N-3 FATTY ACIDS AND JUVENILE TRAUMATIC BRAIN INJURY: EFFECTS OF DIETARY N-3 FATTY ACID CONTENT, N-3 FATTY ACID STATUS, AND ORALLY DOSED FISH OIL ON SENSORIMOTOR AND BIOCHEMICAL OUTCOMES

BY

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Submitted to the graduate degree program in Pharmacology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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EFFECTS OF DIETARY N-3 FATTY ACID CONTENT, N-3 FATTY ACID STATUS,

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OUTCOMES

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ABSTRACT

Children under five years of age are at high risk for sustaining traumatic brain injury (TBI) and tend to have poorer outcomes despite greater neuroplasticity in children. Hence, there is a great need to study TBI specifically in models of juvenile injury. Additionally, long chain n-3 polyunsaturated fatty acids (LC-PUFA) are a major component of neural membranes, and accumulate in the brain during late gestation and early childhood. Low dietary content of these essential fatty acids results in decreased n-3 LC-PUFA accumulation in the developing brain. Long-chain n-3 polyunsaturated fatty acids have multiple neuroprotective and anti-inflammatory activities, thus low dietary LC-PUFA content may put children at risk for poorer outcomes after TBI.

The first aim established a juvenile TBI model with consistent, measurable deficits, without debilitating injury or mortality. In order to assess functional outcomes including severity of initial injury and the duration of deficits, a qualitative assessment of common sensorimotor behavioral tests in rats of various sizes and developmental stages (postnatal days 16-45, 35-190 g) was performed. Tests were evaluated for their developmental appropriateness, scalability for growth, necessity for extensive pre-training, and throughput capability. The tests evaluated were grid-walk, automated gait analysis (DigiGait™), rotarod, beam walk, spontaneous forelimb elevation test, and force-plate actometry. Both the rotarod and grid-walk tests were eliminated on their inability to scale for growth of the animal. Rotarod also required several days of pre-training that young animals were unable to perform. DigiGait™ was eliminated due to problems associated with development and inadequate throughput. Beam walk, spontaneous forelimb elevation test, and force-plate actometry, however, are simple,

complementary tests, each measuring a different aspect of motor function that met the criteria for being adequate behavior tests for use in a rodent model of juvenile TBI and were used in later studies.

The second aim investigated the effects of dietary n-3 fatty acid content and, as a consequence, reduced brain fatty acid composition on outcomes of juvenile TBI. Long-Evans rats raised from conception on diets containing adequate n-3 fatty acids (Control) or low in n-3 fatty acids (Deficient), resulting in decreases in brain DHA of 25% and 54%, respectively, were subjected to a controlled cortical impact or sham surgery on postnatal day 17. Rats with decreased brain DHA levels had poorer sensorimotor outcomes, as assessed with force-plate actometry and the spontaneous forelimb elevation test, after TBI. *Ccl2*, *Gfap*, and *Mmp9* mRNA levels, and MMP-2 and -9 enzymatic activities were increased after TBI regardless of brain DHA level. Lesion volume was also not affected by brain DHA level. In contrast, TBI-induced *Timp1* gene expression was lower in rats fed the Deficient diet and was correlated with brain DHA level. These data suggest that decreased brain DHA content contributes to poorer outcomes after TBI through a mechanism involving modulation of *Timp1* gene expression.

The third aim investigated the use of a high dose oral fish oil dosing regimen on biochemical, blood-brain barrier, and sensorimotor outcomes of TBI in a juvenile rat model. Seventeen-day old Long-Evans rats were given a 15 mL/kG fish oil (2.01 g/kg EPA, 1.34 g/kg DHA) or soybean oil dose via oral gavage thirty minutes prior to being subjected to a controlled cortical impact injury or sham surgery. Doses of oil were then administered for seven days after surgery. Fish oil treatment resulted in improved

hindlimb deficits after TBI as assessed with the beam walk test, decreased IgG infiltration into the ipsilateral and contralateral hemispheres, and decreased TBI-induced gene expression of *Mmp9* one day after injury. TBI-induced increases in *Gfap* were also less persistent in rats treated with fish oil. These results indicate that fish oil may improve sensorimotor outcomes after TBI in juveniles by decreasing blood-brain barrier disruption by a mechanism involving decreased gene expression of *Mmp9*, and also modulating glial activation.

In summary, this dissertation established a juvenile model of TBI with persistent, measurable deficits and established three behavioral tools for assessing severity of injury, persistence of deficits, and recovery from TBI. Furthermore, it determined that brain DHA content, not diet, that most influences TBI outcomes and that improved outcomes as a result of greater brain DHA content may be due to increased TBI-induced *Timp1* gene expression. Lastly, it determined that acute fish oil dosing improves functional outcomes after TBI by limiting blood-brain barrier damage by preventing TBI-induced gene expression of *Mmp9* and faster resolution of astrocytosis. Together, these findings support the use of an LC-PUFA-rich diet during gestation and early neonatal life to provide greater neuroprotection in the event of a TBI as well as support the use of fish oil as a therapy for juvenile TBI.

DEDICATION

I dedicate this work herein to my loving parents, William and Karla Russell.

ACKNOWLEDGEMENTS

This work was supported by NIH grants R03-HD059939, center grant HD02528, NCRR-INBRE RR016475, T32-ES007079, T32-HD057850 and the KUMC Biomedical Research Training Program. I would like to give my sincere appreciation to the taxpayers of the United States of America whose tax revenues made my dissertation research possible.

I thank my mentor, Dr. Beth Levant for her advice, patience, and support over the last several years. Her door was always open and she was always willing to drop what she was doing to help, talk, edit, or brainstorm. She has also been an excellent role model of a strong, female scientist in a male-dominated field. I also thank her for her continual support of my pursuit of an alternative career path.

I thank my past and present Dissertation Committee members, Drs. Nancy Berman, Bryan Copple, Jed Lampe, Jim Luyendyk, Ken McCarson, and Tom Pazdernik for their guidance, expertise and advice, not only for conducting scientific research, but also on becoming a scientist and making career choices.

I thank everyone involved with the T32 Neurological and Rehabilitation Sciences training program, especially Drs. Randy Nudo and Sam Enna, and Lynn LeCount and Jane DeLeon. It has been a pleasure to be involved in such a great program.

I thank Michelle Healy Stoffel, my LevLab partner in crime and listening ear. I don't know that we would have survived, especially the last year, without each other.

I thank all the others who have come and gone on our laboratory and have helped me along the way including Heather Spalding, Paul Davis, Guillermo Lona, Dr. Andy Ralya, Katrina Kutchko, Patrick Gregg, and especially Marlies Ozias. It has been

a great joy to get to know Marlies and to go through graduate school and the pains of dissertation writing with her.

I'd also like to thank Michelle Winter, Jordan Brown, Drs. He Yong-Yu, Warren Nothnick, and Aritra Bhattacherjee for their technical support and help to get my protocols up and running. And I also thank Gene Gregory of Dr. Berman's lab for always loaning me supplies when I was running low and for letting me use their qPCR primers.

Lastly, I thank my family. My grandmother, Flossie Kirsch, a life-long educator, would shamelessly brag about me to her church friends. Though she could never remember that I went to KU and not K-State, I know she would have been unbelievably proud of me. And finally, the biggest thank you goes to my parents, Bill and Karla Russell for their love and unwavering support over the last 29 years. They are encouraging and always supportive of whatever decisions I make, from piercing my nose to moving to Alabama. I couldn't have asked for a better set of parents and doggie grandparents.

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
Aβ	Amyloid beta
AA	Arachidonic acid
ALA	Alpha linolenic acid
Akt	Protein kinase B
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
ANOVA	Analysis of variance
ATL	Aspirin-triggered lipoxin
AQP4	Aquaporin-4
B2M	Beta-2 microglobulin
BAD	B-cell associated death promoter
BAX	BCL2-associated X protein
BBB	Blood-brain barrier
BCL-2	B-cell lymphoma-2
BCL-XL	B-cell lymphoma-extra large
BDNF	Brain derived neurotrophic factor
CCI	Controlled cortical impact
CCL2	Chemokine (C-C) motif ligand-2
CNS	Central nervous system
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid
DAI	Diffuse axonal injury
DBS	Diffuse brain swelling
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
Fisher's LSD	Fisher's least significant difference
FPI	Fluid percussion injury
GFAP	Glial fibrillary acidic protein
ICP	Intracranial pressure
IgG	Immunoglobulin G
IκK	Inhibitor of kappa kinase
IL	Interleukin
I/R	Ischemia/reperfusion
ISSFAL	International Society for the Study of Fatty Acids and Lipids
LA	Linoleic acid
LC-PUFA	Long-chain polyunsaturated fatty acid
LOX	Lipoxygenase
MMP	Matrix metalloproteinase
MUFA	Monounsaturated fatty acid
NF-κB	Nuclear factor kappa B
NMDA	*N*-methyl-D-aspartic acid
NPD1	Neuroprotectin D1

Abbreviation	Full Name
PBF	Phosphate buffered formalin
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositide 3-kinase
PMN	Polymorphonuclear leukocytes
PND	Postnatal day
PPAR	Peroxisome proliferator-activated receptor
PtdCho	Phosphatidylcholine
PtdEth	Phosphatidylethanolamine
PtdIns	Phosphatidylinositol
PtdSer	Phosphatidylserine
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
RvD	D-series resolvins
RvE	E-series resolvins
RXR	Retinoid X receptor
SCI	Spinal cord injury
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE	Standard error
SFA	Saturated fatty acid
TIMP	Tissue inhibitor of matrix metalloproteinases
TBI	Traumatic brain injury
TLR4	Toll-like receptor 4
TNF	Tissue necrosis factor

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CHAPTER ONE

BACKGROUND AND INTRODUCTION

1.1 Overview of Traumatic Brain Injuries

The Center for Disease Control and Prevention has described traumatic brain injuries (TBI) as a “silent epidemic” due the high rate of injury, many of whom never seek medical attention, and limited public knowledge. More than 1.4 million people sustain a TBI each year in the United States. Approximately 34% of those occur in children ages 0 to 14. Falls are the leading cause of TBI and these rates are highest for children ages 0 to 4 and adults 75 years and older. Very young children, ages 0 to 4 also have the highest rate of TBI-related emergency room visits (1,035 per 100,000) (Faul et al., 2010). And, despite increased neuronal plasticity in this group, they often have a worsened outcome following injury compared to adults, making this age group of particular importance to study (Luerssen et al., 1988; Prins and Hovda, 2003).

