of injury severity (Hosseini and Lifshitz 2009). The injury hub was removed and the brain was inspected for uniform herniation and integrity of the dura. The dura was intact in all mice; none were excluded as technical failures. The incision was cleaned using saline and closed using sutures. Moderate brain-injured mice had righting reflex recovery times greater than six minutes

and a positive fencing response. Sham injured mice recovered a righting reflex within 20 seconds. After spontaneously righting, mice were placed in a heated recovery cage and monitored until ambulatory (approximately 5 to 15 minutes) before being returned to their piezoelectric sleep cage. Adequate measures were taken to minimize pain or discomfort.

4.3.3 Pharmacological Treatment

Mice were treated with sterile saline (100µl, 0.9% NaCL), 100ng 17(R)-Resolvin D1 (AT-RvD1; Item # 13060, Cayman Chemical, Ann Arbor, MI), or 100ng Resolvin E1 (RvE1; Item # 10007848, Cayman Chemical, Ann Arbor, MI). Resolvins were administered intraperitoneally in 100µl of sterile saline for seven consecutive days beginning three days before mFPI (see Fig. 1). The seven day dosing schedule was selected to evaluate proof-of-principle for resolvin efficacy by covering both prophylactic and treatment approaches. Dosing was selected with regard to published studies using protectins in cerebral ischemia in mice(Bazan, Eady et al. 2012).

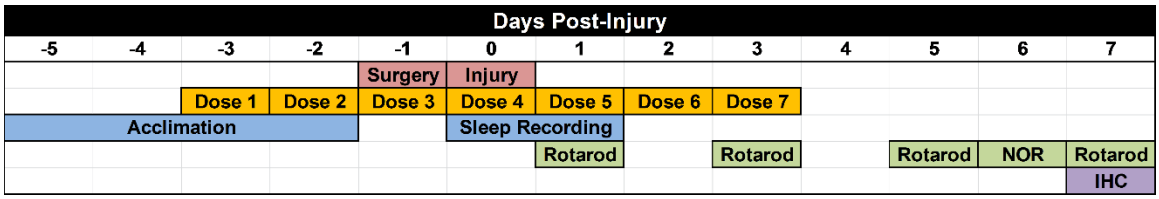

Figure 4.1. Study Design.

Mice were acclimated to their individual sleep cages before surgery and injury (blue). Mice were pre-treated with saline, AT-RvD1, or RvE1, for three days before surgery, and received post-injury injections for 4 consecutive days (orange). All mice received a midline craniectomy (red). One day post-surgery mice received either midline fluid percussion brain injury or sham injury (red). Immediately following injury, mice were placed back in their sleep cage and post-traumatic sleep was non-invasively recorded for 24 hours (blue). Following injury, mice were assessed for sensorimotor function using the rotarod task (1, 3, 5, 7 days post-injury). Cognitive performance was assessed using novel object recognition (NOR) at 6 days post-injury. Following behavior tasks, tissue was collected at 7 days post-injury and prepared for neuroinflammation analysis using immunohistochemistry (IHC; purple).

4.3.4 Sleep Recordings

The non-invasive sleep cage system (Signal Solutions, Lexington, KY) used in this study consisted of 16 separate units which simultaneously monitor sleep and wake states, as previously published (Donohue, Medonza et al. 2008; Mang, Nicod et al. 2014; Rowe, Harrison et al. 2014). Each cage unit housed a single mouse inside 18 x 18 centimeter walled compartments with attached food and water structures(Donohue, Medonza et al. 2008). The cages had open bottoms resting on Polyvinylidine Difluoride (PVDF) sensors serving as the cage floor (Donohue, Medonza et al. 2008). The non-invasive high-throughput PVDF sensors were coupled to an input differential amplifier and pressure signals were generated and classified by the classifier as motions consistent with either activity related to wake or inactivity and regular breathing movements associated with sleep (Donohue, Medonza et al. 2008; Rowe, Harrison et al. 2014). Briefly, sleep was characterized primarily by periodic (3 Hz) and regular amplitude signals recorded from the PVDF sensors, typical of respiration from a sleeping mouse. In contrast, signals characteristic of wake were both the absence of characteristic sleep signals and higher amplitude, irregular signals associated with volitional movements, even during quiet wake. The piezoelectric signals in two second epochs were classified by a linear discriminant classifier algorithm based on multiple signal variables to assign a binary label of “sleep” or “wake” (Donohue, Medonza et al. 2008). Mice sleep in a polycyclic manner (often more than 40 sleep episodes per hour if short arousals are recorded) (McShane, Galante et al. 2010) and therefore mouse sleep was quantified as the minutes spent sleeping per hour, presented as a percentage for each hour.

The current study was designed to avoid initial post-traumatic sleep recordings during a light transition period. As a result, sleep recordings began 6 hours into the light period, covered the light:dark transition, and continued for 6 hours into the dark period. Data collected from the cage system were binned over specified time periods (e.g. 1 hour) using the average of percent sleep, as well as binned by length of individual bouts of sleep and the median bout lengths were calculated. Where applicable, sleep metrics were compared between 6 hours of light and 6 hours of dark.

4.3.5 Behavioral Testing

Rotarod: Sensorimotor function was assessed using the Economex Rotarod system from Columbus Instruments (Columbus, OH). Mice were pre-trained for three consecutive days (60 sec at 4 RPM for 3 trials) prior to injury, following pre-injury drug treatment. For the test (1, 3, 5, 7 days post-injury), mice were placed on the rod with a starting speed of 4 RPM, and rod rotation speed was continuously increased over 5 minutes up to a max speed of 28 RPM, as previously published (Bachstetter, Rowe et al. 2013; Harrison, Rowe et al. 2014; Rowe, Harrison et al. 2014). The trial ended when the mouse fell from the rod or 5 minutes elapsed. Two trials were performed at each time point. Data are presented as latency to fall in seconds (average of two trials).

Novel Object Recognition (NOR): Cognitive impairment was tested using the NOR test as previously published (Ennaceur and Aggleton 1997; Han, Tong et al. 2011; Rowe, Harrison et al. 2014). The test consisted of three phases: habituation, training, and testing. On day 6 post-injury, mice were placed in an open field (42 cm, 21 cm, 21 cm) for one hour of habituation. Mice were removed and two identical objects were placed in

opposing quadrants of the field for the training phase. Mice were placed in the center of the open field and given 5 minutes to explore the objects. Following training, mice were returned to their piezoelectric sleep cages. Testing began 4 hours after training. One familiar object was placed in an original location and one novel object was placed in the opposing quadrant of the open field. Mice were placed into the center and given 5 minutes to explore. For testing, the times spent actively investigating the novel and familiar object were quantified. Investigation of an object included the mice sniffing, touching, or climbing onto an object while facing the object. If an animal climbed onto an object and sniffed into the air, this time was not calculated into the exploration of the novel object. Testing data are displayed as the percentage of total investigation time spent with each object and as a discrimination index (DI) in which $DI = (T_{novel} - T_{familiar})/(T_{novel} + T_{familiar})$.

4.3.6 Analysis of Microglial Activation

Tissue Preparation: At 7 days post-injury or sham operation, mice were given an overdose of sodium pentobarbital (i.p.) and transcardially perfused with 4% paraformaldehyde after a phosphate buffered saline (PBS) flush. Brains were removed and placed in 4% paraformaldehyde overnight. Brains were immersed in serial dilutions (15% and 30%) of sucrose for 24 hours each. The brains were removed from the 30% sucrose and frozen at -20° C. After freezing, brains were cryosectioned in the coronal plane at 20µm, mounted onto glass slides, and stored at -80° C.

Iba-1 Immunofluorescence: Slides were removed from -80°C, placed in an oven at 60°C for approximately 4 hours and then rinsed three times for 5 minutes each in PBS.

Next, the slides were incubated in 4% goat serum blocking solution for 1 hour. Slides were incubated with the primary antibody (rabbit anti-ionized calcium binding adaptor molecule 1, IBA-1; 1:1000, Item # 0199-19741, Wako Chemicals, Richmond, VA) and stored at 4°C overnight. Slides were rinsed three times in PBS and the secondary antibody (biotinylated horse anti-rabbit; 1:250, Vector Laboratories, Burlingame, CA) was added and slides were incubated on a rocker at room temperature for 1 hour. Slides were washed in PBS three times for 5 minutes each and tertiary stain was applied (streptavidin Alexa© Fluor 594; 1:1000, Jackson Immunoresearch, Westgrove, PA) and slides were incubated for 1 hour at room temperature. Lastly, slides were rinsed three times in PBS and coverslipped with anti-fade medium (Fluoromount G; Southern Biotech, Birmingham, AL). Sections were examined for microglial activation in response to brain-injury using a Zeiss LSM 710 Confocal Laser Scanning Microscope with attached digital camera.

Microglial Identification & Semi-Quantification: Stained sections (from 3-4 mice per group) were analyzed following Iba-1 staining to determine the proportion of microglial morphologies post-injury. The area of interest, the primary somatosensory barrel fields (S1BF), was chosen based on previous work demonstrating a multi-focal concentration of neuropathology and microglial activation in the S1BF following midline fluid percussion brain injury in the rat (Cao, Thomas et al. 2012; Lifshitz and Lisembee 2012; Ziebell, Taylor et al. 2012). Sections were screened using a Zeiss (AXIO imager A2) microscope with attached digital camera (AxioCam MRc5). Images were captured with proprietary Zen software (Carl Zeiss, Germany) at 20X magnification. The area of

interest was examined in both hemispheres and in two different coronal planes: an anterior section (approximately Bregma -1.555mm) and a posterior section (approximately Bregma -2.255mm). A total of 4 photos per brain per area of interest were analyzed. Photomicrographs were analyzed in Image J software (National Institutes of Health, Bethesda, MD). On each photomicrograph, 250,000 pixel^2 grid lines were placed and quantification was limited to 4 pre-defined boxes. Microglia within these boxes were classified as either having ramified (small soma, high defined processes), activated (hypertrophied soma, fewer processes), or rod (soma twice as long as width in one direction) morphologies. A minimum of 50 microglia were counted per section per region. Sham treatment groups were combined.

4.3.7 Statistical Analysis

Data are shown as mean ± SEM and analyzed using GraphPad Prism 6, with statistical significance assigned when $p<0.05$, unless otherwise indicated. For each test, shams receiving each treatment were statistically compared. No differences were detected between sham groups (treated and vehicle). To maintain comparable group sizes, a pooled sham group (n=12) was constructed using equal numbers of mice randomly selected from each treatment. Differences in righting reflex times were measured with a one-way analysis of variance (ANOVA). Differences in mortality were evaluated by chi-square analysis between both resolvin and saline-treated brain-injured groups. Percent sleep was analyzed using a repeated measure two-way ANOVA. Cumulative sleep measured in minutes and cumulative bout lengths of sleep were analyzed with a one-way ANOVA. All significant sleep data were further analyzed using Tukey's multiple

comparisons test. Differences in rotarod performance at 24 hours post-injury were determined with a one-way ANOVA followed by Dunnett's multiple comparisons test. Differences in motor performance following TBI over the first week post-injury were determined with a repeated measure two-way ANOVA followed by Dunnett's multiple comparisons test. Differences in time spent exploring the novel versus familiar object were determined for each treatment group by paired t-test, using a modified p value of 0.0125 to account for four separate tests. Proportional differences in rod microglial morphologies were subjected to chi-square analysis (Fisher's exact test) in which significance was defined by a modified p value of 0.0083 to correct for the 6 tests made within each morphological comparison. Statistical values are included in the figure legends.

4.4 RESULTS

4.4.1 Resolvin treatment did not influence the initial induction of diffuse TBI

We have previously reported suppression of the righting reflex response in mice following mFPI (Rowe, Harrison et al. 2014) as an injury-induced deficit and indicator of injury severity. Diffuse brain injury resulted in a significant suppression of the righting reflex in brain-injured mice, compared to anesthetized, uninjured shams, regardless of drug treatment (Fig 2). A one-way ANOVA comparing only brain-injured treatment groups showed no significant difference of righting reflex times, indicating that all brain-injured mice received injuries of similar severities. Of the 73 mice in this study, 48 were brain-injured. As a result of injury, 19 mice died (40% mortality). Of the 18 mortalities, 5 saline-treated mice died within 15 minutes of injury (33% mortality); 4 AT-RvD1-treated

mice died within 15 minutes of injury (27% mortality); 9 RvE1-treated animals died within 15 minutes of injury (50% mortality); all presumably from respiratory arrest. Differences in acute post-injury mortality were compared with chi-square analysis, revealing no significant treatment effects on mortality.

4.4.2 RvE1 increased TBI-induced post-traumatic sleep

As expected, we found that uninjured shams, regardless of drug treatment, slept similarly (data not shown). During the first 12 hours following injury, a repeated-measure two-way ANOVA revealed a significant main effect of both time and treatment group on percent sleep. Further, Tukey's multiple comparisons test revealed that RvE1-treated mice slept significantly more than all other groups during the first 12 hours following injury (Fig 3A). A more detailed analysis was performed by calculating the number of cumulative minutes spent sleeping during the first light and dark periods after brain injury. RvE1-treated brain-injured mice slept significantly more during the light period immediately following injury compared to saline-treated brain-injured mice (Fig 3B). During the following dark period, RvE1-treated brain-injured mice continued to sleep significantly more compared to uninjured sham and both other brain-injured groups (Fig 3C). Overall, brain-injured mice treated with RvE1 slept significantly more than other brain-injured mice, since uninjured mice treated with RvE1 slept similar to the remaining uninjured mice, indicating a brain injury rather than a treatment effect.