TBI fall into three categories: mild, moderate, and severe. Mild TBI, or concussions, are characterized by a brief change in mental status or consciousness. Clinical symptoms of mild TBI include lightheadedness, headache, confusion, dizziness, blurred vision, and tinnitus, difficulty with memory, concentration, and attention, among others. Severe TBI is classified by an extended period of unconsciousness or amnesia after the injury. Moderate TBI any injuries between the mild and severe. Symptoms of a moderate/severe TBI include a constant, worsening headache, vomiting/nausea, convulsions or seizures, dilation of one or both pupils, loss of coordination, slurred speech, and others (National Institute of Neurologic Disorders and Stroke, 2002) .

TBI results in two types of injury—primary and secondary. Primary injury is damage that occurs at the moment of trauma as a result of compressing, stretching, and tearing tissues and blood vessels. Primary injury of TBI occurs rapidly and is untreatable. Upon neuronal injury, cells are damaged and torn and release a variety of

molecules into the extracellular space, including cytokines, bradykinin, proteases, and others (Nortje and Menon, 2004). These molecules then activate the immune response and surrounding glial cells to repair damage and also initiates the secondary injury (Arvin et al., 1996).

Secondary injury is the delayed insult that results from processes initiated by the trauma of the primary injury. The many processes that occur to produce secondary injury are diagramed in **Figure 1-1** and will be elaborated upon further. These processes, although depicted in a linear fashion, most often occur in parallel and interact with one another. Secondary injury occurs over time and is the main target of current TBI therapies.

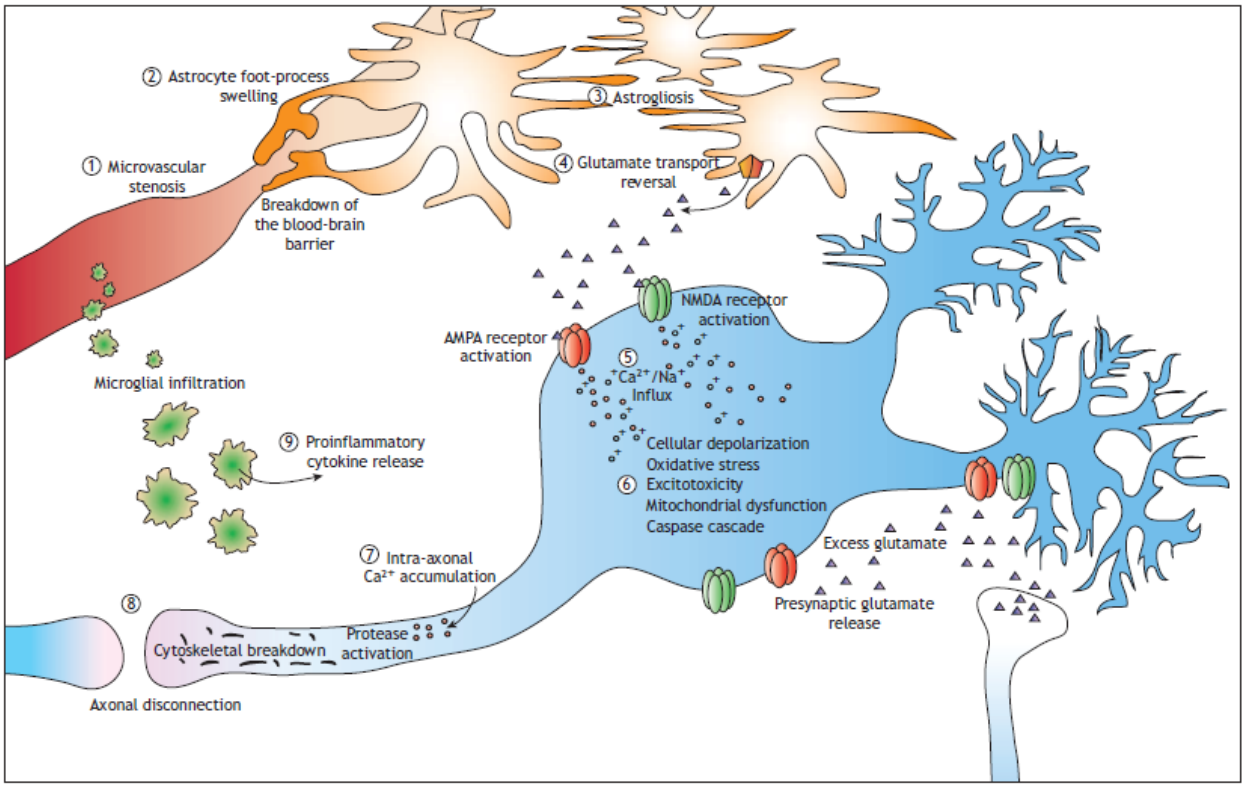

Figure 1-1. **The main processes associated with progression of secondary injury after TBI.** 1) Microvascular stenosis, 2) Breakdown of the blood-brain barrier due to astrocyte swelling and shear forces, 3) Activation and proliferation of astrocytes resulting in 4) reversal of glutamate transport, 5) Activation of AMPA and NMDA receptors by extracellular glutamate causing and influx of Ca^{2+} and Na^{+} into the cell resulting in 6) cellular depolarization, oxidative stress, mitochondrial dysfunction, and activation of the caspase cascade. 7) Intraxonal calcium accumulation causes activation of proteases and 8) breakdown of the cytoskeleton that releases chemoattractant molecules into the extracellular milieu attracting microglia to the site of injury and 9) release pro-inflammatory cytokines. Reprinted with permission from (Park et al., 2008).

1.2 Pathophysiology of TBI

The pathophysiology of TBI is very extensive and well documented. **Figure 1-1** describes some of the major processes occurring after TBI. Briefly, development of secondary injury is initiated by decreased blood flow to the injured area and disruption of the blood-brain barrier (BBB) caused by the initial insult. This can occur if shear force from the injury is great enough to damage vessels causing a loss of vascularization and/or agents released from damaged cells activating astrocytes. This causes the astrocytes to swell around vessels and restrict blood flow. Processes of secondary injury include an early necrotic phase and long term apoptotic phase, neuroinflammation, excitotoxicity, and mitochondrial dysfunction resulting in formation of reactive oxygen species (ROS) (Nortje and Menon, 2004).

Microglia and astrocytes, the two types of glial cells located in the central nervous system (CNS), play an important role in the restoration of normal function after TBI but also have potential damaging roles as well (Nakajima and Kohsaka, 2004; Laird et al., 2008). In the uninjured brain, astrocytes perform several functions including structural and metabolic support of neurons, assisting endothelial cells in the formation of the BBB, and modulation of blood flow (Kandel et al., 2000). The main role of microglia, the brain's resident macrophage, is defense. Microglia scavenge invading microorganisms and dead cells and also act as innate immune cells (Kandel et al., 2000). Upon TBI, damaged cells release factors including glutamate, ATP, ROS, and intracellular proteins into the extracellular milieu that activate astrocytes and microglia. Reactive astrocytes form glial scars that restrict tissue damage, helping to spare neighboring non-injured tissue from the spreading secondary injury (Fitch and Silver,

1997; Rolls et al., 2009). However, by restricting tissue damage, glial scars also inhibit neurite outgrowth and axonal plasticity from surviving neurons, thereby inhibiting regeneration (Bush et al., 1999; Liu et al., 2008).

Reactive astrocytes have also been shown to repair the BBB following both brain and spinal cord injury (SCI) (Bush et al., 1999; Faulkner et al., 2004). As a potential repair mechanism, microglia and reactive astrocytes release growth factors and matrix metalloproteinases (MMPs) at the site of injury to promote neuroplasticity and clean up cellular debris and secrete cytokines to initiate an inflammatory response.

Astrocytes, and particularly microglia, initiate the immune inflammatory response; this inflammatory response is both beneficial and harmful. During inflammation, leukocytes and peripheral macrophages infiltrate the site of injury through the perturbed BBB and further exacerbate and sustain the inflammatory response (Morganti-Kossman et al., 2005). Cytokines and chemokines such as tumor necrosis factor (TNF), interleukin (IL) -6, -1α and -1β, have been shown to be elevated in the hours and days after TBI in both rodent and humans (detectable in cerebral spinal fluid) indicating initiation of an inflammatory response following TBI (Morganti-Kossmann et al., 2002; Morganti-Kossmann et al., 2007; Harting et al., 2008).

Astrocytes are also involved in the formation of edema following injury. Astrocytes, but not neurons, express aquaporin-4 (AQP4) (Verkman, 2008). AQP4 is an important water channel that transports water in and out of the brain (Wolburg et al., 2009). Astrocytes swell after injury due to increased ion uptake, causing water influx to maintain the osmotic gradient (Wolburg et al., 2009; Yukutake and Yasui, 2009). The increase in water in the brain following TBI is very serious and must be monitored

closely. Edema causes an increase in intracranial pressure (ICP), which compresses of blood vessels, reduces tissue blood flow and oxygenation, and can ultimately lead to herniations that may crush vital centers in the brain including those involved with respiratory and cardiac functions.

Excitotoxicity also occurs upon injury to the brain. Release of the excitatory amino acids glutamate and aspartate activate 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) and *N*-methyl-D-aspartic acid (NMDA) receptors causing influx of calcium and sodium into the cell (Yi and Hazell, 2006). This causes cells to depolarize and fire unnecessarily. Excitotoxicity and primary injury also lead to mitochondrial damage. Mitochondria are important for maintaining energy supplies and intracellular calcium homeostasis as well as generating and detoxifying reactive oxygen species. When mitochondria become damaged and these important processes are disrupted, there is an increase in reactive oxygen species, caspases are released and activated, mitochondria swell, and ATP production and respiration are decreased all of which lead to cell death and cause the secondary injury after TBI to spread to neighboring tissues. This is very detrimental as neurons cannot divide or regenerate to replace damaged or lost neurons. For a review of mitochondrial dysfunction after TBI in the mature and immature brain, see Robertson (2004).

Mitochondrial dysfunction also results in diffuse axonal injury (DAI), another process causing the spread of secondary injury after TBI. Initially it was thought that DAI is the result of axons being mechanically torn at the moment of injury. Newer studies, however, have demonstrated otherwise. Instead, DAI is more likely to be caused by the drastic increase in intracellular calcium after injury (Park et al., 2008).

The calcium increase results in activation of caspases and proteases (e.g. calpain) that degrade the axonal cytoskeleton (Walker et al., 2009) causing failure of axonal transport, axonal swelling, and ultimately, disconnection. The timeline of development of many processes associated with secondary injury is shown in **Figure 1-2**.

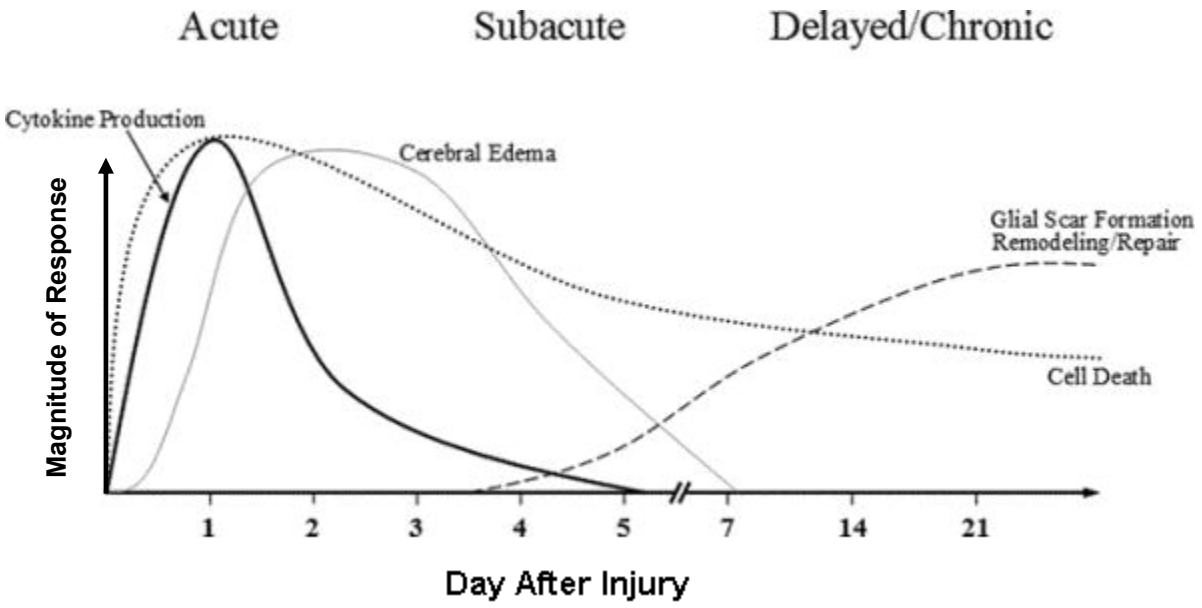

Figure 1-2. Timing in days of cytokine production, cerebral edema, scar formation, and delayed cell death after TBI. Modified from and reprinted with permission from Wolters Kluwer Health: The Journal of Trauma: Injury, Infection, and Critical Care (Walker et al., 2009).

1.4.1 MMPs and their Endogenous Inhibitors

MMPs are a family of zinc-dependent endopeptidases that degrade extracellular matrix proteins and activate an array of bioactive molecules. These enzymes are key factors in bone and heart remodeling, cancer metastasis, edema, inflammation, and other processes. For a review of MMPs see Nagase, Visse et al. (2006). After injury, MMPs are secreted by reactive astrocytes and activated to degrade damaged extracellular matrix proteins and other cellular debris, so that new matrix may be laid. MMPs can also worsen damage. Prolonged activation of MMPs, particularly MMP-2, and -9, contributes to BBB disruption leading to vascular edema following injury and increased intracranial pressure (Shigemori et al., 2006; Sifringer et al., 2007; Vilalta et al., 2008).

MMP-2 and MMP-9 are the only gelatinases in the family of at least 23 (human) MMPs. MMP-2 and MMP-9 digest type IV, V, and XI collagens. MMP-2, but not MMP-9, additionally digests type I, II, and III collagens. Also, both MMPs activate or inactivate a variety of bioactive molecules. Both MMP-2 and MMP-9 cleave the pro-forms of TNFα, IL-1β, transforming growth factor (TGF)- β, and others (Gearing et al., 1994; Gottschall and Deb, 1996; Schonbeck et al., 1998; Yu and Stamenkovic, 2000). Transcription of a pro-MMP-2 and pro-MMP-9 is regulated by nuclear factor kappa B (NF-κB) (Gottschall and Deb, 1996). The pro- forms are cleaved to active forms by each other and other MMPs when necessary (Nagase et al., 2006).

MMPs are endogenously inhibited by a family of four proteins termed tissue inhibitors of matrix metalloproteinases (TIMP). All four TIMP family members are expressed in the brain (Pagenstecher et al., 1998). TIMP-1, which has the broadest

substrate specificity, inhibits MMPs in a 1:1 ratio by binding to the MMP active site (Gomis-Ruth et al., 1997). TIMP-1 also has anti-apoptotic and growth factor properties that are independent of its MMP-inhibiting ability (Hayakawa et al., 1992; Gardner and Ghorpade, 2003; Jourquin et al., 2005), and are thought to occur through interactions with cell surface receptors including CD63 (Strongin et al., 1995)

1.4.2 Pathophysiology Unique to Juvenile TBI

While the above pathophysiology occurs in all brain injuries, there are some unique biochemical and structural elements of the immature brain that may make juveniles more susceptible to worsened TBI. For example, diffuse brain swelling (DBS) more often occurs in children after TBI than in adults (Bruce et al., 1981). Children who demonstrate DBS have a threefold higher rate of mortality than those who don't exhibit DBS (Aldrich et al., 1992; Adelson et al., 1998). This may be due to the naturally higher water content in juvenile brains as well as an inability to properly manage the water after TBI. Both juvenile (newborn and 14 days old) rat and piglet brains have increased water content compared to older animals (seven weeks and 14 weeks) (Dobbing, 1981). AQP4, the water channel expressed by astrocytes is not fully developed until adulthood. Seven days old rats have only 2% of the adult level of AQP4 and rats at post-natal day (PND) 14 and PND 28 have 25% and 60% of adult AQP4 levels, respectfully (Wen et al., 1999).

There are also variable changes in cerebral blood flow following TBI in the juvenile brain. Biagas and colleagues (1996) reported regional increases in cerebral blood flow in immature and mature rats subjected to TBI compared to aged rats.

However, retrospective studies in humans claim the opposite. Adelson and colleagues (1997) reported hypoperfusion and ischemia during the first 24 hours after injury in the immature brain, which was associated with a poorer outcome compared to the adult brain.

The immature brain is also subject to greater damage from ROS generated by TBI than is the adult brain. This is due to yet-fully-developed ROS defense systems, like decreased glutathione peroxidase activity (Fullerton et al., 1998). The immature brain also has increased levels of free iron compared to the mature brain, which causes the formation of hydroxyl radicals from peroxide in a Fenton-like reaction (Ferriero, 2001; Blomgren et al., 2003). Free radicals then cause lipid peroxidation, protein and DNA oxidation, and general cellular dysregulation.

Infants and children also have less protection from mechanical impact than do adults due to a more compliant skull and sutures. This causes an increased mechanical load that is then transferred to the brain tissue and also results in large changes in cranial shapes compared to adults (Thibault and Margulies, 1998; Margulies and Thibault, 2000). All of these differences together contribute to juveniles having worsened outcomes after TBI.

1.3 Current Therapies for TBI

One of the difficulties in treating TBI is that treatment is often not sought until days after the injury, by which time most of the damage has occurred. When patients do seek treatment the main course of action is to lower intracranial pressure through sedation and analgesia, draining cerebrospinal fluid (CSF), administering mannitol or a

hyperosmolar solution of 3% saline, or performing a craniectomy (Huh and Raghupathi, 2009). Medically-induced comas are also in practice in patients with severe TBI because of the lower oxygen demand of a comatose brain, which then, in theory, would reduce post-TBI oxidative damage. However, currently there are no FDA-approved pharmacologic therapies to treat secondary damage resulting from TBI in either children or adults. With so many processes occurring after TBI, there is little hope for a single treatment. Instead, using several process-targeted therapies may be more realistic. Numerous compounds have shown to be beneficial in pre-clinical trials but then go on to fail in clinical trials.

1.5 Modeling TBI

There are many difficulties in studying TBI, one of them being choosing an appropriate model. Currently there are several models of TBI in use. Modeling juvenile brain injuries adds the additional complexities of choosing an appropriate age and injury severity, properly evaluating the recovery in rapidly developing animals, and the use of anesthesia. The most common juvenile age to injure in rats is PND 17 which is roughly the equivalent to a human toddler; however, PND 3-14 and 26-32 are also widely used (Prins and Hovda, 2003). A wide range of injury parameters and severities are also used, varying in diameter, duration, and velocity of impact, making cross-comparison between juvenile studies and comparisons with adult studies difficult.

There is also debate on the effect of anesthesia on juvenile TBI models. Anesthesia, of course must be used during these experiments to comply with animal use regulations; however, there is an abundant body of literature to suggest age-related

differences in response to anesthesia in rodents and humans that may alter outcomes of TBI including variations in cerebral vasodilation and cerebral blood flow, ketone body uptake, and respiratory, cardiovascular, and thermoregulatory functions (Settergren et al., 1980; Cohen et al., 1990; Brussel et al., 1991). Also, different anesthetics are used between groups of researchers, most commonly ketamine, isoflurane, or halothane, which each may have different effects on juvenile TBIs and thus further complicate cross-comparisons between studies. For a complete review on the difficulties of modeling juvenile TBI, see Prins and Hovda (2003). The four most commonly used models, the weight-drop model (Feeny model), the impact acceleration model (Marmarou model), the fluid percussion model (FPI), and the controlled cortical impact (CCI) model are briefly described below and in **Figure 1-3** (Prins and Hovda, 2003; Morales et al., 2005).

1.5.1 Weight-Drop Models

The weight-drop model involves dropping a weight down a cylindrical tube onto a footplate resting on the animal's head. There are two versions of the weight-drop model—the Feeny model, which removes part of the cranium and rests the footplate directly on the dura, and the Marmarou model (also called the impact acceleration model), which leaves the cranium intact to prevent penetration of the weight. In both cases, changing the mass of the weight or the distance from which it is released modifies the severity of injury. The Feeny model generates a focal contusion whereas the Marmarou model creates a more diffuse injury often used to study diffuse brain swelling.

1.4.2 FPI Model

The FPI model creates a diffuse brain injury. This model involves injecting a bolus of saline into the epidural space. Briefly, after a craniotomy is performed, a pendulum is released from a predetermined height and hits a fluid reservoir which then causes a release of a fluid bolus that strikes the intact dural surface (Sullivan et al. 1976). Depending on the volume of the fluid bolus, or the height from which the pendulum is released, a range of injury severities can be created. Mild injury is meant to mimic a concussion where as a more severe FPI generates hemorrhages, contusions, and diffuse axonal injury (Dixon et al., 1987; Povlishock and Christman, 1995).