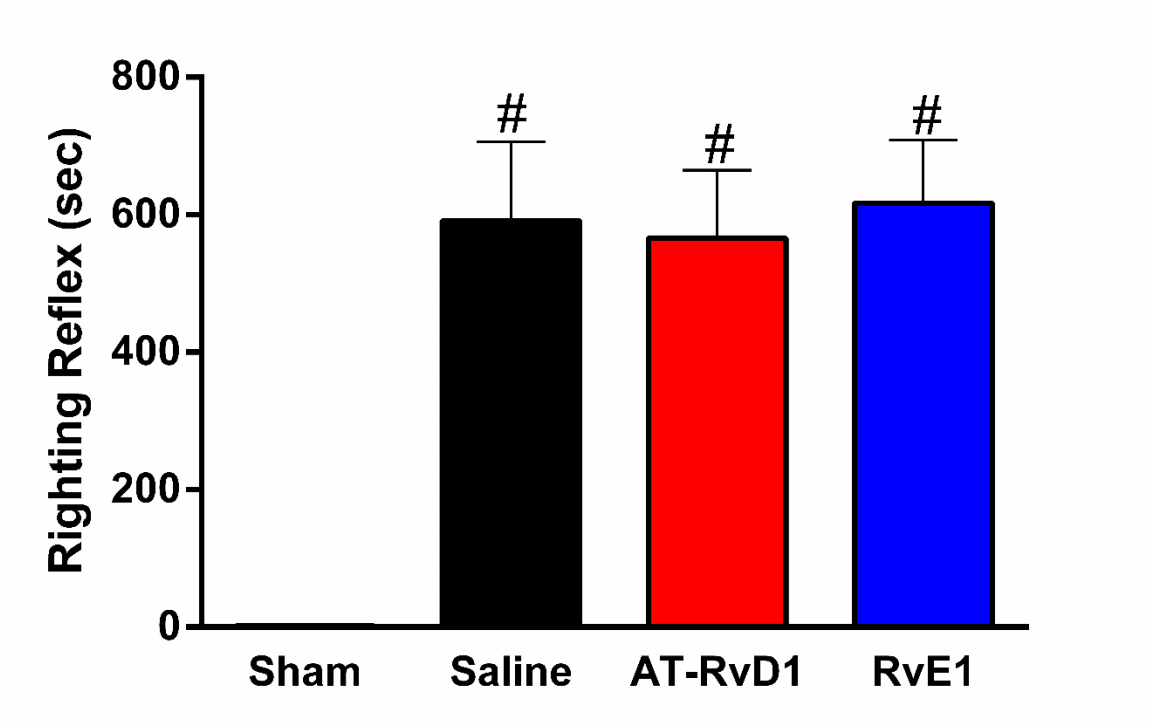

Figure 4.2. TBI suppressed righting reflex regardless of drug treatment.

Diffuse TBI resulted in a significant suppression of the righting reflex (mean ±SEM; $F(3,34)=15.09$, $p<0.0001$) compared to uninjured shams. Further, by excluding the uninjured shams, the one-way ANOVA revealed no significant differences between brain-injured mice, regardless of treatment ($F(2,23)=0.06$; $p=0.9$). (sham n=12, saline-treated injury n=9, AT-RvD1-treated injury n=9, RvE1-treated injury n=8; #, $p<0.05$ compared to sham).

Further, post-traumatic sleep showed a significant injury effect on number of sleep bouts during the first light period post-injury (Fig 3D). Post-hoc analysis indicated that regardless of treatment, all brain-injured groups slept significantly more sleep bouts compared to uninjured shams (Fig 3D). Overall, there was a group effect on the number of bouts in the dark period (Fig 3E). Tukey's multiple comparison test revealed RvE1-treated brain-injured mice had significantly more bouts during the dark period compared to uninjured shams, saline-treated brain-injured mice, and AT-RvD1-treated brain-injured mice. These data show that RvE1 increased sleep through an increase in the number of sleep bouts, but not necessarily the length of the sleep bout.

4.4.3 AT-RvD1 mitigated TBI-induced sensorimotor deficits on the rotarod task

To assess motor function, the rotarod task was used as previously published (Bachstetter, Rowe et al. 2013; Harrison, Rowe et al. 2014; Rowe, Harrison et al. 2014). Motor function was tested as the latency to stay on the rotarod over 7 days post-injury, with significant effects on both time post-injury and between groups. Tukey's post-hoc analysis indicated that compared to uninjured shams, brain-injured mice treated with saline or RvE1 demonstrated significantly shorter latencies to fall from the rotarod. However, AT-RvD1-treated brain-injured mice did not show a significant deficit compared to uninjured shams and displayed significantly longer latencies than RvE1-treated mice. Overall, diffuse brain injury reduced motor function measured on the rotarod task. AT-RvD1-treated brain-injured mice did not show injury-induced impairments measured by latency (Fig 4). While AT-RvD1-treated mice did not perform significantly better than saline-treated mice, they did not demonstrate a deficit in

comparison to sham. Together, these data suggest that AT-RvD1 improved or accelerated recovery from TBI, but perhaps not to an uninjured level.

4.4.4 TBI-induced cognitive impairment was improved with AT-RvD1 treatment

Cognitive function was measured using the novel object recognition task (Antunes and Biala 2012). Paired t-tests indicated significant differences between time spent exploring the novel and familiar objects for uninjured shams and AT-RvD1-treated brain- injured mice, indicating recall of the familiar object (Fig 5A). However, saline-treated and RvE1-treated brain-injured mice spent equivalent times investigating both objects, indicating no recall of the familiar object (Fig 5A). Statistical comparisons between investigation times of the groups were not performed. A significant difference in investigation of novel and familiar was treated as the criterion for 'recognition;' only sham and AT-RvD1-treated groups reached recognition criterion. To further analyze cognitive performance on the NOR task, the discrimination index was calculated as the time spent with the novel object minus the time spent with the familiar object divided by the total time spent exploring for each mouse. There was no significant difference in the discrimination index between groups (Fig 5B).

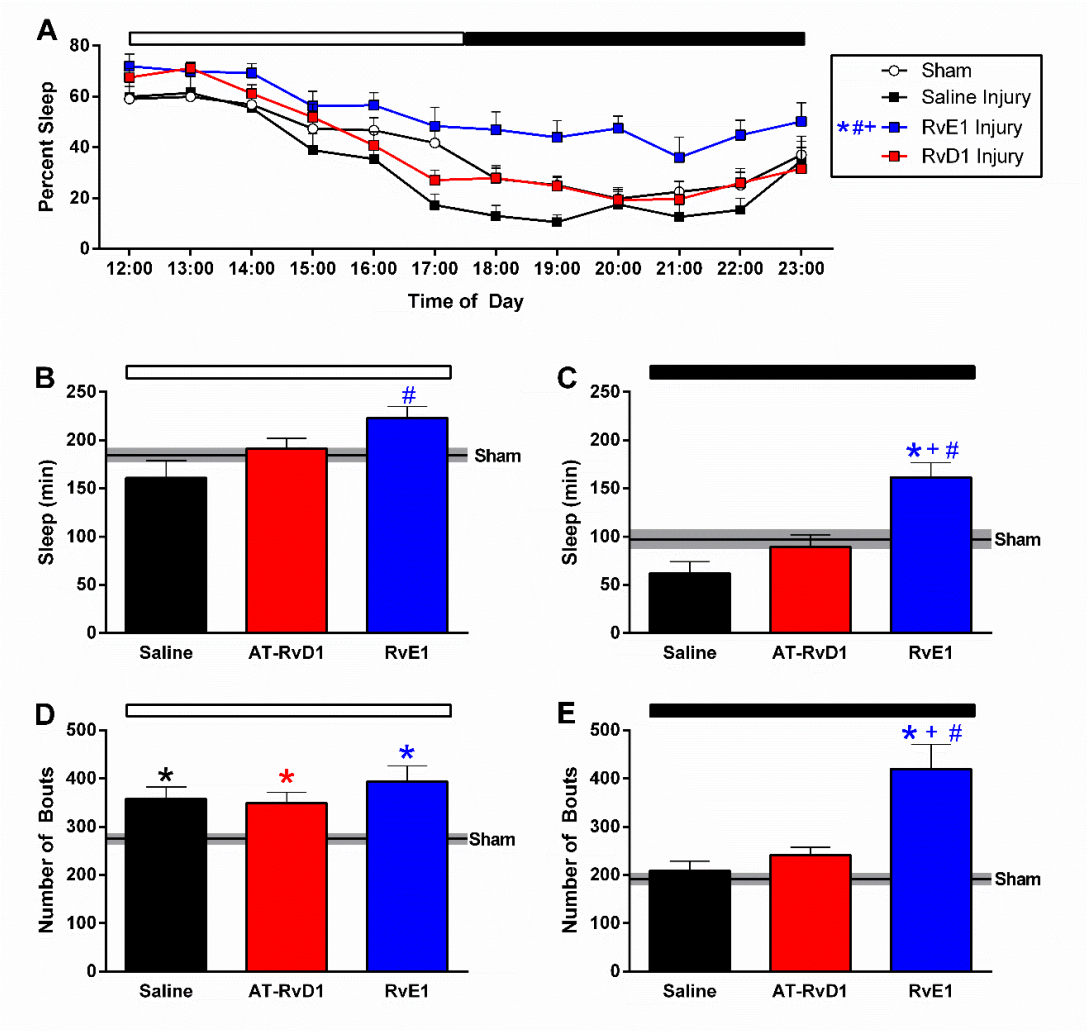

Figure 4.3. RvE1 increased post-traumatic sleep.

During the first 12 hours following injury **(A)**, a two-way ANOVA revealed a significant main effect of treatment group on sleep ($F(3,31)=7.6$, $p<0.0006$). Specifically, Tukey's multiple comparison test indicated that RvE1-treated mice slept significantly more than all other groups. RvE1-treated brain-injured mice slept more during the first light **(B)** and dark **(C)** period following injury. RvE1-treated brain-injured mice slept significantly more compared to saline-treated brain-injured mice in the light period ($F(2,20)=5.0$, $p=0.02$) and dark period ($F(3,31)=9.1$, $p=0.0002$) immediately following injury. Post-traumatic sleep showed a significant injury effect on number of sleep bouts during the first light period **(D)** ($F(3,31)=7.0$, $p=0.001$) and dark period **(E)** ($F(3,31)=16.3$, $p<0.0001$). (saline-treated injury n=7, AT-RvD1-treated injury n=9, RvE1-treated injury n=7, sham n=12) (*, $p<0.05$ compared to sham; #, $p<0.05$ compared to vehicle; +, $p<0.05$ compared to AT-RvD1)

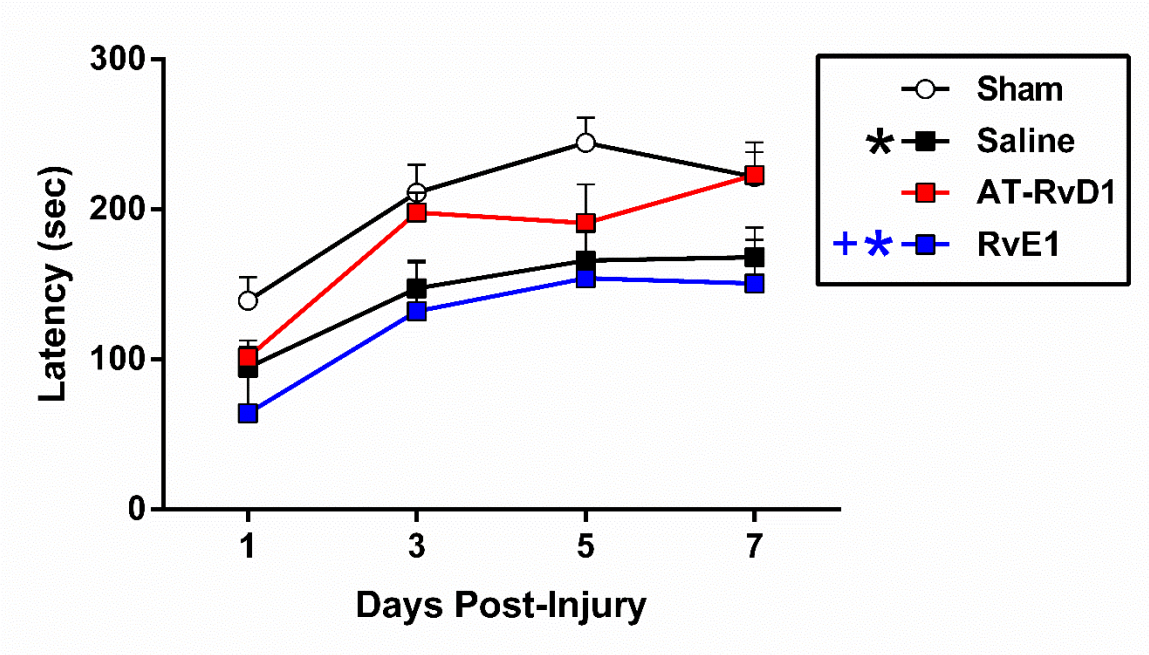

Figure 4.4. AT-RvD1 prevented TBI-induced motor deficits measured by the rotarod task.

There was a significant effect of time after injury on motor performance $F(3,99)=32.0$, $p<0.0001$). There was also a significant group effect on latency to stay on the rotarod ($F(3,33)=4.6$, $p=0.008$). Tukey's post-hoc analysis indicated that compared to uninjured shams, brain-injured mice treated with saline or RvE1 demonstrated significantly shorter latencies to fall from the rotarod. However, AT-RvD1-treated brain-injured mice performed comparably to uninjured shams and displayed significantly longer latencies than RvE1-treated mice. saline-treated injury n=11, AT-RvD1-treated injury n=6, RvE1-treated n=8, sham n=12). (*, $p<0.05$ compared to sham; +, $p<0.05$ compared to AT-RvD1)

4.4.5 RvE1 increased the proportion of ramified microglia and decreased the proportion of rod microglia in the sensory cortex

Resolvin treatment did not alter the proportions of microglia phenotypes among saline and treated sham animals in somatosensory cortex (chi-square, $p>0.05$; data not shown). All brain-injured groups demonstrated a lower proportion of ramified microglia than uninjured sham mice at 7 days post-injury (chi-square, $p<0.0001$). RvE1-treated brain-injured mice had a significantly higher proportion of ramified microglia compared to saline-treated ($p=0.0001$) and AT-RvD1 mice ($p<0.0001$). All brain-injured groups demonstrated a greater proportion of activated microglia than uninjured sham mice (chi-square, $p<0.0001$). RvE1-treated brain-injured mice had a significantly lower proportion of activated microglia than AT-RvD1 treated mice ($p=0.005$). As expected, sham mice demonstrated no rod microglia. RvE1-treated brain-injured mice had a significantly lower proportion of rod microglia than saline-treated brain-injured mice ($p=0.0002$). These data show RvE1 significantly altered the inflammatory profile of microglia in somatosensory cortex, whereas microglial activation after AT-RvD1 treatment remained unchanged from the untreated condition.

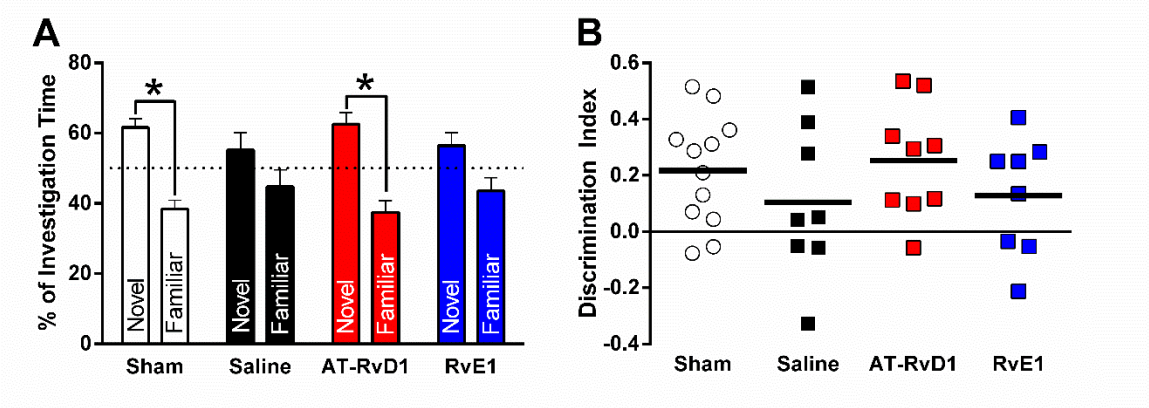

Figure 4.5. AT-RvD1 prevented TBI-induced cognitive impairment measured by novel object recognition.