1.4.3 CCI Model

The CCI model is similar to the Feeny weight-drop model in that it utilizes direct impact onto exposed dura to create a focal injury. CCI injury, however, is delivered by a pneumatic piston allowing for greater control of injury including velocity and duration of impact. Interchangeable tips are also available to vary the diameter of impact.

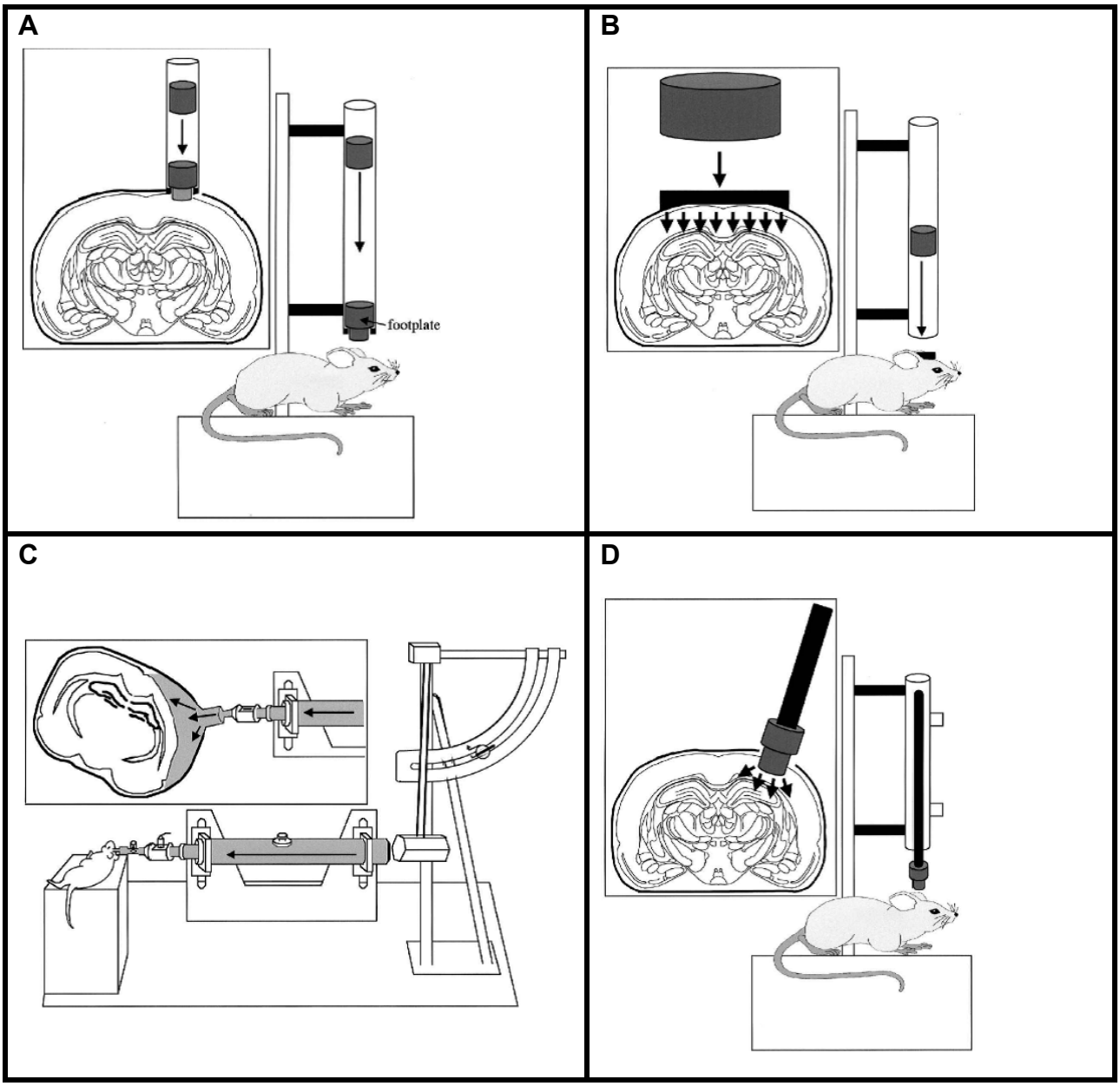

Figure 1-3. Models of TBI. In the case of the Feeny (A) and Marmarou (B) models of TBI, a weight is dropped down a hollow tube onto a foot plate. In the Feeny model, the plate makes direct contact with the dural surface of the brain. In the Marmarou model, the cranium is left intact and the footplate distributes the impact across the cranial surface and prevents penetration into the tissue. In the FPI model (C) after a

craniotomy is performed, a pendulum is released from a predetermined height to strike a reservoir of saline positioned over dural surface of the brain. Fluid is then released to strike the surface of the brain. In the CCI model (D), a pneumatic impactor is used to strike the surface of the brain which creates a more reproducible focal injury than does the weight-drop model. Modified and reprinted with permission from Mary Ann Liebert, Inc.: Journal of Neurotrauma:(Prins and Hovda, 2003).

1.5 Polyunsaturated Fatty Acids: Synthesis and Accretion

The n-3 series of long-chain polyunsaturated fatty acids (LC-PUFAs) are synthesized from the dietary essential fatty acid alpha-linolenic acid (ALA) and are of great importance in developing and maintaining optimal brain function (Willatts et al., 1998; Birch et al., 2000). ALA undergoes several elongation and desaturation steps in the liver before ultimately being converted to docosahexaenoic acid (DHA) and transported to cellular membranes. The counterpart to the n-3 series of fatty acids, the n-6 series, utilizes the same enzymes to convert linoleic acid (LA) to n-6 docosapentaenoic acid (n-6 DPA). See **Figure 1-4**.

When consumed in the diet, LC-PUFAs become incorporated into the phospholipids of cellular membranes. Brain tissue contains three major categories of lipids: cholesterol, sphingolipids (sphingomyelin, cerebrosides, sulfatides, gangliosides), and glycerophospholipids (phosphatidylcholine [PtdCho], phosphatidylethanolamine [PtdEth], phosphatidylinositol [PtdIns], and phosphatidylserine [PtdSer]) (Suzuki, 1972).

Glycerophospholipids contain a glycerol backbone with an unsaturated fatty acid at the second position carbon and a phosphobase (choline, ethanolamine, serine, or inositol) at the third position carbon. Sphingolipids contain ceramide linked to phosphocholine through the primary hydroxyl group. These lipids provide neural membranes with stability, fluidity, and permeability and are also required for proper function of receptors, transporters, integral membrane proteins, and ion-channels (Farooqui et al., 2000). In the brain, the phospholipids are unequally distributed across cellular membranes. PtdEth, PtdSer, and PtdIns are concentrated in the inner leaflet of the membrane whereas PtdCho and sphingomyelin are concentrated in the outer leaflet (Farooqui et al., 2000).

DHA constitutes approximately 15% of weight of the total fatty acids in the brain of rats and >33% of the total fatty acids in the retina and is mainly found on the *sn*-2 position of PtdEth and PtdSer (Sinclair, 1975). In human brain gray matter, DHA accounts for approximately 24% of acyl groups in PtdEth and 37% of acyl groups in PtdSer (Salem, 1986). The n-6 LC-PUFA, arachidonic acid (AA) is distributed evenly in the gray and white matter and among the different cell types in the brain. DHA, however, is highly enriched in neuronal and synaptic membranes (Farooqui et al., 2000).

DHA content of the membranes can significantly alter basic properties of the cellular membrane. DHA is sterically incompatible with cholesterol and has been shown to alter fatty acid chain order and fluidity, ion permeability, elastic compressibility, resident protein function, phase behavior, and fusion of membranes (Stillwell and Wassall, 2003; Wassall and Stillwell, 2008). Preferential incorporation of DHA into

PtdEth and PtdSer on the inner leaflet of neuronal and synaptic membranes and incompatibility with cholesterol allows for formation of DHA-rich/cholesterol-poor and DHA-poor/cholesterol-rich lipid rafts. Lipid rafts are specialized membrane microdomains that allow for selectivity of proteins within the membrane and play an important role in compartmentalization and modulation of cell signaling (Farooqui et al., 2000).

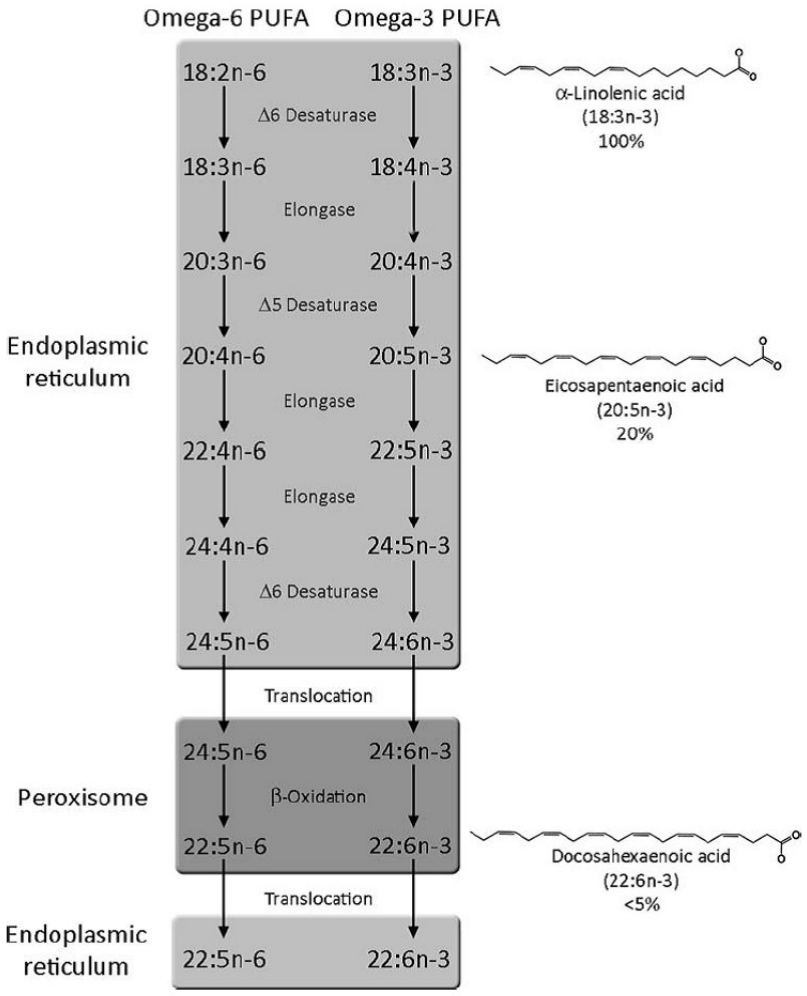

Figure 1-4. Summary of omega-3 and omega-6 PUFA biosynthetic pathways. The pathways proceed through a series of desaturation and elongation steps in the endoplasmic reticulum until 24:5n6 and 24:6n3 are formed, at which time they are translocated to the peroxisome and shortened by C2 by one cycle of the β-oxidation pathway to form 22:5n6 and 22:6n3 (DHA), respectively. These are then translocated back to the endoplasmic reticulum. The relative efficiencies of the omega-3 PUFA conversion process are shown to the right of the pathways. For futher details refer to the text. Reprinted with permission from Springer Science + Business Media: NeuroMolecular Medicine (Dyall and Michael-Titus, 2008).

1.5.1 DHA Accretion and the Western Diet

Because DHA cannot be synthesized *de novo* in mammals, ALA or any of the n-3 fatty acids, must be consumed in the diet. Some foods high in n-3 fatty acids include cold-water fish such as salmon and sardines, canola oil, and walnuts. However, Western diets, particularly those in the United States are very low in n-3 fatty acids, and have an n-6/n-3 as high as 16.7/1 (Simopoulos, 2003). Multiples studies report that a high n-6/n-3 ratio promotes numerous disease states including heart disease, cancer, increased inflammation, and autoimmune diseases (Simopoulos, 2002). Lower dietary n-6/n-3 ratios have been shown to have beneficial effects including decreasing the mortality of cardiovascular disease, reducing cell proliferation in colorectal cancer, decreasing risk of breast cancer, and decreasing inflammatory states associated with rheumatoid arthritis and asthma (Haworth and Levy, 2007; Calder and Yaqoob, 2009; Fetterman and Zdanowicz, 2009; Lavie et al., 2009).

In humans, the main period of DHA accumulation occurs in late gestation and early childhood while turnover continues throughout life (Clandinin et al., 1980a; Clandinin et al., 1980b; Hadley et al., 2009). Rats, however, are more immature at birth and DHA accumulates with a pronounced spike during the last three days of gestation and continues through weaning (Kishimoto et al., 1965; Green and Yavin, 1996). During this time, DHA is supplied to growing fetuses by the mothers' dietary consumption and to infants in breast milk. Recently, infant formula has been supplemented with DHA and eicosapentaenoic acid [EPA, 20:5(n3)]. However, studies in infant baboon suggest that formula supplementation is still insufficient in raising brain DHA levels compared to breastfeeding (Diau et al., 2005; Hsieh et al., 2007).

Adequate DHA is essential for optimal brain and visual development and function. While there are no gross disorders associated with LC-PUFA deficiency, several studies have reported visual and cognitive deficits in children due to a low n-3 diet and benefits inferred by an n-3 supplementation during pregnancy (McNamara and Carlson, 2006). Infants from mothers supplemented with DHA during pregnancy had significantly improved visual acuity at 4 and 6 months of age (Judge et al., 2007). Also, several randomized controlled studies reported impaired mental performance and visual function in otherwise healthy term infants with a lack of dietary DHA (Willatts et al., 1998; Birch et al., 2000). Studies with rhesus monkeys with an n-3 deficient diet during gestation and early postnatal development demonstrated reduced DHA levels in the retina and cerebral cortex, psychomotor and cognitive deficits, and impaired visual function (Neuringer et al., 1984; Neuringer et al., 1986).

1.6 Recommended Intakes of LC-PUFAs

Despite the multiple worsened disease states associated with a high n-6/n-3 ratio, the Food and Drug Administration has no formal dietary recommendation of LC-PUFAs. The International Society for the Study of Fatty Acids and Lipids (ISSFAL), however, has recommended that normal, healthy individuals consume 2% of daily energy of the n-6 LA and 0.7% daily energy of the n-3 ALA. To improve cardiovascular health, ISSFAL recommends a 500 mg/day minimum intake of EPA and DHA combined (ISSFAL, 2004). Because of the increased LC-PUFA demand by a growing fetus and infant, it is recommended that pregnant or lactating mothers consume at least 200 mg of DHA/day (Koletzko et al., 2007).

1.7 Phospholipases

Before n-3 fatty acids can act as signaling molecules, they must first be cleaved from the membrane. This is achieved through a family of enzymes called phospholipases. Phospholipases, first identified in snake venom, are a family of enzymes that cleave phospholipids into fatty acids and other lipophilic molecules through hydrolysis. There are four classes of phospholipases—A, B, C, and D; each distinguished by the site of hydrolysis on the phospholipid. N-3 and n-6 fatty acids are preferentially cleaved by the cytosolic form of phospholipase A2 ($cPLA_2$) allowing for formation of DHA and AA-derived signaling molecules including prostaglandins, eicosanoids, docosanoids, and maresins (Burke and Dennis, 2009; Serhan et al., 2009).

1.8 DHA Mechanisms of Action

There are several mechanisms by which DHA exerts its neuroprotective properties that are relevant to TBI. DHA itself is anti-inflammatory but can also be metabolized in to a wide variety of anti-inflammatory and pro-inflammation resolving molecules. DHA also is anti-apoptotic, anti-excitotoxic, and has anti-oxidant properties. Each of these processes is described herein.

1.8.1 DHA and Inflammatory Signaling

Inflammation is the body's response to harmful stimuli including cell damage, pathogens, or irritants. The purpose of inflammation is to remove the damaging stimulus and initiate healing. In the brain, the inflammatory response is initiated by microglia. Currently, there are three general mechanisms by which n-3 fatty acids (DHA

and EPA, specifically) alter the inflammatory response. They 1) alter lipid raft formation, 2) compete with AA and are synthesized into unique anti-inflammatory molecules, and 3) modify cell signaling and alter pro-inflammatory gene expression (Chapkin et al., 2009).

Cytokines are small peptides involved in modulating and amplifying an acute or chronic inflammatory state. Transcription of many cytokines is regulated by the NF-κB pathway. Upon a ligand binding to a Toll-like receptor the inhibitor of kappa B kinase (IκK) complex is formed. The IκK complex consists of IκKα and/or IκKβ catalytic subunits and two of the scaffolding molecule NF-κB essential modulator. The IκK complex phosphorylates IκB, the NF-κB inhibitor. IκB is then degraded by the proteosome, allowing the freed NF-κB to translocate to the nucleus and activate target genes regulated by κB sites. Target genes include IL-1, IL-6, TNFα, MMPs and others. These proteins have several important roles including promoting T-cell and B-cell activation (which release more cytokines), increasing vascular permeability, inducing apoptosis, and attracting leukocytes to the site of injury (Arvin et al., 1996).

Many of the beneficial actions of DHA are thought to come from its ability to inhibit toll-like receptor 4 (TLR4), decrease IkB phosphorylation, and interact with various nuclear receptors which then initiate the NF-κB-mediated inflammatory response. Free n-3 fatty acids, cleaved from membrane phospholipids by $cPLA_2$, can directly inhibit TLR4 to prevent activation of IκK and ultimately translocation of NF-κB into the nucleus (Lee et al., 2003; Weatherill et al., 2005). DHA also inhibits TLR4 receptor dimerization in the membrane, which is required for activation (Wong et al., 2009).

Several nuclear receptors are also influenced by n-3 fatty acids. The peroxisome proliferator-activated receptors (PPAR) alpha and gamma and retinoid X receptor (RXR) nuclear receptors are activated by DHA and EPA at micromolar concentrations (Kliewer et al., 1997; Xu et al., 1999; de Urquiza et al., 2000; Fan et al., 2003). Activated peroxisome proliferator-activated receptors have been shown to transrepress the NF-κB-mediated inflammatory response (Pascual et al., 2005). Several studies have documented the ability of n-3 fatty acids to decrease cytokine production. *In vitro*, cells pre-treated with DHA before lipopolysaccharide, a TLR4 agonist, decreases protein levels of IL-12p70, IL-6, and decreases transcription of NF-κB and cyclooxygenase-2 (COX-2) (Lee et al., 2003; Weatherill et al., 2005). Also, prostaglandin E_2 production, as a response to COX-2 activity, was decreased in humans supplemented with 15 g/day of fish oil for four weeks (Lee et al., 2003).

Another part of the inflammatory response involves the cleavage of the n-6 fatty acid AA from the membrane by $cPLA_2$ and its conversion into the potent pro-inflammatory molecules 2-series prostanoids (thromboxanes and prostaglandins) and 4-series leukotrienes by COX-2 and lipoxygenase (LOX), respectively. However, not all AA-derived molecules are pro-inflammatory. For example, lipoxins and aspirin-triggered lipoxin (ATL) share many of the same endogenous anti-inflammatory and pro-resolving properties; however, ATL is longer acting and resists rapid dehydrogenation. Briefly, lipoxins and ATL have been shown to inhibit entry of polymorphonuclear leukocytes into the site of injury, as well as reduce vascular permeability, and stimulate clearance of apoptotic neutrophils via macrophages (Maderna et al., 2005). For a complete review of actions of lipoxins and ATL, see Serhan, Yacoubian et al. (2008b).

Previously, it was thought that the anti-inflammatory properties exhibited by DHA were due to DHA's competition with AA; more DHA meant less AA. While this is true to an extent, it was recently discovered that DHA and EPA themselves are substrates for COX-2 and LOX and serve as a precursors to several unique inflammatory and immunoregulatory molecules, termed resolvins and protectins (Serhan et al., 2002). As with the AA-derived lipoxins, there are also aspirin-triggered and non-aspirin-triggered resolvins. During resolution of inflammation, acetylated COX-2 coverts EPA to 18R-HEPE, which is then oxygenated and undergoes epoxide hydrolysis and rearrangement into Resolvin E1 (RvE1). RvE1 acts to down-regulate NF-κB activity by binding to the ChemR23 G-protein coupled receptor and has demonstrated very potent anti-inflammatory properties *in vivo.* Administration of RvE1 has been shown to reduce leukocyte and neutrophil migration, activate resolution earlier and decreases the number of several pro-inflammatory cytokines and chemokines during resolution of inflammation in murine peritonitis (Bannenberg et al., 2005). Similarly to EPA, DHA in the presence of aspirin can be converted into the *17R* D-series of resolvins. These resolvins have been shown to decrease IL-1β secretion from glioma cells and reduce leukocyte migration in murine periotonitis (Marcheselli et al., 2003; Bannenberg et al., 2005).