Differences in time spent exploring the novel versus familiar object were determined for each treatment group by paired t-test, using a modified p value of 0.0125 to account for four separate tests. These tests revealed a significant differences between investigation time of novel and familiar objects among sham ($t(11)=4.6$, $p=0.0008$) and AT-RvD1 treated brain-injured mice ($t(8)=3.8$, $p=0.006$) **(A)**, indicating recall of the familiar object. However, saline-treated ($t(7)=1.1$, $p=0.3$) and RvE1-treated brain-injured mice ($t(7)=1.7$, $p=0.1$) spent similar times investigating both objects indicating no recall of the familiar object.The discrimination index **(B)** was calculated as the time spent with the novel object minus the time spent with the familiar object divided by the total time spent exploring. While sham and AT-RvD1 trended toward object recognition, there was no significant difference in discrimination indices between groups ($F(3,33)=0.9$, $p=0.4$). (saline-treated injury n=8, AT-RvD1-treated injury n=9, RvE1-treated injury n=8, shams n=12) (*, $p<0.0125$ between novel and familiar)

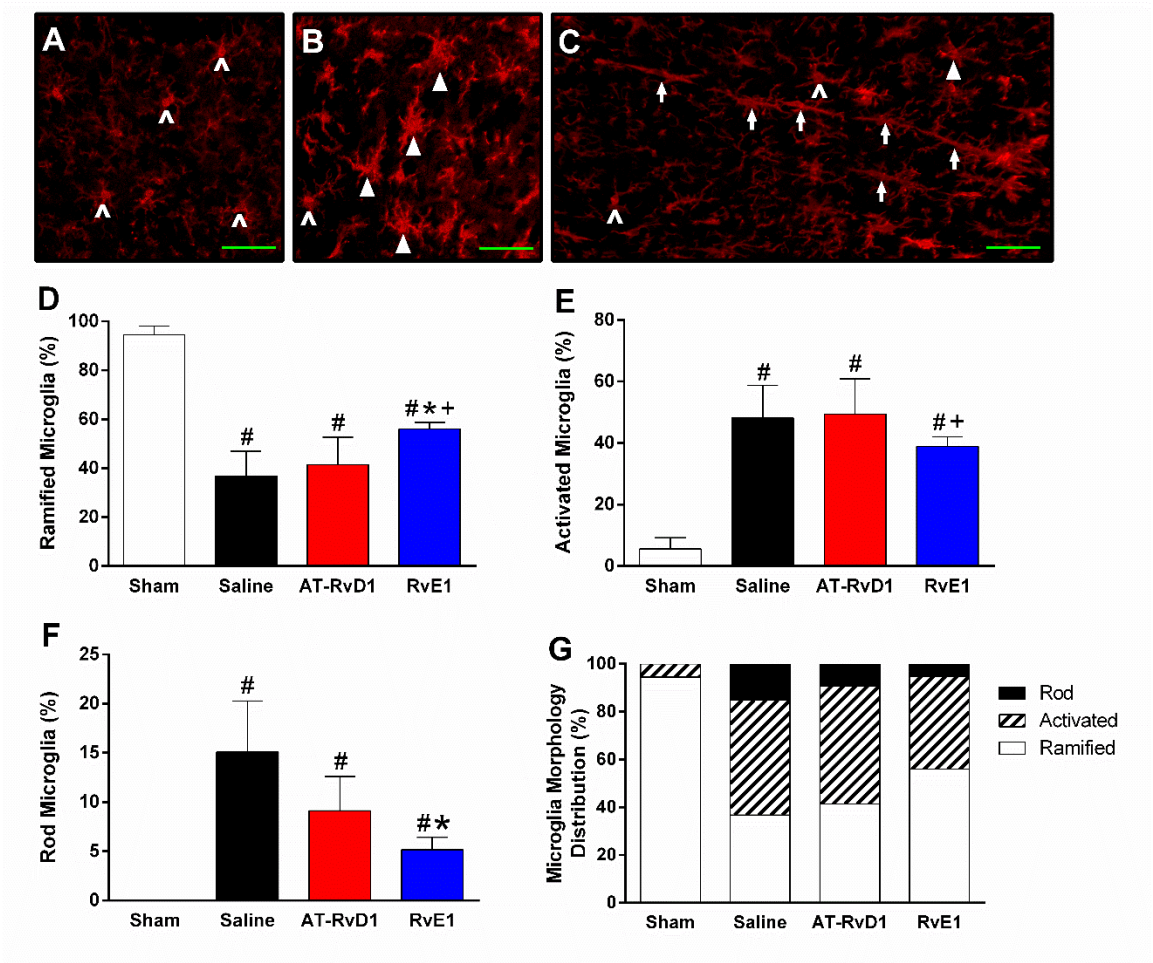

Figure 4.6. RvE1 increased the proportion of ramified microglia and decreased the proportion of rod microglia.

Representative microscopic fields abundant with **(A)** ramified microglia (carets), **(B)** activated microglia (arrowheads), and **(C)** rod microglia (arrows) in the primary somatosensory barrel fields at 7 days post-injury. **(D)** Sham mice demonstrated a higher proportion of ramified microglia than all brain-injured groups ($p<0.0001$). RvE1-treated brain-injured mice had a significantly higher proportion of ramified microglia compared to saline-treated ($p=0.0001$) and AT-RvD1 mice ($p<0.0001$). **(E)** Sham mice demonstrated a lower proportion of activated microglia than all brain-injured groups ($p<0.0001$). RvE1-treated brain-injured mice had a significantly lower proportion of activated microglia than AT-RvD1 treated mice ($p=0.005$). **(F)** Sham mice demonstrated a lower proportion of rod microglia than all brain-injured groups ($p<0.0001$). RvE1-treated brain-injured mice had a significantly lower proportion of rod microglia than saline-treated brain-injured mice ($p=0.0002$). **(G)** A combined representation of observed microglial morphological distributions. (*, $p<0.05$ compared to sham; #, $p<0.05$ compared to vehicle; +, $p<0.05$ compared to AT-RvD1)

4.5 DISCUSSION

The current study was designed with the proof-of-principle goals to: 1) test the administration of SPMs AT-RvD1 and RvE1 for therapeutic effect on translational outcomes of motor and cognitive function; 2) investigate a potential mechanism for SPM interactions in the injured brain by evaluating their effects upon microglial activation; 3) further probe the relationship between sleep and inflammation following diffuse brain injury in the mouse. One SPM, AT-RvD1, showed significant efficacy in mitigating the motor and cognitive deficits resulting from diffuse TBI. The other, RvE1, elicited a dramatic increase in post-traumatic sleep and tempered microglia activation without significantly influencing behavioral outcome compared to saline treatment. The divergence in histological (microglial activation) and physiological (sleep activity) outcomes from functional (motor and cognitive performance) outcomes suggests that microglial reactivity may contribute to post-traumatic sleep and that AT-RvD1 may achieve therapeutic efficacy through means other than inflammation resolution alone. Overall, SPMs demonstrate proof-of-principle as a therapeutic approach for diffuse brain injury, where additional studies are warranted, as described below.

Diffuse brain injury in humans results in long-term disabilities, including motor and cognitive impairments, which appreciably diminish quality of life (Arciniegas, Topkoff et al. 2000; Masel and DeWitt 2010). These injury-induced impairments can be modeled in rodents. The rotarod test has been used in the evaluation of motor function in rats (Hamm 2001) and mice (Fenn, Gensel et al. 2013; Harrison, Rowe et al. 2014; Rowe, Harrison et al. 2014), demonstrating impairments to remain on the accelerating rod

following diffuse TBI. In the current study, saline-treated brain-injured mice exhibited significant rotarod impairment compared to sham-injured mice. This impairment was also evident in RvE1-treated mice, but not in AT-RvD1-treated mice. Similar results were found in cognitive performance using the novel object recognition (NOR) test, where sham-injured and brain-injured AT-RvD1-treated mice investigated novel objects significantly more than familiar objects—indicating short term episodic recognition (Ennaceur and Delacour 1988)—but saline-treated and RvE1-treated brain-injured mice failed to recognize the familiar object. Although no literature is available on the effects of SPMs on functional outcome following TBI, there are several studies which show that DHA and fish oil (containing both EPA and DHA) supplementation attenuate TBI-induced motor (Desai, Kevala et al. 2014; Russell, Berman et al. 2014) and cognitive (Wu, Ying et al. 2011; Wu, Ying et al. 2013) deficits. The current set of studies were undertaken, because the neuroprotective effects of DHA and fish oil may be in part due to actions of their SPM derivatives, including the resolvins, lipoxins, and protectins. We selected two resolvins to evaluate in diffuse brain injury. To this effect, one study tested the therapeutic efficacy of aspirin-triggered neuroprotectin D1 (AT-NPD1), an SPM derived from DHA, in a rat model of experimental stroke. One i.v. dose of AT-NPD1 at three hours after onset of experimental stroke was sufficient to improve performance on a battery of tests of neurological function compared to rats treated with saline (Bazan, Eady et al. 2012). These data, in light of the functional improvements elicited by AT-RvD1 in the present manuscript, suggest that SPM derivatives may mediate the protective effects of omega-3 fatty acids.

Clinical studies have provided evidence that TBI contributes to disorders in sleep regulation, including chronic sleep disturbance as well as excessive daytime sleepiness (Baumann, Werth et al. 2007; Castriotta, Wilde et al. 2007; Kempf, Werth et al. 2010; Baumann 2012). Chronic sleep issues in the rodent are unclear, with evidence both for (Lim, Elkind et al. 2013; Hazra, Macolino et al. 2014) and against (Rowe, Harrison et al. 2014) sustained sleep issues. We have previously demonstrated that sleep is increased in the first 3-6 hours following diffuse TBI in the mouse (post-traumatic sleep) compared to sham, when injury was induced 3 hours after light onset (Rowe, Striz et al. 2014). In the present study, diffuse brain injury was delayed until 6 hours after the light onset and did not result in increased post-traumatic sleep among vehicle-treated mice. Therefore, the time of injury relative to the light:dark transition may influence post-traumatic sleep more than the time of day. Similarly, another study subjected mice to CCI and found that TBI resulted in acute decreases in wakefulness (Willie, Lim et al. 2012). It is unclear the cellular benefit or detriment of this acute post-traumatic sleep on recovery following TBI, however, we have previously shown immediate disruption of post-traumatic sleep does not worsen injury-induced motor or cognitive deficits (Rowe, Harrison et al. 2014) in the mouse. While the role of post-traumatic sleep in the pathophysiology of TBI remains unclear, data support a mechanistic link between inflammation and the induction of sleep (Krueger, Takahashi et al. 1995; Krueger, Obal et al. 2001; Krueger, Rector et al. 2007; Rowe, Striz et al. 2014). SPMs including RvE1 and AT-RvD1 may resolve inflammation resulting from TBI, as incorporated into the extended drug delivery design, and subsequently attenuate post-traumatic sleep disorders.

Presently, RvE1 treatment of TBI significantly increased post-traumatic sleep, without functional improvement, compared to saline-treated and AT-RvD1-treated brain-injured mice. Further, microglial activation showed a lower proportion of rod microglia, with a higher proportion of ramified microglia, at 7 days post-injury when treated with RvE. The increase in sleep behavior and attenuation of the post-acute inflammatory response appear contradictory. However, inflammation and, more recently, microglial activation have been implicated in sleep regulation (for review, see (Ingiosi, Opp et al. 2013)). It has been proposed that some drugs, such as methamphetamine, exert sleep regulatory effects through actions on microglia (Wisor, Schmidt et al. 2011). For example, genetic depletion of microglia attenuated the wake-promoting effect of methamphetamine in mice, suggesting that the presence of microglia contributes to sleep regulation following cellular stress (Wisor, Schmidt et al. 2011). Clinically, intracellular monocyte pro-inflammatory cytokine production was correlated with sleep modulation (Thomas, Motivala et al. 2011). In the present study, post-traumatic sleep could increase in RvE1-treated mice with reduced post-acute microglial activation because of the delay between measurements, non-inflammatory sleep pressures, or unknown physiological mechanisms that also contributed to the higher mortality in RvE1 mice. Further, analysis of microglia morphology is only one indirect index of inflammation; direct measurement of inflammation-mediating cytokines would improve our understanding of the cellular processes involved. It remains likely that increased post-traumatic sleep of RvE1-treated mice, possibly a protective response, may promote resolution of inflammation as indicated by fewer activated rod microglia, albeit without functional benefit. Since sleep

disruption results in microglial activation (Hsu, Lee et al. 2003) without worsening functional outcome after diffuse TBI in mice (Rowe, Harrison et al. 2014), the effect of disrupting post-traumatic sleep in RvE1 treated mice would be intriguing.

While electroencephalographic (EEG) recording offers insight into sleep quality and stage, the piezoelectric sleep monitoring system offers a non-invasive approach to only quantify wakefulness and sleep durations, concordant with EEG analysis (Mang, Nicod et al. 2014). It is unclear how TBI or resolvin treatment may independently alter respiratory rate and confound the algorithmic detection of sleep, but we have previously demonstrated that the piezoelectric sleep system detects discrete bouts of 3 Hz breathing classified as sleep following mFPI. Further, post-traumatic sleep was not continuous and was interleaved with periods classified as wake and movement(Rowe, Striz et al. 2014). Sham-injured mice treated with either RvE1 or AT-RvD1 did not show changes in sleep pattern compared to vehicle treated mice.

Of the SPMs investigated in the current study, RvE1 solely tempered microglial reactivity while AT-RvD1 solely reduced the functional consequences of diffuse brain injury. Because selective results were obtained, the mechanism through which SPMs imparts functional benefit may not be through resolution of inflammation alone. In various experimental paradigms, resolvins and protectins have been implicated in reducing the deleterious effects of oxidative stress (Wang, Yuan et al. 2014), a recognized component of TBI pathophysiology. Further research is warranted to determine the mechanism(s) through which AT-RvD1 improved functional outcome from TBI. Regardless, the omega-3 fatty acids and likely their derivatives can impart

functional recovery from TBI (Hasadsri, Wang et al. 2013), indicating that each derivative may target a specific pathophysiological process in a combination therapy manner.

A systematic isobolographic approach could combine the two (or more) treatments in varying concentrations for different groups of animals to better study synergistic drug effects (Tallarida 2012). This method has been suggested as a valid method of detecting beneficial drug-drug interactions in the development of therapies for TBI (Shear and Tortella 2013). An isobolographic analysis of RvE1 and AT-RvD1 could potentially shed light on the interactions among post-traumatic sleep, inflammation, and functional outcome. Yet, this study may serve to caution against dissecting a pleotropic drug at the expense of separating specific effects and mechanisms.

As the first study to specifically assess the effects of resolvins in experimental TBI, several factors should be taken into consideration in future studies. The current proof of principle design could be refined to optimize the resolvin dosing with respect to the time of injury. Additionally, no literature is available on the most effective or biologically applicable dosing of synthetic resolvins with respect to acute neurological injury. One study administered AT-RvD1 (i.v. 300ng/mouse) in a mouse model of fibromyalgia (Klein, Sperotto et al. 2014) while another study administered the comparable docosanoid neuroprotectin D1 (i.v. at 333 µg/kg) in a rat model of cerebral ischemia (Bazan, Eady et al. 2012). The current study falls between these wide margins of 12.5 µg/kg and 333 µg/kg respectively with a cumulative intraperitoneal dose of

~30µg/kg (700 ng/mouse). Further studies are warranted on the most appropriate administration and dosing schedules of lipid mediators.

4.6 CONCLUSIONS

In the diffuse brain-injured mouse, AT-RvD1 treatment, but not RvE1, mitigated motor and cognitive deficits. RvE1 treatment significantly increased post-traumatic sleep in comparison to all other groups. RvE1 treated mice displayed a higher proportion of ramified microglia and lower proportion of rod microglia in the cortex compared to saline or AT-RvD1 treated brain-injured mice. Increased post-traumatic sleep in the RvE1-treated brain-injured mice may be associated with active resolution of acute inflammation. We demonstrate that modulation of post-traumatic sleep and the inflammatory response to TBI seen in RvE1-treated mice were accomplished independently of improvement in motor and cognitive outcome as seen in AT-RvD1-treated mice. This suggests AT-RvD1 may impart neuroprotection through mechanisms other than inflammation resolution alone.

4.7 ACKNOWLEDGMENTS

I would like to thank all collaborators on this project: Rachel Rowe for her contribution to study design, experiment execution, data interpretation, and manuscript preparation; Jonathan Lifshitz for his contribution to study design, data interpretation, and manuscript preparation, Bruce O'Hara, P. David Adelson, Timothy Ellis, and Nicole Yee for their contributions to data interpretation and manuscript preparation.

CHAPTER 5

REMOTE ISCHEMIC CONDITIONING FOLLOWING DIFFUSE TBI IN THE MOUSE: ASSESSMENT OF FUNCTIONAL OUTCOME AND IDENTIFICATION OF PLASMA PROTEIN CORRELATES

5.1 ABSTRACT

Millions of Americans sustain traumatic brain injuries (TBI) each year without seeking professional medical care. The secondary injury cascades of TBI begin immediately following injury and can persist for days to weeks. There is a need for practical therapies to prevent further secondary injury which can be applied while in transit to a medical facility or by an individual who would otherwise not seek medical care. Recent studies have shown remote ischemic conditioning (RIC) to have wide ranging protective effects in studies from stroke to cardiovascular infarct. This low-cost, universally-available, nontoxic therapy could be used to lessen the chronic consequences of TBI. RIC requires only a tourniquet or blood pressure cuff to transiently restrict blood flow to a limb in approximately four cycles of restriction and reperfusion. In the current study, we test RIC efficacy on functional outcome after fluid percussion brain injury in the mouse. Further, we use untargeted mass spectrometry proteomic analysis of plasma from RIC-treated mice to identify molecular correlates this therapeutic intervention. Diffuse brain injury in the mouse resulted in acute post-injury sensorimotor impairments regardless of RIC treatment. No TBI-induced cognitive or affective deficits were detected by 14 days post-injury. Proteomic analysis identified multiple proteins impacted by treatment group (TBI and RIC), including promising candidate proteins which appear

selectively responsive to RIC. These results represent a first-of-kind objective evaluation of potential RIC protein correlates and serve to inform future studies of the presently unclear mechanism underlying RIC efficacy.

5.2 INTRODUCTION

In the United States alone, traumatic brain injury (TBI) affects approximately 1.7 million individuals each year (Faul, Xu et al. 2010). No pharmacological or other treatments are available for individuals who suffer the lifelong neurological morbidities associated with TBI; clinical interventions have focused on prevention and symptom treatment rather than restoration of function after TBI. The lingering consequences of TBI include cognitive, motor, and emotional dysfunction. In this exploratory chapter, we assess a low-cost, universally-available, nontoxic therapy which could be used to lessen the chronic consequences of TBI – Remote Ischemic Conditioning (RIC). RIC requires only a tourniquet or blood pressure cuff to transiently restrict blood flow to a limb in approximately four cycles of restriction and reperfusion. From this simple intervention, we propose that restorative and regenerative bioactive molecules serve to reduce the secondary injury processes which would otherwise exacerbate the injury and worsen outcome. In addition to assessing RIC efficacy in a battery of behavioral tests following diffuse brain injury in the mouse, the current manuscript uses untargeted discovery proteomic analysis of plasma to elucidate circulating protein correlates of RIC.

Minor ischemic injury in the brain protects from subsequent severe ischemic events (Shamloo, Rytter et al. 1999; Shamloo and Wieloch 1999; Shamloo and Wieloch 1999; Tomasevic, Shamloo et al. 1999). In humans, though, even a transient ischemic attack can result in chronic cognitive impairment (van Rooij, Schaapsmeerders et al. 2014). To translate a minor ischemic event into a safe therapy for various acute conditions, RIC is broadly defined as transiently occluding blood flow to an organ or

body part to impart protection in a distant organ or body part. Literature on RIC is varied in terms of the applied location, intended target of therapy, and timing of experimental RIC. RIC has been applied to the lower limb (Cheung, Kharbanda et al. 2006; Dave, Saul et al. 2006; Peng, Guo et al. 2012; Sun, Tong et al. 2012), upper limb (Gunaydin, Cakici et al. 2000; Kharbanda, Mortensen et al. 2002), kidney (Pell, Baxter et al. 1998), and liver (Peralta, Serafin et al. 2003), among others to protect the brain (Dave, Saul et al. 2006; Peng, Guo et al. 2012; Sun, Tong et al. 2012) and heart (Oxman, Arad et al. 1997; Cheung, Kharbanda et al. 2006), primarily. For cerebral ischemia, the timing of RIC has included a spectrum of pre-insult (Wang, Birch et al. 2010; Wang, Yu et al. 2013) and post-insult (Peng, Guo et al. 2012; Pignataro, Esposito et al. 2013) sequences to achieve efficacy. In a proof-of-principle clinical study that illustrated the feasibility of RIC as a pre-hospital therapy, 333 patients with suspected myocardial infarction were treated with RIC or no RIC while in transit to the hospital. Simply, a blood pressure cuff was placed on the arm and inflated through four 5 minute cycles interleaved with 5 minute reperfusion intervals. Patients treated with RIC had significantly greater cardiac salvage rates and did not experience any RIC-related adverse effects (Botker, Kharbanda et al. 2010). These laboratory and clinical studies establish the need for investigation of RIC as an early intervention for TBI.

It is likely that RIC has both long-lasting and fast-acting effects based on experimental studies demonstrating efficacy of both pre- and post-injury application. This first quality may make it ideal as a routine prophylactic conditioning regimen for individuals at high risk for TBI, including active-duty military personnel and athletes.

The second quality may make it ideal to implement for pre-hospital care at the scene of an injury or during transport. The current study includes translationally relevant measures of outcome, focusing on cognitive, motor, and affective dysfunction to detect both acute and chronic morbidities following diffuse TBI in the mouse. Additionally, we propose to explore the mechanisms of RIC action through discovery proteomics. Currently, the mechanism(s) through which RIC imparts protection is unclear (Tapuria, Kumar et al. 2008). Conflicting reports suggest that RIC may work through nitric oxide (Kuntscher, Kastell et al. 2003), opioid receptors (Addison, Neligan et al. 2003), potassium ATP channels (Xia, Herijgers et al. 2003), inflammatory cytokines (Harkin, Barros D'Sa et al. 2002), or free radicals (Chen, Wu et al. 2005). A vital first step in understanding RIC mechanism(s) is the unbiased identification of circulating plasma protein correlates of RIC. In an open-ended hypothesis-generating approach, here we use mass spectrometry to perform untargeted proteomic analysis of plasma from RIC-treated mice. Protein and pathway candidates identified as enriched by RIC will allow for follow-up hypotheses regarding the mechanistic underpinnings of the therapy. These hypotheses are anticipated to be testable using positive and/or negative genetic manipulation of identified proteins to understand their role in RIC efficacy. Ultimately, RIC could serve as a cost-effective and feasible therapy to restore function after TBI and other acute neurological conditions.

5.3 METHODS

5.3.1 Animals

Male C57BL/6 mice (Harlan Laboratories, Inc., Indianapolis, IN) were used for behavioral and mass spectrometry experiments (n=47). Mice were housed in a 12 h

light/12h dark cycle at a constant temperature (23°C ± 2° C) with food and water available *ad libitum* according to the Association for Assessment and Accreditation of Laboratory Animal Care International. All mice used in this study were group housed. Mice were acclimated to their environment following shipment for at least three days prior to any experiments. After surgery, mice were evaluated daily during post-operative care by a physical examination and documentation of each animal's condition including weight. Animal care was approved by the University of Arizona Institutional Animal Care and Use Committee.

5.3.2 Midline Fluid Percussion Injury

Mice (20-24g) were subjected to midline fluid percussion injury (mFPI) consistent with methods previously described (Lifshitz 2008; Harrison, Rowe et al. 2014; Rowe, Harrison et al. 2014; Rowe, Harrison et al. 2014; Rowe, Striz et al. 2014). Group sizes are indicated in the results section and figure legends for individual studies. Mice were anesthetized using 5% isoflurane in 100% oxygen for five minutes and the head of the mouse was placed in a stereotaxic frame with continuously delivered isoflurane at 2.5% via nosecone. While anesthetized, body temperature was maintained using a Deltaphase® isothermal heating pad (Braintree Scientific Inc., Braintree, MA). A midline incision was made exposing bregma and lambda, and fascia was removed from the surface of the skull. A trephine (3 mm outer diameter) was used for the craniotomy, centered on the sagittal suture between bregma and lambda without disruption of the dura. An injury cap prepared from the female portion of a Luer-Loc needle hub was fixed over the craniotomy using cyanoacrylate gel and methyl-methacrylate (Hygenic Corp.,

Akron, OH). The incision was sutured at the anterior and posterior edges and topical Lidocaine ointment was applied. The injury hub was closed using a Luer-Loc cap and mice were placed in a heated recovery cage and monitored until ambulatory before being returned to their home cage.

For injury induction 24 hours post-surgery, mice were re-anesthetized with 5% isoflurane delivered for five minutes. The cap was removed from the injury-hub assembly and the dura was visually inspected through the hub to make sure it was intact with no debris. The hub was then filled with normal saline and attached to an extension tube connected to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). An injury of moderate severity for our injury model (1.4 atm) was administered by releasing the pendulum onto the fluid-filled cylinder. Sham-injured mice underwent the same procedure except the pendulum was not released. Mice were monitored for the presence of a forearm fencing response and righting reflex times were recorded for the injured mice as indicators of injury severity (Hosseini and Lifshitz 2009). The righting reflex time is the total time from the initial impact until the mouse spontaneously rights itself from a supine position. The fencing response is a tonic posturing characterized by extension and flexion of opposite arms that has been validated as an overt indicator of injury severity (Hosseini and Lifshitz 2009). The injury hub was removed and the brain was inspected for uniform herniation and integrity of the dura. The dura was intact in all mice; none were excluded as technical failures. The incision was cleaned using saline and closed using sutures. Moderate brain-injured mice had righting reflex recovery times greater than six minutes

and a positive fencing response. Sham injured mice recovered a righting reflex within 20 seconds. After spontaneously righting, mice were placed in a heated recovery cage and monitored until ambulatory (approximately 5 to 15 minutes) before being returned to their home cage. Adequate measures were taken to minimize pain or discomfort.

5.3.3 Remote Ischemic Conditioning

Remote ischemic conditioning was administered 15 to 30 minutes following recovery of righting reflex after mFPI. Anesthesia was induced using 5% isoflurane in 100% oxygen for three minutes. Mice were placed in a supine position and light anesthesia was maintained throughout the RIC procedure using 1.5% isoflurane in 100% oxygen via nosecone. RIC was applied using a disposable plastic coated wire tie (100mm L x 2mm diameter). With the left mouse hindlimb extended at the knee, a wire tie was wrapped around the hindlimb as proximally to the hip as possible. Pressure was applied by twisting the wire tie approximately four times clockwise. RIC was administered in four cycles composed of 5 minutes RIC and 5 minutes reperfusion. Mice were monitored while recovering from anesthesia before being returned to their home cage. Non-RIC treated sham and TBI groups received equal anesthesia treatment without wire tie application. For additional information on the development and further refinement of non-invasive rodent RIC procedures, see Appendix B.

5.3.4 Behavioral Testing

Mice were tested for sensorimotor function using the rotarod and neurological severity score at 1 and 7 days post-injury. Cognitive and affective function were assessed

using novel object recognition, open field test, elevated plus maze, and forced swim test at 14 days post-injury.

Rotarod: Sensorimotor function was assessed using the Economex Rotarod system from Columbus Instruments (Columbus, OH). Mice were pre-trained for three consecutive days prior to injury: day one included habituation to a non-accelerating rod at 5RPM while days two and three of training were conducted as normal test days with day 3 scores used as baseline performance. For the test (1 and 7 days post-injury), mice were placed on the rod with a starting speed of 5 RPM, and rod rotation speed was continuously increased by 0.2 RPM per second. The trial concluded when the mouse fell from the rod. Three trials were performed at each time point. Data are presented as latency to fall in seconds (average of two highest latencies from a single time point).

Neurological Severity Score (NSS): Post-traumatic neurological impairments were assessed using an 8-point NSS paradigm adapted from those previously used in experimental models of TBI (Chen, Constantini et al. 1996; Semple, Bye et al. 2010; Pleasant, Carlson et al. 2011; Ziebell, Bye et al. 2011). One point was given for failure on an individual task, and no points were given if a mouse completed a task successfully. Mice were observed for hind limb flexion, startle reflex, and seeking behavior (presence of these behaviors was considered successful task completion). Mice traversed in sequence, 3, 2, and 1 centimeter beams. The beams were elevated and mice were given 1 minute to travel 30 centimeters on the beams. The task was scored as a success if the mouse traveled 30 centimeters with normal forelimb and hindlimb position (forelimb/hindlimb did not fall from the beam). Mice were also required to balance on a

0.5 centimeter beam and a 0.5 centimeter round rod for 3 seconds in a stationary position with front paws between hind paws. Non-parametric data are presented as a composite score ranging from 0 to 8 representing performance on all tasks combined. High final NSS scores were indicative of task failure and interpreted as neurological impairment.