DHA, in the absence of aspirin, can be converted by COX-2 into the 17S resolvins (RvD1-RvD4) which also exhibit anti-inflammatory, pro-resolving properties (Serhan et al., 2002). In side-by-side comparisons, equal amounts of RvE series, RvD series, or aspirin-triggered-RvD series resolvins all caused a 50% reduction in polymorphonuclear leukocyte (PMN) infiltration in murine peritonitis. This is in contrast

to indomethacin, a widely used non-selective COX inhibitor, which only decreased leukocyte infiltration by 25% at the same dose (Serhan et al., 2002).

DHA can also form non-aspirin triggered docosatrienes. In this case, DHA is converted 17S-H(p)DHA by 15-lipoxygenase. 17S-H(p)DHA can then undergo enzymatic hydrolysis to form Neuroprotectin D1 (NPD1) when in neural tissues (Marcheselli et al., 2003). NPD1 exhibits tremendous anti-inflammatory properties *in vivo* and *in vitro*. Ten nM of synthetic NPD1 decreased human neutrophil transmigration *in vitro* by 50% (Marcheselli et al., 2003). NPD1 also reduced PMN infiltration by 40% at a dose of 1 ng/mouse in murine peritonitis. It also shortened the interval of inflammation resolution, down-regulated pro-inflammatory cytokines and up-regulated anti-inflammatory cytokines (Marcheselli et al., 2003). NPD1 also reduced both retinal damage and limited ischemic damage in model of kidney injury and stroke (Marcheselli et al., 2003; Mukherjee et al., 2004; Duffield et al., 2006; Bazan et al., 2012).

More recently, activated macrophages have been implicated in the formation of another group of DHA-derived anti-inflammatory mediators, the maresins. Maresins have potent anti-inflammatory and pro-resolving activity with potency similar to RvE1 and NPD1 (Serhan et al., 2009).

In general, there are more n-3-derived anti-inflammatory molecules than there are n-6-derived ones, suggesting that the ratio of n-3 to n-6 PUFAs in the brain may play an important role in regulating an inflammatory response after injury. See **Figure 1-5** for a diagram of the synthesis of fatty acid-derived inflammatory mediators.

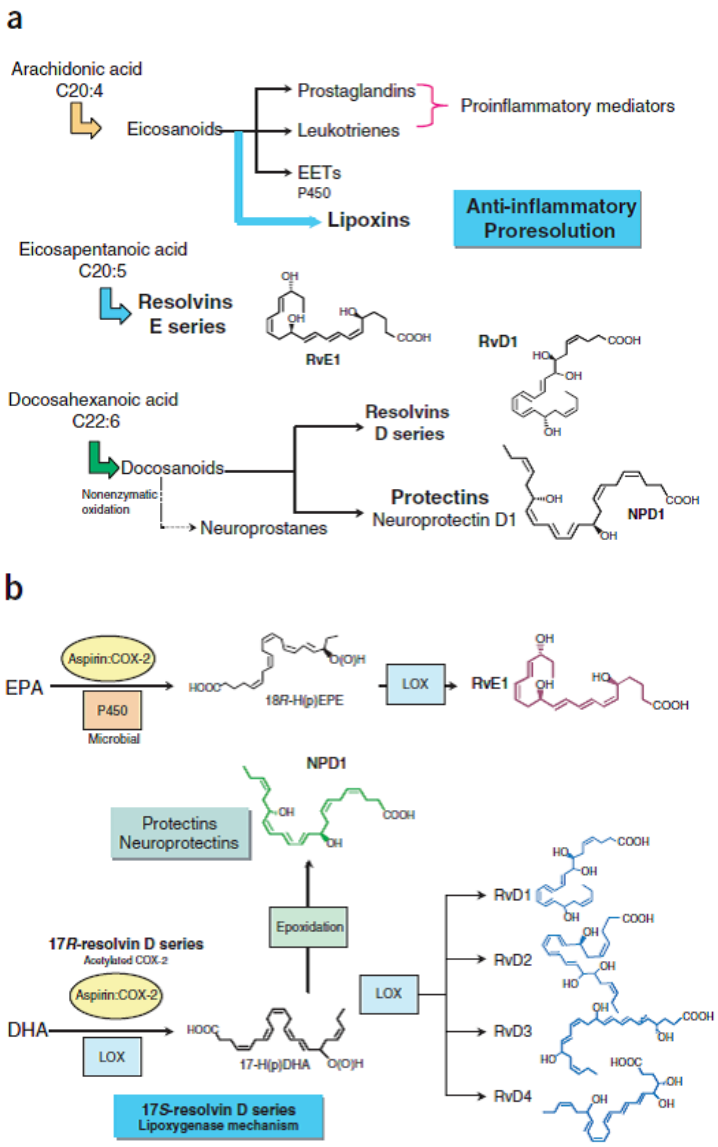

Figure 1-5. Fatty acid-derived bioactive lipid mediators. (a) Arachidonic acid is the precursor of eicosanoids, which have distinct functions as proinflammatory mediators. Lipoxins are also generated from AA but are anti-inflammatory and promote resolution. The n-3 fatty acids EPA and DHA are converted into the anti-inflammatory E1 (RvE1) and D1 (RvD1) resolvins, respectively. DHA can also form neuroprotectin D1 and neuroprostanes, also both anti-inflammatory. **(b)** In the presence of aspirin, EPA and

DHA form aspirin-triggered E1 and D series resolvins, respectively which vary slightly in structure from their non-aspirin triggered counterparts, but still are anti-inflammatory. Besides RvD1, in the presence of aspirin, DHA can also form RvD2, D3, and D4 and neuroprotection D1. Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology (Serhan and Savill, 2005).

1.8.2 DHA and Apoptosis

DHA is also thought to be involved in apoptosis, a critical process of neurodegeneration after TBI. The DHA-derived NPD1 has been shown to decrease oxidative stress and apoptotic DNA damage in culture and also up-regulate anti-apoptotic proteins (B-cell lymphoma-2 [*Bcl2*] and B-cell lymphoma-extra large [*BclxL*]) and down-regulate pro-apoptotic proteins (Bcl-2-associated X protein [*Bax*] and B-cell-associated death promoter [*Bad*]) after ischemia/reperfusion (I/R) (Bazan, 2005). NPD1 also inhibits caspase-3 activity, an important apoptosis initiating molecule, and IL-1-mediated expression of COX-2 in retinal pigment epithelial cells (Mukherjee et al., 2004; Mukherjee et al., 2007). N-3 supplementation has also shown to significantly reduced DNA fragmentation, and caspase-3 and *Bax* levels as well as increase *Bcl2* and *BclxL* levels in the cerebellum of rat pups in a model of hypothyroidism-induced neuronal apoptosis (Sinha et al., 2009). DHA also inhibits soluble β-amyloid oligomer-mediated neuronal apoptosis and significantly increases neuronal survival by preventing cytoskeleton perturbations, caspase activation, and promoting extra signal-related kinase pathways (Florent et al., 2006).

DHA can also influence apoptosis and cell survival through its incorporation in to the membranes and altering phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt) signaling. PI3K/Akt is a well-studied anti-apoptotic pathway overactive in cancer cells (Fresno Vara et al., 2004). DHA is preferentially incorporation into PtdSer in the inner leaflet of the membrane bilayer. This facilitates translocationof Akt resulting in efficient phosphorylation and activation of Akt. Akt initiates a series of signaling cascades that, ultimately, suppresses caspase-3 activation and cell death (Akbar et al., 2005). Conversely, DHA-depleted membranes slow translocation and phosphorylation of Akt (Akbar and Kim, 2002; Akbar et al., 2005).

1.8.3 DHA and Excitotoxicity

Besides being anti-inflammatory and anti-apoptotic, DHA has anti-excitotoxic and anti-oxidant properties, which contribute to its overall neuroprotective profile. DHA reduces endothelial COX-2 induction through inhibiting NADPH oxidase and protein kinase Cε (Massaro et al., 2006). DHA up-regulates γ-glutamyl-cysteinyl ligase and glutathione reductase activities in human fibroblasts, thereby enhancing the antioxidant response (Arab et al., 2006). Dietary depletion of DHA activates caspases and decreases NMDA receptors in Tg2576 mouse brain and DHA supplementation partially protects the mice from NMDA receptor subunit loss (Calon et al., 2005). DHA also induces antioxidant defense mechanisms by enhancing cerebral activities of catalase and glutathione peroxidase and increasing levels of glutathione in the cerebral cortex (Hossain et al., 1998). DHA supplementation reduced ROS in the hippocampus of amyloid beta (Aβ)-infused and aged rats (Hossain et al., 1998; Hossain et al., 1999).

Additionally, infant rats receiving an LC-PUFA-enriched formula were protected against against NMDA-induced excitotoxic degeneration of cholinergic neurons (Hogyes et al., 2003).

1.9 DHA in CNS injury

Several studies have demonstrated the benefits of an n-3 fatty acid-enriched diet or administering DHA in adults before or after CNS injuries, including TBI, SCI and I/R. In 2004, Wu and colleagues demonstrated beneficial effects of a DHA- and EPA-enriched diet when given after FPI. A DHA and EPA-enriched diet after FPI normalized levels of brain derived neurotrophic factor (BDNF), and its downstream effectors cAMP response element binding protein (CREB), and synapsin I (Wu et al., 2004). Injured animals on the diet also had reduced oxidative damage and improved learning compared to those on the control diet. There are, however, flaws with this study. The authors claim all benefits are the result of DHA rather than EPA, a claim that cannot be substantiated due to lack of DHA-only or EPA-only diet controls. Also, the claim that the fish oil diet normalized BDNF levels after injury cannot also be made because the fish oil diet also increased BDNF in sham animals.

DHA has also shown promise in the treatment of spinal cord injuries. Huang and King and colleagues have investigated the benefits of DHA both as diet and post-injury administration in a model of adult spinal cord injuries. Post-injury DHA and DHA administration in conjunction with a DHA-enriched diet increased neuron and oligodendrocyte survival, improved behavioral recovery, and decreased the axonal injury and macrophage and microglial recruitment to the site of injury (Huang et al.,

2007). DHA administration after injury alone decreased lipid, protein and DNA/RNA oxidation and decreased COX-2. In a similar study by the same group, post-injury treatment with n-3 fatty acids improved and n-6 fatty acids worsened outcomes after SCI (King et al., 2006). These data suggest that, like in TBI, DHA provides neuroprotection by many mechanisms in SCI, including reducing oxidative stress, protecting white matter tracts, and modulating the inflammatory response. DHA supplementation in humans suffering spinal cord injuries was also beneficial in improving strength and stamina (Javierre et al., 2006).