Novel Object Recognition (NOR): Cognitive impairment was tested using the NOR test as previously published (Ennaceur and Aggleton 1997; Han, Tong et al. 2011; Rowe, Harrison et al. 2014). The test consisted of three phases: habituation, training, and testing. On day 6 post-injury, mice were placed in an open field (40 cm x 40 cm x 30 cm, L x W x H) for fifteen minutes of habituation. Mice were removed and two identical objects were placed in opposing quadrants of the field for the training phase. Mice were returned to the center of the open field and given 5 minutes to explore the objects. Following training, mice were returned to their home cages. Testing began 4 hours after training. One familiar object was placed in an original location and one novel object was placed in the opposing quadrant of the open field. Mice were placed into the center and given 5 minutes to explore. Exploration behaviors were scored during testing using Noldus EthoVision XT (Noldus Information Technology Inc., Leesburg, VA). Custom zones approximately 2cm larger than each object were placed and exploration of an object was defined as the duration the mouse nose point was detected in this zone. Testing data are displayed as a discrimination index (DI) in which $DI = (T_{novel} - T_{familiar})/(T_{novel} + T_{familiar})$. A positive DI indicates greater exploration time of the novel object compared to the familiar object.

Open field test: The open field task was used to assess anxiety-like behavior. At 14 days after injury, mice were placed in the center of an open field (40cm x 40cm x 30cm, L x W x H) and allowed to explore freely for 5 min. Their movement was tracked by an overhead camera and the distance traveled, time spent in the center of the arena (20 × 20 cm), and number of entries into the center were calculated using EthoVision XT (Noldus Information Technology Inc., Leesburg, Va., USA).

Forced swim test: Depression-like behavior was assessed using the forced swim task. Mice were placed into glass cylinders (20cm x 15cm, L x Diameter) filled with water (25°C) for 6 min. The first minute was excluded from analysis as an acclimation phase. Mice were recorded using an overhead camera. Videos were scored by an individual blinded to treatment groups for time spent mobile vs immobile.

Elevated plus maze: Anxiety-like behavior was assessed using the elevated plus maze. Mice were placed into the center of a custom elevated plus maze comprised of two enclosed arms (25cm high walls) and two open arms of 8cm width meeting at a neutral zone (8cm x 8cm, L x W). Mice were recorded using an overhead camera while exploring the maze for 5 minutes. Noldus EthoVision XT (Noldus Information Technology Inc., Leesburg, Va., USA) was used to track the cumulative duration mice spent in closed and open arms.

5.3.5 Untargeted Proteomic Analysis

Blood was collected from the same cohort of mice from behavioral studies via cheek bleed 24 hours following the administration of RIC (and TBI or sham). Blood was collected into EDTA-coated microcentrifuge tubes and immediately stored on ice. Plasma

was separated by centrifugation at 3,000 x g for 10 minutes and immediately stored at -80C until use. High abundance proteins were removed from plasma samples using Seppro Mouse M7 spin columns (Sigma-Aldrich, St. Louis, Missouri) to increase resolution of low abundance proteins of interest. A buffer exchange was conducted to remove unwanted buffers from the Seppro column prior to digestion. Protein concentration was quantified using Pierce BCA (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein were brought into in-solution digestion, starting with urea to denature the proteins. Dithiothreitol (DTT) was added to reduce the proteins, followed by iodoacetemide to alkylate the proteins. Trypsin Gold was used to digest the proteins into peptides (Promega Corporation, Madison, WI). Samples were desalted to remove digestion agents and remaining salts. Protein concentration was quantified again by BCA and all injections were aligned to 1ug of peptides.

LC-MS/MS data were acquired on a tribrid quadrupole-ion trap-Orbitrap instrument (Orbitrap Fusion Lumos, Thermo, San Jose, CA) interfaced with a nanoAcquity UPLC system (Waters, Millford-MA). Samples were first loaded on a trapping column (Acclaim PepMap 100 C18, 75 µm ID * 2 cm, 3 µm particle size, 100 Å pore size) and washed for 10 minutes with 99.5% Solvent A (0.1% formic acid in water) and 0.5% Solvent B (0.1% formic acid in acetonitrile) at a flow rate of 4 µl/min. The trapped peptides were transferred to an analytical column (PepMap RSLC C18, 50 µm ID * 15 cm, 2 µm particle size, 100 Å pore size) and eluted at a flow rate of 300 nl/min using the following gradient: 3% to 7% B in 1 minute, 7% to 25% B in 72 minutes, 25% B to 45% B in 10 minutes, 45% to 90% B in 0.5 minutes, 90% B for 1 minute, 3% B in

0.5 minute and re-equilibration for 10 minutes. Data-dependent acquisition was performed in Top Speed mode with a duty cycle of 3 seconds and following parameters: spray voltage of 2100V, ion transfer tube temperature of 275 °C, survey scan in the Orbitrap at a resolution of 120K at 200 m/z, scan range of 400-1500 m/z, AGC target of 2E5 and maximum ion injection time of 50 ms. Every parent scan was followed by a daughter scan using High Energy Collision (HCD) dissociation of top abundant peaks and detection in the iontrap with the following settings: quadrupole isolation mode, isolation window at 1.4 m/z, AGC target of 5E3 with maximum ion injection time of 35 ms and HCD collision energy of 35%. Dynamic exclusion was set to 60 seconds. Twelve standard controls of *E. coli* proteome analysis were conducted in which no anomalous variation was detected (data not shown). Peptide spectrum peaks were used to calculate area under the curve for relative abundance comparisons. Protein identification and relative quantification was conducted with Proteome Discoverer v2.1 (Thermo Fisher Scientific, Waltham, MA) and Mascot v2.3 (Matrix Science, Boston, MA) using the Mouse SwissProt 2015 database.

5.3.5 Statistical Analysis

Data shown were analyzed using GraphPad Prism 6, with statistical significance assigned when $p<0.05$, unless otherwise indicated. For each behavioral test, shams receiving each treatment were statistically compared. No differences were detected between sham groups (RIC and no-RIC). To maintain comparable group sizes for these studies, a pooled sham group was constructed by combining all mice from each group. Differences in open field, elevated plus maze, and novel object recognition were

determined with a one-way ANOVA followed by Dunnett's multiple comparisons test. Differences in rotarod performance following TBI over the first week post-injury were determined with a repeated measure two-way ANOVA followed by Dunnett's multiple comparisons test. Differences in neurological severity scores were determined at each testing time point by Kruskal-Wallis test. Differences in relative abundance (area under the curve) of proteins identified by mass spectrometry were determined between groups by Mann-Whitney U test.

5.4 RESULTS

5.4.1 Diffuse TBI resulted in acute sensorimotor impairment regardless of RIC treatment

Sensorimotor function was assessed at days 1 and 7 post-injury using the Neurological Severity Score (NSS) and rotarod tests (Figure 5.1). At 1 day post-injury, there was a significant main effect of treatment on the NSS ($KW(3,39)=13.91$, $p=0.001$) with post-hoc analysis indicating significantly higher NSS scores among TBI and TBI+RIC groups when compared to sham ($p<0.05$). While the main effect of treatment remained significant at 7 days post-injury in the NSS ($KW(3,39)=8.477$), post-hoc analysis indicated that neither TBI nor TBI+RIC were individually significantly higher than sham ($p>0.05$). Similarly, the rotarod test showed an overall effect of treatment group on latency ($F(2,35)=3.314$, $p=0.0481$) with specific effects indicating latencies from both TBI and TBI+RIC groups were lower than sham at 1 day post-injury ($p<0.05$). These specific effects dissipated by 7 days post-injury when both groups returned to sham level ($p<0.05$).

5.4.2 Diffuse TBI did not result in cognitive or affective impairments at 14 days post-injury

To test for potential therapeutic effects of RIC, mice were subjected to a battery of cognitive and affective behavioral tests at 14 days post-injury (Figure 5.2). All groups spent more time exploring novel rather than familiar objects with no effect of TBI or RIC treatment ($F(2,35)=0.7944$, $p=0.460$). No anxiety-like behaviors were detected among brain-injured groups compared to sham as measured by time in the center of the open field test ($F(2,35)=0.4097$, $p=0.667$) or open arms of the elevated plus maze ($F(2,35)=0.9961$, $p=0.905$). Demonstrating no TBI-induced depression-like behaviors, all groups performed similarly in time spent immobile during the forced swim test ($F(2,35)=0.1634$, $p=0.850$).

5.4.3 Mass spectrometry identified candidate protein correlates of RIC in plasma

Untargeted proteomic analysis was performed by mass spectrometry of plasma collected 24 hours following RIC administration. 565 unique proteins were identified among all samples with an average of 227 unique proteins identified per sample. Four analyses were conducted to identify proteins which were most increased or decreased in response to RIC among sham and brain-injured mice. For each of these comparisons, the ten proteins with the greatest absolute value fold-change were identified along with proteins identified exclusively in one comparison group. For sham mice, these data are presented for proteins which were increased (Table 5.1) and decreased (Table 5.2) among RIC treated mice. For brain-injured mice, these data are presented for proteins which were increased (Table 5.3) and decreased (Table 5.4) among RIC treated mice.

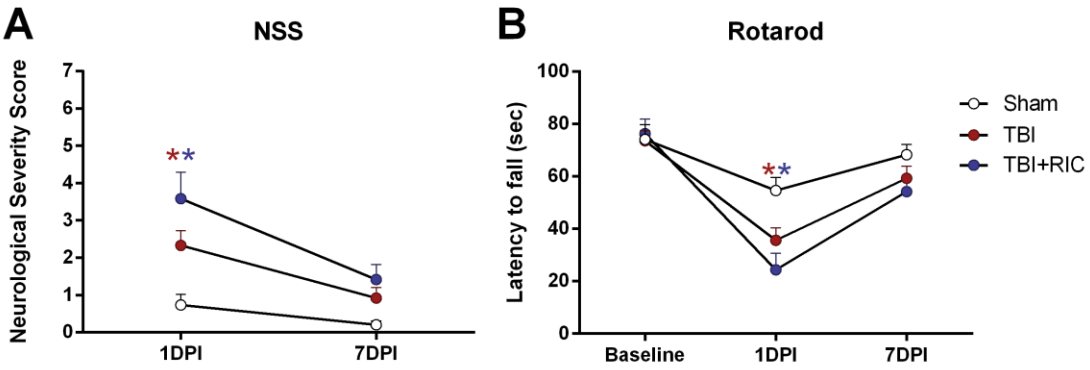

Figure 5.1. Diffuse TBI resulted in acute sensorimotor deficits regardless of RIC treatment.

The Neurological Severity Score (NSS) indicated sensorimotor impairment among both TBI and TBI+RIC groups compared to sham at 1 day post-injury which resolved by 7 days post-injury (A). Similarly, the rotarod detected acute motor deficits among TBI and TBI+RIC groups which resolved by 7 days post-injury regardless of treatment (B). (Sham n=14, TBI n=12, TBI+RIC n=12)

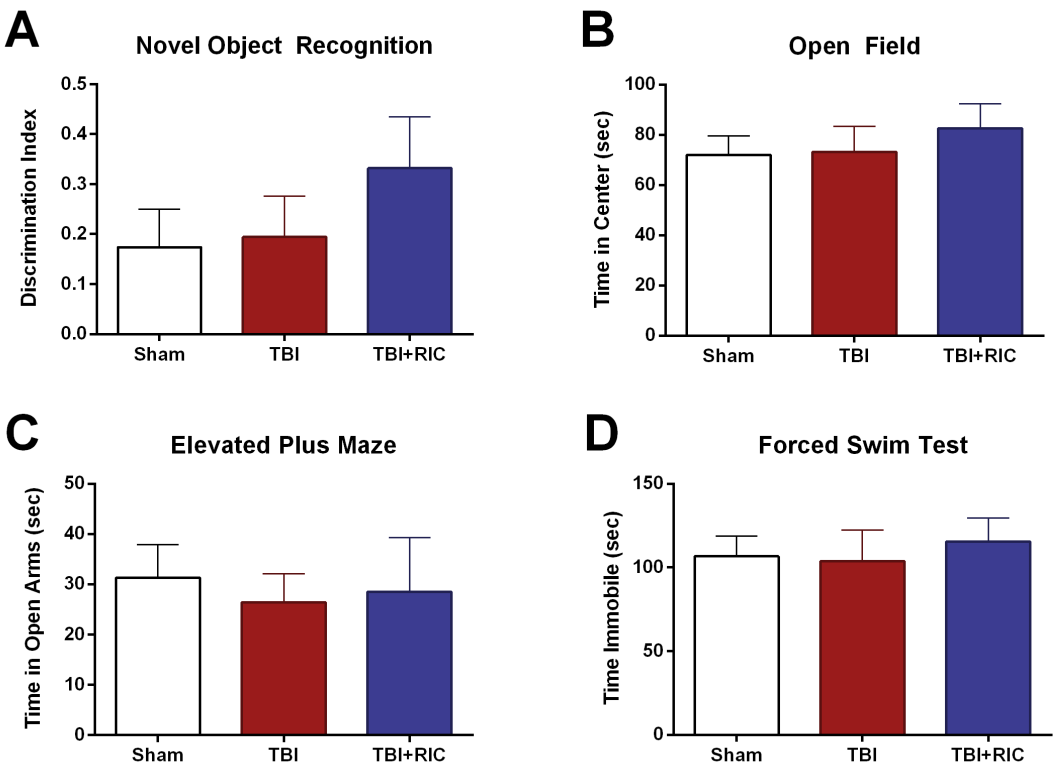

Figure 5.2. Diffuse TBI did not result in cognitive or affective impairment at 14 days-post injury.

All groups of mice investigated novel objects more than familiar objects presented in the novel object recognition (NOR) task, indicating no cognitive impairment (A). No TBI- or RIC-induced changes in anxiety-like behavior were detected in the open field test (B) or elevated plus maze (C). No TBI- or RIC-induced changes in depression-like behaviors were indicated by the forced swim test (D). (Sham n=14, TBI n=12, TBI+RIC n=12)

A Mann-Whitney U test indicated that RIC resulted in increased selenium-binding protein 1 (Fold-change=10.7, U=13.50, p=0.0256) and regenerating islet-derived protein 3-gamma (Fold-change=3.5, U=12.00, p=0.0348) among sham mice. Keratin, type II cytoskeletal 5, was identified as significantly decreased following RIC among sham mice (Fold-change=-63.8, U=9.00, p=0.0070). In the context of TBI, mice treated with RIC demonstrated significantly higher levels of selenium-binding protein 1 (fold-change=90.2, U=27.50, p=0.0072), adipocyte fatty acid binding protein (Fold-change=60.1, U=27.50, p=0.0072), and bisphosphoglycerate mutase (Fold-change=26.1, U=38.50, p=0.0363).

Two proteins were identified as being particularly responsive to RIC regardless of TBI. Selenium-binding protein 1 and adipocyte fatty acid-binding protein were each identified in more than 50% of RIC plasma samples (Figure 5.3). Fatty acid-binding protein was identified in 71.4% of total RIC samples (Sham+RIC and TBI+RIC) and only 21.7% of non-RIC samples (Naïve, Sham, and TBI). Similarly, selenium-binding protein 1 was identified in 61.9% of total RIC samples but only 13.0% of non-RIC samples. Significant overall group effects were detected among selenium-binding protein 1 (KW(5,44)=19.44, p=0.0007) and fatty-acid binding protein (KW(5,44)=12.06, p=0.0025).

Table 5.1. Proteins increased in Sham+RIC vs Sham groups.

Protein	Fold Change	p-value
Uteroglobin	12.8	0.1406
Hyaluronan-binding protein 2	12.7	0.7128
Selenium-binding protein 1	**10.7**	**0.0256**
Ig gamma-2B chain C region	10.7	0.7128
Fructose-1,6-bisphosphatase 1	6.4	0.4667
Phosphoglycerate mutase 1	5.4	0.7128
Sorbitol dehydrogenase	4.8	> 0.9999
BPI fold-containing family A member 2	4.7	0.5692
Proteasome subunit beta type-1	**4.7**	**0.0350**
Regenerating islet-derived protein 3-gamma	**3.5**	**0.0348**
Identified only in Sham+RIC	**% Samples**	
Afamin	100.0%	
Proteoglycan 4	50.0%	
Neuroplastin	37.5%	
Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	37.5%	

The top ten proteins most increased in relative abundance among Sham+RIC treated mice in comparison to Sham mice are displayed with respective fold increases. Four proteins were identified in Sham+RIC mice but not Sham mice. Resulting statistical p-values from the Mann-Whitney U test are presented.

Table 5.2. Proteins decreased in Sham+RIC vs Sham groups.

Protein	Fold Change	p-value
Keratin, type II cytoskeletal 5	**-63.8**	**0.0070**
Cathepsin Z	**-59.1**	**0.0256**
Dynein heavy chain 12, axonemal	-13.7	0.7333
Proteasome subunit alpha type-2	-13.2	0.4667
Keratin, type II cytoskeletal 2 oral	-4.8	0.4517
Keratin, type II cytoskeletal 79	-4.1	0.3538
Neuropilin-1	-3.5	0.2000
Ig heavy chain V region M167	-3.4	0.4126
Insulin-like growth factor-binding protein 4	-3.3	0.1515
Protein S100-A8	-3.1	0.3231

Identified only in Sham (no RIC)	% Samples
Isoform 3 of Afamin	100.0%
Isoform C of Proteoglycan 4	75.0%
Platelet-activating factor acetylhydrolase	50.0%
Cytoskeleton-associated protein 2	50.0%
Ig gamma-1 chain C region, membrane-bound form	50.0%
RUN domain-containing protein 3A	37.5%
Transcription factor IIIB 50 kDa subunit	50.0%
Thrombospondin-1	37.5%
Vigilin	37.5%
Corepressor interacting with RBPJ 1	37.5%
Myosin-1	37.5%
Adhesion G protein-coupled receptor F5	37.5%

The top ten proteins most decreased in relative abundance among Sham+RIC treated mice in comparison to Sham mice are displayed with respective fold decreases. Twelve proteins were identified in Sham mice but not Sham+RIC mice. Resulting statistical p-values from the Mann-Whitney U test are presented.

Table 5.3. Proteins increased in TBI+RIC vs TBI groups.

Protein	Fold Change	p-value
Selenium-binding protein 1	**90.2**	**0.0072**
Cofilin-1	77.2	0.1694
Alpha-enolase	69.7	0.0728
Fatty acid-binding protein, adipocyte	**60.1**	**0.0072**
Phosphoglycerate kinase 1	41.7	0.7064
Alpha-synuclein	31.7	0.3614
Proteasome subunit beta type-3	29.0	0.7064
S-formylglutathione hydrolase	29.0	0.3614
Bisphosphoglycerate mutase	**26.1**	**0.0363**
Peroxiredoxin-1	15.2	0.6730
Identified only in TBI+RIC	**% Samples**	
Isoform 2 of Ig mu chain C region	100.0%	
Isoform C of Proteoglycan 4	84.6%	
Actin, alpha cardiac muscle 1	76.9%	
Protein S100-A8	38.5%	
Neuroplastin	30.8%	
Peroxiredoxin-6	30.8%	
Coagulation factor XI	30.8%	
Protein CDV3	30.8%	
Parvalbumin alpha	30.8%	
Osteopontin	23.1%	
Transcobalamin-2	23.1%	
Acyl-CoA-binding protein	23.1%	
Phosphoglycerate mutase 1	23.1%	
Napsin-A	23.1%	
Beta-enolase	23.1%	
Isoform 2 of Adenylosuccinate synthetase isozyme 1	23.1%	
Stress-induced-phosphoprotein 1	23.1%	
Thymosin beta-4	23.1%	
Chitinase-like protein 3	23.1%	
Vasopressin-neurophysin 2-copeptin	23.1%	
Vasorin	23.1%	

The top ten proteins most increased in relative abundance among TBI+RIC treated mice in comparison to TBI mice are displayed with respective fold increases. Twenty one proteins were identified in TBI+RIC mice but not TBI mice. Resulting statistical p-values from the Mann-Whitney U test are presented.

Table 5.4. Proteins decreased in TBI+RIC vs TBI groups.

Protein	Fold Change	p-value
Keratin, type II cytoskeletal 5	**-321.1**	**0.0005**
Ig heavy chain V region M167	-38.6	0.0715
Platelet-activating factor acetylhydrolase	-26.2	0.1925
Keratin, type II cytoskeletal 2 oral	-21.0	0.0715
Serine protease inhibitor Kazal-type 3	-18.2	> 0.9999
Vitamin K-dependent protein Z	-11.3	0.2103
BPI fold-containing family A member 2	-10.1	0.9618
Flavin reductase (NADPH)	-8.7	0.4495
Hyaluronan-binding protein 2	-8.4	0.9108
Complement C1q subcomponent subunit A	**-8.2**	**0.0407**
Identified only in TBI (no RIC)	**% Samples**	
Ig mu chain C region	100.0%	
Proteoglycan 4	90.0%	
Actin, alpha skeletal muscle	80.0%	
Corepressor interacting with RBPJ 1	60.0%	
Transcription factor IIIB 50 kDa subunit	60.0%	

The top ten proteins most decreased in relative abundance among TBI+RIC treated mice in comparison to TBI mice are displayed with respective fold decreases. Five proteins were identified in TBI mice but not TBI+RIC mice. Resulting statistical p-values from the Mann-Whitney U test are presented.

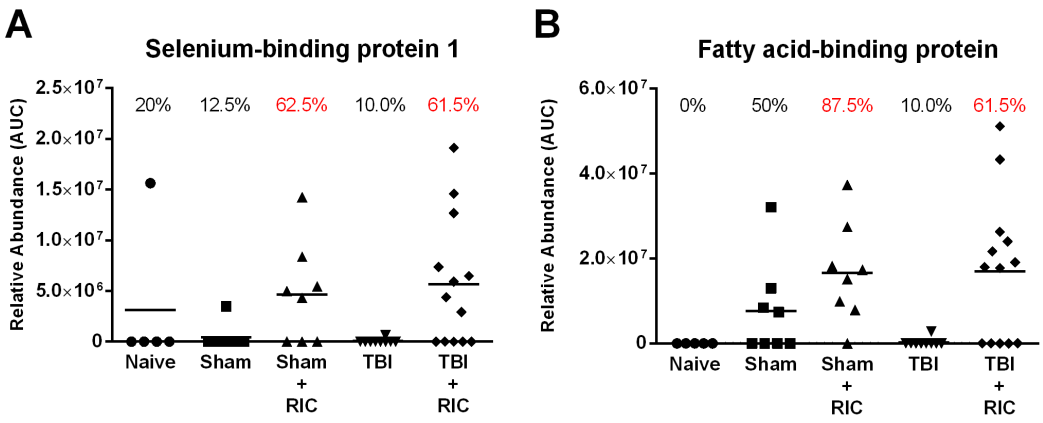

Figure 5.3. Selenium-binding protein 1 and fatty-acid binding protein are selectively responsive to RIC.

Relative abundance of selenium-binding protein 1 (A) and fatty-acid binding protein (B) are indicated for all samples. Percentage of samples in which the protein of interest was identified is displayed with those of RIC-treated groups shown in red. (Naïve n=5, Sham n=8, Sham+RIC n=8, TBI n=10, TBI+RIC n=13)

5.5 DISCUSSION

The current study was designed with two aims: (1) assess the therapeutic potential of RIC in a mouse model of diffuse TBI and (2) identify molecular correlates of RIC. RIC did not prevent very acute sensorimotor impairments resulting from diffuse TBI. The lack of TBI-induced cognitive or affective deficits at the selected time points precluded assessment of RIC efficacy in these domains. Despite the lack of positive proof of RIC efficacy in improving behavioral outcome in this model of TBI, the body of literature supporting RIC efficacy in multiple domains warranted further study of RIC mechanism. Untargeted proteomic analysis identified multiple proteins differentially impacted by TBI and/or RIC, including promising candidate proteins which responded selectively to RIC regardless of TBI. Overall, these findings lay a vital groundwork for advancing understandings of RIC mechanism by starting with an objective and hypothesis-generating analytical chemistry approach.

The findings of acute sensorimotor dysfunction from the current study reflect previous findings from our group (Harrison, Rowe et al. 2014; Harrison, Rowe et al. 2015). Though previous findings from our group have demonstrated cognitive impairment resulting from diffuse TBI in the mouse (Harrison, Rowe et al. 2015), these studies were conducted at earlier time points within one week of injury as opposed to two weeks in the current study. The later time point was selected for the current study to assess more translationally relevant long-term outcome. Few studies have addressed the effect of RIC on functional outcome from brain injury. A recent study of closed head injury in mice indicated that RIC prevented acute motor deficits in the rotarod and acute

cognitive deficits in the NOR test (Sandweiss, Azim et al. 2017). One important limitation of this study was the exclusion of any non-brain-injured controls for comparison. Differences in findings from the current study could also be due to differences in the time course of cognitive impairment assessment, with the aforementioned study testing only within the first week post-injury. A study using the bilateral common carotid artery occlusion (BCCAO) model of cerebral ischemia/reperfusion injury indicated no effect of RIC on spontaneous motor activity but a protective effect of RIC on associative learning (Wang, Zhang et al. 2017). Differences in injury models, time of behavioral testing, and timing and method of RIC application make comparisons among the few functional studies of RIC after brain injury difficult and warrants additional study.

Selenium-binding protein 1 was identified as significantly increased among RIC-treated mice regardless of TBI. Selenium-binding protein 1 has not previously been studied specifically in the context of either RIC or TBI. There are numerous studies demonstrating increased levels of selenium-binding protein 1 among patients with schizophrenia (Kanazawa, Chana et al. 2008; Udawela, Money et al. 2015) which could indicate either its involvement in brain disease or potential for use as a neuropsychiatric biomarker. Selenium-binding protein 1 knockout mice have shown no gross phenotypic changes but levels of tumor-related genes, including Notch1, were increased in its absence (Tsujimoto, Ishida et al. 2013). A recent study demonstrated that notch pathway activation may be neuroprotective following TBI (Wang, Zhang et al. 2015), a finding which would suggest that a RIC-induced increase selenium-binding protein 1 may not be

a major neuroprotective mechanism. Dietary supplementation with elemental selenium has shown to attenuate impairments in functional outcome and mitochondrial damage following controlled cortical impact TBI in rats (Crowdus Meyer 2015). Further investigation is warranted to elucidate whether RIC-induced increase in selenium-binding protein 1 could act synergistically with dietary selenium supplementation after TBI.

Adipocyte fatty acid binding protein (fatty acid binding protein 4, fabp4), currently shown as selectively increased by RIC in the context of TBI, has not been studied in either RIC or TBI. Other members of the fatty acid binding protein family, particularly fatty acid binding protein 5, have been demonstrated to mediate the blood-brain-barrier transport of DHA (Pan, Scanlon et al. 2015; Figueroa, Serrano-Illan et al. 2016). Further, administration of small interfering RNA to silence fatty acid binding protein 5 was shown to suppress the neuroprotective effect of DHA on outcome from experimental spinal cord injury in rats (Figueroa, Serrano-Illan et al. 2016). It is plausible that RIC could play a role in elevating fatty acid binding proteins which would facilitate DHA uptake in the brain and further improve the efficacy of omega-3 fatty acid supplementation demonstrated in Chapter 3.

The current study includes the first untargeted proteomic analysis of RIC plasma to date. While there have been many investigations of potential RIC mechanisms, they have largely focused on targeted analysis of molecules of interest using traditional molecular biology approaches (for review, see (Tapuria, Kumar et al. 2008)). These approaches can provide valuable insight and have resulted in a wide array of proposed mechanistic pathways, but are subject to experimenter bias in the determination of targets

of interest. Untargeted proteomic analysis is not subject to this bias and allows for objective identification of RIC-driven changes in the plasma proteome. It is plausible that RIC efficacy is not entirely dependent upon the proteome, though, and accordingly a recent untargeted metabolomic study identified α-hydroxybutyrate as a potential cardioprotective factor resulting from RIC (Laursen, Hansen et al. 2017). A general cellular pathway and secondary injury mechanism currently identified as impacted by RIC and TBI is inflammation. Studies of RIC in models of sepsis (Turner, Brabb et al. 2011; Calder 2017) have similarly indicated potential anti-inflammatory effects. Studies, including those from our group, have also indicated beneficial effects of reducing neuroinflammation following TBI (Harrison, Rowe et al. 2015). Future confirmation of candidate protein correlates of RIC including selenium-binding protein 1 and fatty acid-binding protein by traditional molecular biology approaches will lay the groundwork for this new avenue of RIC mechanism exploration.

5.6 CONCLUSIONS

Despite inconclusive findings on the neuroprotective effects of RIC in the currently tested model of diffuse TBI, we were able to identify preliminary molecular correlates of RIC in the context of TBI. As the first study of its kind to objectively assess RIC-driven changes in the plasma proteome, the current study provides a broad framework within which potential mechanisms of RIC efficacy can begin to be placed. With top matches of proteins impacted by RIC being associated with previously proposed pathways of inflammation and oxidative stress responses, these findings do not stand alone but rather complement our cumulative understanding of this therapeutic approach.