Despite demonstrated benefits of administering DHA post-injury in SCI models, DHA administration in the area of adult I/R has been contradictory depending on treatment time. Pan and colleagues (2009) investigated the effects of various pretreatment regimens and doses of DHA on outcomes of adult I/R. At a high dose and in all treatment regimens (one hour, three days, or daily administration for 6 weeks), DHA decreased infarct volume, brain water content, BBB disruption, IL-6 levels, caspase-3 and myeloperoxidase activity, and levels of malondialdehyde, a product of lipid peroxidation. However, in an earlier study by the same group (Yang et al., 2007), post-treatment of I/R with DHA was detrimental. Post-treatment with DHA increased infarct volume, BBB disruption, water content, myeloperoxidase and caspase-3 activity, lipid peroxidation, and oxidative stress and decreased motor activity after I/R.

Even though progress has been made to investigate the potential benefits of LC-PUFAs in CNS injuries, no studies have investigated these effects in juvenile models, a time during which DHA is accumulating and risk of TBI is high. Additionally, no studies

have determined whether diet or tissue LC-PUFA composition has more influence on TBI outcomes.

CHAPTER TWO

STATEMENT OF PURPOSE

2.1 Significance and Objectives

Young children are at high risk for sustaining TBI and have poorer outcomes than adults despite having greater neuroplasticity. Additionally, juveniles have unique therapeutic needs compared to the average adult including unique pathophysiologic processes, differential gene expression, and differential drug metabolism (Maxwell, 2012; Pinto et al., 2012a; Pinto et al., 2012b). N-3 polyunsaturated fatty acids are a major component of neural membranes and accumulate in the brain during late gestation and early childhood (Clandinin et al., 1980a; Clandinin et al., 1980b). Low dietary content of these essential fatty acids results in decreased n-3 LC-PUFA accumulation in the developing brain. N-3 LC-PUFAs have multiple neuroprotective and anti-inflammatory activities (Serhan et al., 2008b; Serhan et al., 2009; Orr et al., 2012), thus low dietary n-3 LC-PUFA content, during a time at which they're lacking a full complement of brain DHA, may put children at risk for poorer outcomes after TBI. Additionally, the neuroprotective properties, low toxicity, and high bioavailability of n-3 LC-PUFAs make them an attractive therapeutic for neural injuries. While studies using n-3 LC-PUFA as a therapeutic strategy in adult models of neural injuries have been done and show benefit (Javierre et al., 2006; Huang et al., 2007; Wu et al., 2007; Pan et al., 2009; Bailes and Mills, 2010), no studies have looked at acute n-3 LC-PUFA supplementation in juvenile models of injury, including TBI. Thus, the **OBJECTIVES** of this project were to:

1. **Establish a model of juvenile TBI with consistent injury and measurable sensorimotor deficits.** A qualitative comparison of six behavioral tests was assessed in rats of various sizes and developmental

stages. Male and female rats were also assessed for potential sex differences in sensorimotor response to TBI.

2. **Determine whether or not dietary n-3 fatty acid intake and/or brain fatty acid status influence recovery from juvenile TBI.** Sensorimotor and biochemical outcomes of TBI were assessed in rats with one of three levels of brain DHA and consuming a control or n-3 LC-PUFA deficient diet.

3. **Investigate the use of acute fish oil dosing as a therapeutic option to improve recovery from juvenile TBI.** Sensorimotor and biochemical outcomes of TBI were assessed in rats treated acutely with fish oil.

2.2 Rationale for Using a Rat Model of Juvenile TBI

Using young rats to model juvenile TBI is well established (Prins and Hovda, 2003). The most widely used injury day in the field is PND 17. A PND 17 rat is at the approximate developmental stage as a human toddler with regard to motor function and brain development (Rice and Barone, 2000; Prins and Hovda, 2003). With regard to DHA accretion, human toddlers and PND 17 rats are also very similar. Humans accumulate most brain DHA during the third trimester of gestation (Clandinin et al., 1980b) and throughout early childhood (Clandinin et al., 1980a). Likewise, rats have a dramatic spike in DHA accretion beginning 5 days before birth that continues through weaning (Green et al., 1999).

A PND 17 rat weighs approximately 35 g, larger than the average adult mouse. Therefore, using rats provides much more tissue allowing for multiple types analysis for

which sample preparation procedures may not be compatible. Additionally, subtle changes in motor function are more easily detectible in rats because they are larger.

2.3 Rationale for the CCI Model and Sham Surgery

Controlled cortical impact is a widely used procedure to model focal brain injuries, especially in juvenile models of TBI (Adelson et al., 1996; Adelson et al., 1998; Appelberg et al., 2009). A very specific, controlled injury to a select area of tissue can be produced using CCI. In this case, the sensorimotor cortex was injured allowing us to measure motor deficits as a measure of injury severity and recovery. Other models of TBI, such as fluid percussion, produce diffuse, wide-spread injuries which may cause deficits that can be more difficult to quantify (Prins and Hovda, 2003).

Sham procedures involving the use of a trephine or drill to produce craniotomy have been shown to cause brain injury distinct from that caused by impact (Cole et al., 2011). Cortical damage induced by TBI significantly outweighs damage caused by the craniotomy (Wu et al., 2013); nevertheless, to avoid potential experimental confounds, the sham surgery consisted of a scalp incision with no craniotomy or impact from the CCI device. Thus, the experiments utilized two experimental conditions: no injury to the skull or brain or craniotomy with contusion injury to the brain.

2.4 Aims and Rationale for Endpoints Chosen

2.4.1 Specific Aim 1

The goal of Aim 1 was to establish a juvenile TBI model with consistently reproducible behavioral deficits as well as establish a battery of sensorimotor behavioral

tests capable of assessing recovery throughout the rapid growth and development of a juvenile rat. While establishing this model, we also investigated potential sex differences in sensorimotor deficits after TBI. It was **hypothesized** that**:**

- Suitable sensorimotor tests could be identified or created to assess behavioral recovery after TBI in juvenile rats
- There would be no sex differences in sensorimotor deficits after TBI.

This study is presented in Chapter 4.

2.4.1.1 Rationale for Aim 1 Endpoints

Rats received a CCI injury to the sensorimotor cortex thus enabling us to assess motor deficits as a function of injury and recovery. As such, motor tests assessing forelimb and hindlimb function, balance, gait, and locomotion and related behaviors were selected for evaluation.

With the anticipation that the sensorimotor tests would be used to assess the effects of a TBI occurring on PND 17 for at least 28 days after injury in later studies, the initial evaluation of the sensorimotor tests examined the use of each procedure with rats ranging in age from as early as PND 14 (allowing for as many as three days of pre-training, depending on the test) through PND 45 (35-170 g). Rats' body size, motor coordination, and eyesight change drastically and rapidly during the juvenile and adolescent period; therefore, it was important to establish reliable behavioral tests that could assess function from two weeks of age through adulthood. Tests were evaluated based on four criteria: the necessity for pre-training to learn the task before injury, ability to scale the test to accommodate animals of varying sizes, whether the task was developmentally appropriate for rats at all of the relevant ages, and the throughput

capacity including the labor required to analyze the data. Scalability was determined empirically by testing the ability of rats of each age group to perform each test on each available size of apparatus.

Both male and female rats were used when evaluating behavioral tests assess potential hormonal or estrous cycle effects on behavioral outcomes. Though at the time of injury rats are sexually immature, toward the end of the testing period of interest, rats begin to mature. As a result of maturation, sex differences might develop and create a potential confound. If no behavioral sex differences exist, this would allow us to use both sexes in later behavioral studies, if necessary.

2.4.2 Specific Aim 2

The goal of Aim 2 was to determine the effects of dietary n-3 LC-PUFA content and brain fatty acid status on sensorimotor and molecular outcomes of a TBI in juvenile rats. Previous studies showed that, in addition to diet, affects offspring brain fatty acid composition. By breeding two sequential litters of rats on a Control and Low N-3 diet, we can produce rats with three levels of brain DHA, with both litters consuming the same diets. This allowed us to investigate the “dose-response” effects of brain DHA content on the motor and biochemical outcomes of juvenile TBI. Three possible outcomes were **hypothesized**:

- Severity of biochemical and sensorimotor outcomes will not be influenced by diet or brain fatty acid content such that all injured rats, regardless of diet or litter, will have similar outcomes.

- Severity of biochemical and sensorimotor outcomes **will correlate with diet, not brain DHA,** such that injured rats fed the low n-3 diet will have worsened outcomes. This would indicate outcomes of TBI are a function of the amount of n-3 fatty acids consumed in the diet and not of the fatty acid status of the brain.
- Severity of biochemical and sensorimotor outcomes **will correlate with brain DHA content**, such that rats with greater decreases in brain DHA will have the worst outcomes; thus indicating outcomes after TBI are a function of tissue DHA levels rather than dietary n-3 content, since both litters consume the same diets

This study is presented in Chapter 5.

2.4.2.1 Rationale for Aim 2 Endpoints

This was the first study of the effects of dietary n-3 LC-PUFA content on TBI outcomes in a juvenile rat model. As such, endpoints providing information on multiple injury processes were chosen to provide a comprehensive overview that would form the basis for subsequent targeted studies of implicated processes.

Previous studies have shown that maternal diets deficient in n-3 LC-PUFAs decrease the DHA content of the offspring and that this effect increases when animals are maintained on n-3 LC-PUFA-deficient diets for multiple pregnancies or generations (Favreliere et al., 1998; Levant et al., 2006c; Ozias et al., 2007). The technique of breeding multiple litters from a single dam on a deficient diet has been used extensively by our laboratory (Levant et al., 2006c; Ozias et al., 2007; Levant et al., 2010) to

produce pups with varying degrees of brain DHA without the confound of using multiple n-3 LC-PUFA deficient diets to produce multiple levels of brain DHA content. This diet and breeding procedure allows for the determination of dose-response effects of brain DHA on content on the outcomes of TBI while avoiding potentially confounding effects of using different diets.

It was established in Aim 1 that no sex differences with regard to behavioral outcomes after TBI are detectable with our tests; however, sex differences on biochemical outcomes were not assessed and may exist. To eliminate the confound of potential hormonal or estrous cycle effects, only male rats were used in Aim 2. Additionally, males have a higher incidence of TBI than females making using only males more clinically relevant (Faul et al., 2010).

Behavior was assessed in a repeated measures design weekly through 28 days after injury to assess the initial magnitude of the injury as well as recovery or persistence of effects using tests identified as suitable in Aim 1. This also allowed us to assess not only the severity of the initial injury but also the rate of recovery. To make the best use of all rats in the study, rats used for behavioral testing were used to measure lesion volume after completion of the behavioral testing on day 28.