It is unlikely that any one protein or molecule drives the neuro-, cardio-, and otherwise protective effects of RIC. Ongoing analyses on the currently presented data set will be aimed at narrowing down pathways of cellular processes which promote these protective effects. At the culmination of this hypothesis-generating approach, fatty acid binding proteins appear to be a promising avenue of RIC mechanism which could facilitate the transport of vital fatty acids across the blood-brain-barrier following TBI. A preliminary study is warranted to test whether RIC increases brain DHA content after acute DHA supplementation. Parallel studies could be conducted in available fatty acid binding protein knockout mice (Furuhashi and Hotamisligil 2008) to detect whether these molecules are required for RIC facilitation of DHA uptake. Finally, the role of fatty acid binding proteins in RIC efficacy could be determined by conducting a RIC+TBI study in fatty acid binding protein knockout mice. If this family of molecules is necessary for RIC efficacy, any neuroprotective effects or positive influence on functional outcome would be nullified in the transgenic mice.

5.7 ACKNOWLEDGMENTS

I would like to thank all collaborators on this project: Rachel Rowe for her contribution to study design, experiment execution, and data interpretation; Jonathan Lifshitz and Patrick Pirrotte for their contribution to study design, data interpretation, and chapter preparation; Ritin Sharma, Marissa Saltzman, and Krystine Garcia Mansfield for their contributions to experiment execution and data interpretation.

CHAPTER 6

CONCLUSIONS

Practical therapies are needed for the millions of TBI sufferers who would not otherwise seek treatment. These at-home or on-the-field interventions require special consideration for convenience and safety such that an individual unsure of whether to seek medical attention would feel comfortable self-administering a practical therapy as a safeguard. These therapeutic approaches do not replace professional medical care, but hopefully can be used in conjunction with clinical recommendations to improve public health. A score card for evaluation of practical therapies at a glance has been developed and applied to the treatments from each chapter of this dissertation (Table 6.1).

The current body of work assessed four classes of potential practical therapies for diffuse TBI. The first of these, OTC analgesics, showed little difference in outcome from TBI in mice (Table 6.2). This negative result, however, was complemented by the finding that acute administration of ibuprofen and acetaminophen did not adversely affect outcome. This is an important result since these pain relievers are largely taken to treat the acute symptoms of diffuse TBI, such as headache. While OTC analgesics did not show much promise as a practical therapy, results suggest that these common medications do not exacerbate ongoing secondary injury processes when taken acutely for pain relief.

The present study of omega-3 fatty acids detailed in Chapter 3 is the first of its kind to assess a comprehensive timeline of supplementation prior to and following experimental TBI (Table 6.3). Results showed that dietary supplementation with DHA either prior to or

following diffuse TBI decreased post-acute inflammatory responses and decreased injury-induced sensory sensitivity. The unique study design also indicated that DHA administered in the weeks immediately following TBI provided more potent anti-inflammatory effects than that taken prior to injury. This was an unexpected result considering the relatively long time required for DHA to incorporate into cell membranes, but it indicated further promise of DHA as a practical therapy since the window for effective treatment relative to injury appears to encompass both pre-injury and post-injury supplementation.

Treatment with omega-3 derived resolvins E1 (RvE1) and AT-D1 (AT-RvD1) were assessed for their potential to circumvent the lengthy cellular uptake phase required by fatty acid supplementation (Table 6.4). Each resolvin tested demonstrated quite different effects on outcome. RvE1 increased post-injury sleep and attenuated microglial activation in response to diffuse TBI. RvD1 did not affect these two outcome measures, but significantly reduced TBI-induced motor and cognitive impairments. These results indicate that RvD1 may be a candidate practical therapy with the caveat of very limited availability to the public. Though RvE1 did not improve functional outcome, findings suggest that it did attenuate the inflammatory response; further studies are warranted to understand how the impact of RvE1 on secondary injury may be harnessed to a therapeutic effect.

Table 6.1. Practical Therapy Score Card Template

Practical Therapy Score Card	
Safety	
Availability	
Ease of administration	
Cost effectiveness	
Currently tested efficacy	
e.g. Motor function	
e.g. Affective function	

Symbol	**Description**
~	Inconclusive or unknown
-	Negative effect or quality
0	No effect
+	Positive effect or quality
++	Very positive effect or quality

Treatment with RIC demonstrated little effect on functional outcome from diffuse TBI in mice (Table 6.5). Despite this finding in the current model of TBI, RIC has shown great promise of neuroprotection in models of stroke and focal TBI. For this reason, mass spectrometric analysis of plasma samples from RIC-treated mice was used to identify candidate molecular correlates of RIC. Identification of these candidates represents a vital first step in understanding the neuroprotective mechanisms underlying RIC.

Practical therapies examined in this dissertation were administered in differing protocols: OTC pain relievers once (i.p.) after injury, DHA orally either before or after injury, resolvins (i.p.) both before and after injury, and RIC after injury. While these differing approaches make it difficult to draw one-to-one comparisons between treatments, they also mirror the diversity of treatment regimens in the clinical case. Routes and timing of administration were determined based both on previous literature available and translational considerations for when and how these therapies would be most likely used in the clinical case. Conclusions of efficacy are based on the currently utilized administration regimens and it is plausible that changing route of administration or timing could alter outcomes in any tested approach. When oral administration was indicated in humans but not feasible in rodents, intraperitoneal injection was chosen for its consistency, slower uptake, and liver metabolism similar to oral administration (Turner, Brabb et al. 2011).

Table 6.2. Practical Therapy Score Card: OTC analgesics

Practical Therapy Score Card: OTC analgesics	
Safety	+
Availability	++
Ease of administration	++
Cost effectiveness	++
Currently tested efficacy	
Motor function	0
Inflammatory cytokines	0

Table 6.3. Practical Therapy Score Card: Dietary DHA

Practical Therapy Score Card: Dietary DHA	
Safety	++
Availability	++
Ease of administration	+
Cost effectiveness	+
Currently tested efficacy	
Spatial learning	0
Sensory sensitivity	+
Microglial activation	++

Table 6.4. Practical Therapy Score Card: Resolvins

Practical Therapy Score Card: Resolvins	
Safety	~
Availability	-
Ease of administration	~
Cost effectiveness	-
Currently tested efficacy	
Motor function	++
Cognitive function	++
Microglial activation	0

Table 6.5. Practical Therapy Score Card: RIC

Practical Therapy Score Card: RIC	
Safety	++
Availability	++
Ease of administration	++
Cost effectiveness	++
Currently tested efficacy	
Motor function	0
Affective function	~

Greater understanding of the pharmacokinetic and pharmacodynamic properties of each of these treatments would allow for fine tuning of optimal treatment dosage and timing. Pharmacokinetic preliminary studies of how practical treatments are processed by the body would test how long administered molecules or biomarkers of treatment are present in the plasma and brain. Knowing how quickly these targets dissipate or are metabolized provides insight into timing of administration. Practical therapies such as RIC present unique challenges to pharmacokinetic strategies since there are no definitive biomarkers of the treatment to date (though preliminary candidate proteins have been identified in Chapter 5). Studies of pharmacodynamics are warranted where there is evidence of efficacy in at least one outcome measure. Dose-response studies would be ideal for identifying optimal concentration or dosages of practical therapies which maximize beneficial effect on a predetermined outcome measure and minimize potential risk of harm from excessive dosing. While studies of pharmacokinetics would be difficult with a practical therapy such as RIC, studies of pharmacodynamics are plausible and warranted. In the case of RIC, understanding the optimal duration and number of cycles could be elucidated by their differential effects on biomarkers of TBI or rodent performance on behavioral tests. These approaches, coupled with confirmation of administered compound levels in the brain and plasma provide context to better evaluate efficacy, but also are resource intensive beyond conventional molecular biology techniques.

Similarly to routes of administration, not all chapters of this dissertation use the same outcome measures to evaluate efficacy. There is, however, common ground in that

they all incorporate tests of functional outcome. There is much value in identifying underlying mechanism of treatment efficacy. In some cases, the identification of mechanism could lead to approaches which augment a practical therapy (e.g. dietary supplementation with DHA before RIC administration). Ultimately, though, practical therapies must lessen the consequences of TBI. For this, behavioral testing of functional outcome in multiple domains which mirror the human condition remains paramount in the evaluation of practical therapies.

There currently exists no regulatory body in the U.S. to oversee the advancement and implementation of practical therapies. Nutritional supplements and physiological interventions such as RIC face challenges in implementation—particularly outside of direct clinical advisement. How information about these practical therapies (including what to use them for and when to use them) could be effectively disseminated remains unclear. While physicians may recommend at-home application of practical therapies, this still excludes the large population of individuals never seeking medical attention for diffuse TBI. One potential answer is in a public awareness campaign for thoroughly tested, evidence-based practical therapies for TBI, but this also comes with the risk of inadvertently advocating self-treatment over professional medical attention. It will likely require a coordinated effort of new governmental oversight, public education, and clinical advisement for practical therapies to manifest as improved public health.

Practical therapies do not by definition stand alone and may benefit patients by their addition to other treatments, including other practical therapies. Chapter 5 used mass spectrometry to identify fatty acid binding proteins as a potential protein increased in

plasma by RIC. Research from other groups demonstrating that fatty acid binding proteins can facilitate the transport of DHA across the blood-brain barrier indicate that RIC as described in Chapter 5 may act synergistically with DHA supplementation as described in Chapter 3. One of the practical drawbacks of DHA supplementation is the relatively long period required for DHA cellular uptake and RIC could plausibly shorten this time by allowing greater DHA availability in the brain. The combining of practical therapies detracts from the practicality of the treatment by decreasing the ease of administration but may be warranted for increases in efficacy. Combining therapies introduces further complication in the timeline of therapy application where further research would be warranted to maximize any synergistic effect with regard to timing.

Overall, the findings of this dissertation suggest that practical therapies may serve to fill a gap in contemporary public health by treating otherwise untreated sufferers of diffuse TBI. Omega-3 fatty acid supplementation, SPM administration, and RIC all exhibited at least some positive effect on outcome from TBI in the mouse without any observed negative effects. Advancing the field of practical therapies requires additional rigorous study of treatments from home remedies to cutting edge techniques requiring only resources available to the average person.

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APPENDIX A

IACUC APPROVAL FOR ANIMAL RESEARCH

THE UNIVERSITY OF ARIZONA

Institutional Animal Care and Use Committee
Research Compliance Services

P.O. Box 210101
Tucson, AZ 85721
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Verification of Review and Approval

by the Institutional Animal Care and Use Committee
PHS Assurance Number A3248-01 - USDA Registration Number 86-R-0003

This protocol has been reviewed by the Institutional Animal Care and Review Committee (IACUC) and the work may commence at this time. This approval only authorizes the activities reviewed by the IACUC as described on the final version of the Protocol Form.

Principal Investigator:	**Jonathan Lifshitz**
Department/School:	**Child Health**
Protocol Number:	**13-460**
Title:	**Translational neurotrauma research**
Approval Date:	**09/16/2016**
Expiration Date:	**09/16/2019**
Funding Source(s):	**Departmental** **Arizona Alzheimer's Consortium** **Arizona Biomedical Research Commission** **American Sleep Society** **Diane and Bruce Halle Foundation** **NINDS R03 NS093291** **NINDS R03 NS090013** **NINDS R21 NS096515**
Grant to Protocol Review:	**Congruent**
Additional Notes:	**None**

Sean W. Limesand, PhD
IACUC Chair

APPENDIX B

REMOTE ISCHEMIC CONDITIONING METHOD DEVELOPMENT

Many rodent studies of RIC have used transient femoral artery clamping to induce RIC (Ren, Yan et al. 2009; Peng, Guo et al. 2012; Sandweiss, Azim et al. 2017). It is understandable that many RIC studies to date have been undertaken as proof of principle endeavors, but surgically opening the hind limb decreased the practicality of the treatment and failed to reflect the human RIC paradigm achieved with a blood pressure cuff. With respect to practical treatments for diffuse TBI and testing acute behavioral outcome, we deemed femoral artery clamping too invasive for the studies presented in Chapter 5. Several methodological approaches were pilot tested and have since been refined beyond the approach used in Chapter 5. Here these will be briefly described.

Common twine was chosen as a pilot material for RIC induction for its tensile strength and relatively thick diameter over which the RIC forces could be exerted. Isoflurane anesthesia was achieved and maintained as described in Chapter 5. A short length of twine (~10cm) was tied to a wooden applicator stick (Puritan Medical Products, Guilford, ME) cut to length (~5cm). The loose end of twine was wrapped around the left mouse hind limb and tied to the applicator stick. The applicator stick was twisted approximately two turns clockwise to apply pressure and was secured to the underlying bench with laboratory tape. After 5 minutes of RIC, the twine was cut to allow for reperfusion. This method was simplified by the use of plastic coated wire ties as described in Chapter 5.

Since the conduct of the studies described in Chapter 5, a further refined non-invasive RIC approach was developed by our lab using orthodontic rubber bands. This approach was adapted from a previously reported model of murine hind limb ischemia

used in the study of peripheral artery disease (Crawford, Hashmi et al. 2007). Anesthesia was induced using 5% isoflurane in 100% oxygen for 3 minutes. The mouse was removed from the anesthesia induction chamber and a McGivney hemorrhoidal ligator (MedixPlus Quality Instruments) was used to apply a 1/8" 4.5oz heavy force orthodontic rubber band as proximally to the hip as possible. The mouse was moved to a recovery cage for 5 minutes and the orthodontic rubber band was then clipped to allow for reperfusion. 4.5 minutes following reperfusion, the mouse was reintroduced to 5% isoflurane in 100% oxygen for 30 seconds. A new orthodontic rubber band was applied to begin the second of 4 RIC cycles.

This non-invasive approach to rodent RIC induction is more comparable to human RIC than femoral artery clamping and applies a more consistent force than previous twine and wire tie approaches. Further, the ease of application of the orthodontic rubber bands allows for greatly reduced isoflurane exposure time during the RIC procedure (4.5 minutes total vs 43 minutes total in previous approaches). This is of particular concern in studies of RIC efficacy in neurological injury due to the potentially confounding effects of anesthesia on outcome (Rowe, Harrison et al. 2013). Mice are allowed to wake intermittently during the RIC procedure and show no overt pain or discomfort due to the orthodontic rubber band. Future RIC studies from this group will incorporate this refined non-invasive approach.

APPENDIX C

TABLE OF PRE-CLINICAL EFFICACY OF RIC IN NEUROLOGICAL DISEASE

Table C1. Pre-clinical efficacy of RIC in neurological disease

Citation	Model	Conditioning Location	Protection Target	Type	Protocol	Primary Result
(Sandweiss, Azim et al. 2017)	TBI, mouse (closed skull)	Hindlimb	Brain	Post	6x4min, 2hrs post-injury	Improved cognitive & motor outcome
(Ren, Wang et al. 2015)	Stroke, rat	Hindlimb	Brain	Per	3x10min during MCAO	Decreased lesion volume
(Ren, Wang et al. 2015)	Stroke, rat	Hindlimb	Brain	Per+Post	3x10min daily, 14d	Decreased lesion volume (more than Per alone)
(Qi, Dong et al. 2015)	Stroke, rat	Hindlimb	Brain	Per+Post	Unclear	Decreased mitochondrial damage
(Khan, Hoda et al. 2015)	Vascular cognitive impairment, mouse	Hindlimb	Brain	Post	4x5min, bilateral, 7-21d post-injury	Improved cognitive function, decreased cell death
(Hoda, Bhatia et al. 2014)	Stroke (embolic), mouse	Hindlimb	Brain	Per	4x10min (2hrs after induction)	Prevented mortality
(Shan, Li et al. 2013)	Stroke, rat	Hindlimb	Brain	Per	3x5min	Increased platelet-derived microparticles
(Hasseldam, Hansen-Schwartz et al. 2013)	Stroke, rat	Hindlimb	Brain	Post	3x15min	Decreased marker of ischemia (pimonidazole)
(Yuan, Zhu et al. 2012)	Stroke, rat	Hindlimb	Brain	Post	3x5min daily, 3d	Decreased lesion and behavioral deficits
(Hoda, Siddiqui et al. 2012)	Stroke (embolic), mice	Hindlimb	Brain	Per	5x5min (2hrs after induction)	Improved neurological function
(Hahn, Manlhiot et al. 2011)	Stroke, rat	Hindlimb	Brain	Pre	4x5min before induction	Decreased lesion volume

APPENDIX D

CURRICULUM VITAE

JORDAN L. HARRISON

CURRICULUM VITAE

EDUCATION

PhD, Neuroscience; 2017
Arizona State University, Tempe, AZ

BA, Biology; 2011
Berea College, Berea, KY

EXPERIENCE

Research

2015-2017 **NIH Ruth L. Kirschstein NRSA Predoctoral Fellow**
Neuroscience, Arizona State University. Advisor: Jonathan Lifshitz, PhD

Dissertation Overview: This dissertation identifies and critically examines the efficacy of four classes of practical therapies in improving rodent outcome from experimental diffuse traumatic brain injury. Over-the-counter (OTC) analgesics, omega-3 fatty acids, specialized pro-resolving mediators (SPMs), and remote ischemic conditioning (RIC) were administered before or following midline fluid percussion injury. Behavioral, histological, and molecular analyses were used to assess treatment effects on functional outcome and secondary injury progression. The overall findings suggest that omega-3 fatty acid supplementation, SPM administration, and RIC may serve as effective practical therapies.

2012-2015 **Graduate Research Assistant**
Department of Child Health, University of Arizona College of Medicine—Phoenix, Phoenix, AZ. Advisor: Jonathan Lifshitz, PhD

- Investigated the efficacy of practical therapies for diffuse traumatic brain injury in the mouse.

2011-2012 **Laboratory Technician**
Spinal Cord & Brain Injury Research Center, University of Kentucky College of Medicine, Lexington, KY. Supervisor: Jonathan Lifshitz, PhD

2010 **Undergraduate Research Assistant**
Department of Psychology, Berea College, Berea, KY. Advisor: Robert Smith, PhD

- Investigated interactions among personality, creative self-efficacy, and EEG activity.

2008 **Undergraduate Research Assistant**
Department of Biology, Berea College, Berea, KY. Advisor: Marc Rowley, PhD

- Investigated the feeding pattern decision making system of the larval Tobacco Hornworm *Manduca sexta*.

Teaching

2008-2011 **Teaching Assistant**, Department of Biology, Berea College, Berea, KY

- Assisted in preparation and execution of Anatomy & Physiology I&II labs
- Taught recitation sessions and held open lab hours, 128 contact hrs/semester
- Graded assignments and practical exams

2007-2008 **Teaching Assistant**, Department of Mathematics & Computer Science, Berea College, Berea, KY

- Tutored in a group setting for College Algebra courses, 92 contact hrs/semester
- Graded assignments

PEER-REVIEWED JOURNAL ARTICLES

Butt, CM, **Harrison, JL** Rowe, RK, Jones, J, Salem, N Jr., Lifshitz, J, Pauly, JR. (2017) Dietary docosahexaenoic acid (DHA) alleviates long-term sensory hypersensitivity and inflammation induced by experimental diffuse brain injury in rats. *Current Research: Concussion* (In press)

Rowe RK, Ziebell JM, **Harrison JL**, Law ML, Adelson PD, and J Lifshitz. (2016) Aging with traumatic brain injury: Age at injury effects on behavioral outcome following diffuse brain injury in rats. *Developmental Neuroscience*, 38*(3)*.

Rowe, RK, Ellis, GI, **Harrison, JL**, Bachstetter, AD, Corder, GF, Van Eldik, LJ, Taylor, BK, Marti, F, Lifshitz, J. (2016) Diffuse traumatic brain injury increases inflammatory hyperalgesia and inhibits systemic T cell proliferation and differentiation in the mouse. *Molecular Pain*, 12.

Harrison, JL, Rowe, RK, Lifshitz, J. (2015) Lipid mediators of inflammation in neurological injury: Shifting the balance toward resolution. *Neural Regeneration Research,* 11*(1)*.

Ziebell, JM, Rowe, RK, **Harrison, JL**, Eakin, K, Colburn T, Willyerd, FA, Lifshitz, J. (2015) Experimental diffuse brain injury results in regional alteration of gross vascular morphology independent of neuropathology. *Brain Injury*, 30*(2).*

Harrison, JL, Rowe, RK, Ellis, TW, Yee, NS, O'Hara, BF, Adelson, PD, Lifshitz, J. (2015) Resolvins AT-D1 and E1 differentially impact functional outcome, post-traumatic sleep, and microglial activation following diffuse brain injury in the mouse. *Brain, Behavior, & Immunity*, 47.

Harrison, JL*, Rowe, RK*, O'Hara, BF, Adelson, PD, Lifshitz, J. (2014) Acute over-the-counter pharmacological intervention does not adversely affect behavioral outcome following diffuse traumatic brain injury in the mouse. *Experimental Brain Injury*, 232*(9)*. *Denotes co-first authors.

Rowe, RK*, **Harrison, JL***, O'Hara, BF, Lifshitz, J. (2014) Diffuse brain injury does not affect chronic sleep patterns in the mouse. *Brain Injury*, 28*(4)*. *Denotes co-first authors.

Miremami, JD, Talauliker, PM, **Harrison, JL**, Lifshitz, J. (2014) Neuropathology in sensory, but not motor, brainstem nuclei of the rat whisker circuit after diffuse brain injury. *Somatosensory & Motor Research*, 31*(3)*.

Rowe, RK, **Harrison, JL**, O'Hara, BF, Lifshitz, J. (2014) Recovery of neurological function despite immediate sleep disruption following diffuse brain injury in the mouse: clinical relevance to medically untreated concussion. *SLEEP*, 37*(4)*.

Rowe, RK, **Harrison, JL**, Thomas, TC, Pauly, JR, Adelson, PD, Lifshitz, J. (2013) Anesthetics and analgesics in experimental traumatic brain injury: Selection based on experimental objectives. *Lab Animal*, 42*(8)*.

Ziebell, JM, Taylor, SE, Cao, T, **Harrison, JL**, Lifshitz, J. (2012). Rod microglia: elongation, alignment, and coupling to form trains across the somatosensory cortex after experimental diffuse brain injury. *Journal of Neuroinflammation*, 9*(1)*.

RESEARCH SUPPORT

2015 NIH Ruth L. Kirschstein National Research Service Award
2015-2018
'Remote ischemic conditioning as a pre-hospital therapeutic intervention for traumatic brain injury'
F31-NS090921
$117,252 total direct

HONORS & AWARDS

2016 Graduate Excellence Award, College of Liberal Arts & Sciences, ASU
2016 Travel Grant, School of Life Sciences, ASU
2016 Graduate Training Fellowship, School of Life Sciences, ASU
To attend the Neuroscience School of Advanced Studies (Non-Coding RNA course), Tuscany, Italy
2015 Emeritus Faculty Fellowship, ASU
2015 Travel Award, Society for Neuroscience Global Affairs Committee
To present at the International Brain Research Organization World Congress, Rio de Janeiro, Brazil
2015 Graduate Excellence Award, College of Liberal Arts & Sciences, ASU
2014 Travel Grant, School of Life Sciences, ASU
2014 Travel Grant, National Neurotrauma Society
2013 Travel Grant, School of Life Sciences, ASU
2008 National Honor Society of Phi Kappa Phi
2008 J. Stanton King Award in Science, Berea College
2007 Berea College Full Tuition Scholarship (Four years)
2007 Kentucky Excellence in Education Scholarship (Four years)
2007 Valedictorian, Madison Southern HS

ABSTRACTS & PRESENTATIONS

Harrison, JL, Rowe, RK, Ellis, GI, Bachstetter, AD, Corder, GF, Van Eldik, LJ, Taylor, BK, Marti, F, Lifshitz, J. (2016) Diffuse traumatic brain injury increases inflammatory hyperalgesia and inhibits systemic T cell proliferation and differentiation in the mouse. Poster presentation. 34th Annual Symposium of the National Neurotrauma Society. Lexington, KY.

Rowe, RK, Ziebell, JM, **Harrison, JL**, Law, LM, Adelson, PD, Lifshitz, J. (2016) Aging with Traumatic Brain Injury: Age at injury effects on behavioral outcome following diffuse brain injury in rats. Poster presentation. 34th Annual Symposium of the National Neurotrauma Society. Lexington, KY.

Rowe, RK, **Harrison, JL**, Lifshitz, J. (2016) Post-traumatic sleep as a personalized physiological biomarker of vulnerability to repetitive traumatic brain injury. Poster presentation. 34th Annual Symposium of the National Neurotrauma Society. Lexington, KY.

Harrison, JL. Remote ischemic conditioning as a pre-hospital therapy for traumatic brain injury. Oral presentation. 5th Annual Neuroscience Symposium hosted by Arizona State University and Barrow Neurological Institute. Phoenix, AZ.

Harrison, JL, Rowe, RK, Ellis, TW, Yee, NS, O'Hara, BF, Adelson, PD, Lifshitz, J. (2015) Resolvins AT-D1 and E1 differentially impact functional outcome, post-traumatic sleep, and microglial activation following diffuse brain injury in the mouse. Poster presentation. International Brain Research Organization World Congress, Rio de Janeiro, Brazil.

Harrison, JL, Rowe, RK, Ellis, TW, Yee, NS, O'Hara, BF, Adelson, PD, and Lifshitz, J. (2015) Omega-3 derived lipid mediators differentially impact functional outcome, sleep, and microglial activation after experimental TBI. Poster presentation. National Neurotrauma Symposium, Santa Fe, NM.

Rowe, RK, **Harrison, JL**, Zhang, H, Hesson, DP, Greene, M, and Lifshitz, J. (2015) Novel allosteric inhibitors of TNF-R1 modulate post-traumatic sleep resulting from experimental diffuse TBI in the mouse. Poster presentation. National Neurotrauma Symposium, Santa Fe, NM.

Rowe, RK, **Harrison, JL**, Zhang, H, Hesson, DP, Greene, M, and Lifshitz, J. (2015) Novel allosteric inhibitors of TNF-R1 modulate post-traumatic sleep resulting from experimental diffuse TBI in the mouse. 6th World Congress on Sleep Medicine. Seoul, South Korea.

Rowe, RK, **Harrison, JL**, Zhang, H, Hesson, DP, Greene, M, and Lifshitz, J. (2014) Novel allosteric inhibitors of TNF-R1 modulate post-traumatic sleep resulting from experimental diffuse TBI in the mouse. Society for Neuroscience Annual Meeting, Washintgon, D.C. Selected for press conference, *Sleep and the Brain.*

Harrison, JL, Rowe, RK, Adelson, PD, and Lifshitz, J. (2014) Remote ischemic conditioning as pre-hospital therapeutic intervention for diffuse traumatic brain injury. Poster presentation. National Neurotrauma Symposium, San Francisco, CA.

Ellis, TW, Rowe, RK, **Harrison, JL**, Ziebell, JM, Adelson, PD, and Lifshitz, J. (2014) Midline fluid percussion injury in the developing rodent results in diffuse injury and deviation of the neurovascular unit. Poster presentation. National Neurotrauma Symposium, San Francisco, CA.

Harrison, JL, Rowe, RK, O'Hara, BF, Adelson, PD, and Lifshitz, J. (2014) Acute over-the-counter pharmacological intervention does not adversely affect behavioral outcome following diffuse traumatic brain injury in the mouse. Poster presentation. National Neurotrauma Symposium, San Francisco, CA.

Rowe, RK, **Harrison, JL**, O'Hara, BF, Adelson, PD, and Lifshitz, J. (2013) Immediate post-injury sleep disruption does not affect functional outcome following diffuse brain injury in mice. Poster presentation. National Neurotrauma Symposium, Nashville, TN.

Harrison, JL, Rowe, RK, O'Hara, BF, Adelson, PD, and Lifshitz, J. (2013) Experimental diffuse brain injury does not impact chronic sleep patterns. Poster presentation. National Neurotrauma Symposium, Nashville, TN.

Ellis, TW, Ziebell, JM, Rowe, RK, **Harrison, JL**, Adelson, PD, and Lifshitz, J. (2013) Influence of age on rod-microglia formation following diffuse brain injury. Poster presentation. Phoenix Children's Hospital Annual Research Day, Phoenix, AZ.

Harrison, JL, Rowe, RK, O'Hara BF, and Lifshitz, J. (2012) Immediate post-injury sleep disruption alters the expression of inflammation related genes after diffuse brain injury in the mouse. Poster presentation. National Neurotrauma Symposium. Phoenix, AZ.

Rowe, RK, **Harrison, JL**, Striz, M, Donohue, M, O'Hara, BF, and Lifshitz, J. (2012) Diffuse brain injury increases acute quantitative measures of reparative sleep in the mouse. Poster presentation. National Neurotrauma Symposium. Phoenix, AZ.

Katsumi, Y, Fulton, V, **Harrison, JL**, and Hassan, A. (2010) EEG spectral analysis of neurocognitive style in relation to a remote associations task. Poster presentation. Berea Undergraduate Research Symposium.

Gan, Y, **Harrison, JL**, Stephens, T, and Rowley, M. (2008) The perception of phagostimulants is context dependent in larval *Manduca sexta*. Poster presentation. 94th Annual Meeting of the Kentucky Academy of Sciences, University of Kentucky, Lexington, KY.

SERVICE

2015 Educational Exhibit Organizer
Phoenix Children's Hospital Foundation Teddy Bear Fair Fund Raiser

PROFESSIONAL MEMBERSHIPS

National Neurotrauma Society
Women in Neurotrauma Research
Society for Neuroscience
American Association for the Advancement of Science