For biochemical endpoints, day one after injury was chosen as the optimal time point because multiple injury processes are occurring including necrosis, inflammation, edema, *etc*. Additionally, preliminary data suggested MMP activity after injury may partly be regulated by n-3 fatty acid content of the brain. Matrix metalloproteinase-2 and -9 activities peak approximately 24 hours after TBI (Sifringer et al., 2007). By seven days after injury many of the acute injury processes, including MMP activity, are

concluding but long-term processes like apoptosis and glial scaring are beginning (Sifringer et al., 2007; Walker et al., 2009). Additionally, choosing day one and day seven after injury allowed the assessment of the initial magnitude of the injury as well as persistence of effects, which may be prolonged in rats with low brain DHA.

To best determine the effects of TBI on MMP-2 and MMP-9, both enzymatic activity and mRNA levels were assessed one and seven days after injury. Matrix metalloproteinases, like many other proteases, are transcribed and then translated into zymogens that require cleavage to be fully activated. As such, mRNA levels likely do not directly reflect the level of active protein. Also, MMP-9 is transcriptionally regulated whereas MMP-2 is constitutively expressed and is primarily regulated at the level of enzyme-activation (Strongin et al., 1995; Gottschall and Deb, 1996). Therefore, to get the most accurate profile of MMP level after TBI, both mRNA levels and enzymatic activity were assessed.

Because little is currently known about the mechanism(s) by which brain n-3 fatty acid content would modulate recovery after TBI, it was important to select genes that are representative of the spectrum of physiological processes occurring after TBI. Evaluation of mRNA levels using quantitative polymerase chain reaction (qPCR) was chosen because it enabled the assessment of a large number of mediators from a very small sample. Furthermore, genes were chosen based on processes affected by n-3 administration in other models of neural injury (Wu et al., 2004; King et al., 2006; Huang et al., 2007; Wu et al., 2007; Pan et al., 2009; Wu et al., 2013). Messenger RNAs for *Il-6*, *Il-1*β, and *Tnf*α were chosen based on their involvement with inflammation, a process DHA and EPA are known to modulate in other models of neural injury. Glial fibrillary

acidic protein (*Gfap*) is a marker of injury and glial activation. Chemokine (C-C motif) ligand 2 (*Ccl2*) is an inducible chemoattractant protein secreted by monocytes, macrophages, and dendritic cells after tissue injury to attract other similar cells to the site. Matrix metalloproteinase-2 and MMP-9 are proteases known to be involved in the degradation of the BBB after TBI through degradation of the matrix. Matrix metalloproteinases also activate many pro-inflammatory molecules including IL-1β. TIMP-1 is a broad substrate MMP inhibitor has-apoptotic and growth factor properties (Hayakawa et al., 1992; Gardner and Ghorpade, 2003; Jourquin et al., 2005).

Gene expression and mRNA level does not necessarily correlate with the level of functional protein. There is a significant amount of post-translational modification, stabilization, activation, and degradation that may lead to more or less protein in relation to mRNA levels. Additionally, there are many factors that contribute to mRNA stability which many therefore affect measured mRNA levels. These limitations will be taken into consideration when drawing conclusions about mRNA levels.

2.4.3 Specific Aim 3

The goal of Aim 3 was to examine the effects of acute administration of fish oil on sensorimotor and biochemical outcomes of TBI in juvenile rats. It was **hypothesized** that:

- Acute fish oil administration will improve sensorimotor and biochemical outcomes after TBI. Fish oil administration will improve locomotor function, reduce immunoglobulin G (IgG) infiltration, and improve measures of gene expression after TBI compared to injured rats administered soybean oil.

This study is presented in Chapter 6.

2.4.3.1 Rationale for Aim 3 Endpoints

Aim 3 examines the effects of fish oil dosing on outcomes of TBI. Soybean oil served as the control as that was the oil supplied in the diet to all groups. Thirty minutes prior to receiving a brain injury rats were dosed with the appropriate oil to serve as a "loading dose" and provide possible benefit immediately after TBI. Oil dosing continued once a day to mimic a reasonable human dosing paradigm and also to allow the young rats to consume other necessary nutrients through maternal milk, and later, chow. Rats were dosed with 15 mL/kg of oil, which was approximately 0.5 mL at the time of injury, a volume that could be well tolerated but still provided a substantial dose of n-3 fatty acids.

Several studies have shown the benefit of fish oil, and DHA or EPA alone for the treatment of neural injuries (Bailes and Mills, 2010; Huang et al., 2007; Javierre et al., 2006; Pan et al., 2009; Wu et al., 2007). Both DHA and EPA have neuroprotective properties (Zhang et al., 2011). Therefore, for this initial study, animals were dosed with fish oil, a combination of DHA, EPA, and other n-3 fatty acids to test the effects of n-3 LC-PUFAs generally. This treatment strategy is also highly clinically relevant as nutraceutical fish oil supplements are currently available and could thus be immediately used in patients with TBI should they prove beneficial. The specific contributions of individual n-3 LC-PUFA to the beneficial effects observed after TBI, if any, must be determined in subsequent studies.

Endpoints for Aim 3 were based on the outcomes of Aim 2. Likewise with Aim 2, to eliminate possible sex effects on biochemical outcomes, only male rats were used for this study. Rats underwent behavioral assessment one, four, and seven days after TBI after determining that most behavioral effects were maximal within 24 hours after injury and recovery could be detected within seven days after TBI. To minimize the number of rats used, rats used for behavioral testing were also used to assess BBB damage via IgG immunohistochemistry after completion of the behavioral testing on day seven.

Damage to the BBB was assessed using IgG immunohistochemistry to measure IgG infiltration into the brain. Under normal conditions, IgG is not found in the brain and it can only pass through the BBB when it is damaged. An alternative technique to measure BBB damage would be to inject Evans Blue dye prior to sacrifice. Like IgG, it cannot pass through the BBB unless it is damaged. However, using IgG immunohistochemistry provided additional unaltered sets of sectioned tissue that could be analyzed for other mediators of injury if necessary.

To limit redundancy from Aim 2 to Aim 3, only genes with significant main effect of injury in Aim 2 as indicated by a two way analysis of variance (ANOVA) were measured in Aim 3. Those genes were: *Timp1*, *Mmp2*, *Mmp9*, *Gfap*, and *Ccl2*.

CHAPTER THREE

MATERIALS AND METHODS

Parts of this chapter are reprinted with permission from *Journal of Neuroscience* Methods,199, Russell KL, Kutchko KM, Fowler SC, Berman NE and Levant B, Sensorimotor behavioral tests for use in a juvenile rat model of traumatic brain injury: assessment of sex differences, 214-222, (2008), with permission from Elsevier.

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

3.1 Animals, Husbandry, Diets, and Dosing

Long-Evans rats were housed in a temperature- and humidity-controlled facility with a 14-10 hour light-dark cycle (on at 06:00 h) with *ad libitum* access to food and water. Breeding stock (females 75–85 days; male proven breeders; Harlan Laboratories, Inc. Indianapolis, IN) were obtained a minimum of five days prior to the beginning of the experiment and were handled regularly. Males and females were maintained on a standard laboratory rodent diet (#8604, Harlan Laboratories, Inc., Indianapolis, IN) until mating. At the time of mating, breeding pairs were placed on one of two purified diets (Control or Deficient). Mated females were singly housed and maintained on their diet through two consecutive litters, allowing one week rest after weaning the first litter and mating for the second litter. Litters were culled to eight pups with preference for males on PND 1. Pups received either a CCI injury or sham surgery on PND 17 and were returned to the dam until weaning. Pups were weaned on PND 20 onto their mothers' respective diet and housed in groups of two to four, TBI and sham-injured together, for the remainder of the study.

3.1.1 Experimental Diets

In Aim 1, rats were fed standard laboratory chow (Teklad 8604, Indianapolis, IN). For Aim 2, the Control diet was AIN-93G (Teklad, Indianapolis, IN, which was formulated with unhydrogenated soybean oil (70 g/kg) and contained 4.20 g/kg ALA (18:3n-3) and 33.81 g/kg LA (18:2n-6). It met all current nutrient standards for rat pregnancy and growth (Reeves et al., 1993). The Deficient diet was a custom prepared pelleted diet (Teklad) that was identical to the Control diet except it was prepared with safflower oil (66.5 g/kg) and soybean oil (3.5 g/kg), and thus contained 0.38 g/kg ALA (18:3n-3) and 45.96 g/kg LA (18:2n-6).

For Aim 3, rats were fed Teklad Global diet 2016, which does not contain soy products and thus obviates the potential confound of phytoestrogens in the diet. Fatty acid composition of the Control, Deficient, and Teklad Global 2016 diets are shown in **Table 3-1**.

Table 3-1. Diet fatty acid composition.

Diet Fatty Acid Composition (g/kg)	Control	Deficient	Teklad Global 2016
14:00	0.29	1.23	0.16
16:00	9.84	7.23	6.16
18:00	6.42	5.84	2.29
20:00	0.51	0.19	0.14
22:00	0.33	0.39	0.21
24:00	ND	ND	ND
16:01	ND	0.10	ND
18:01	13.39	10.99	7.38
20:1n-9	0.06	0.16	0.28
18:2n-6	28.68	37.20	17.78
18:3n-3 (ALA)	5.32	0.54	1.59
20:2n-6	0.22	0.17	0.41
20:4n-6	ND	0.62	0.11
20:5n-3 (EPA)	ND	ND	ND
22:5n-3	ND	ND	ND
22:6n-3 (DHA)	ND	ND	ND

ND: not detected

3.1.2 Acute Fish Oil Dosing

Rats were dosed with either 15 mL/kg of fish oil (2.01 g/kg EPA, 1.34 g/kg DHA; Nature Made 1200 mg fish oil capsules, Mission Hills, CA) or unhydrogenated soybean oil via oral gavage 30 minutes prior to the initial TBI or Sham surgery and then daily for seven days. Fish oil contained no traces of Vitamin D and 5.6 pg/mg of oil of Vitamin E (tocopherol) as an antioxidant. On days behavioral testing occurred, rats were dosed after testing. On days of sacrifice, rats were sacrificed approximately six hours after dosing. Soybean oil was chosen as the control as it was the oil used in the Teklad 2016

diet and was already supplied to all groups via the diet. Fatty acid composition of the fish and soybean oils are shown in **Table 3-2**.

Table 3-2. Soybean oil and fish oil fatty acid composition.

Oil Composition (% Total Fatty Acids)	Fish Oil	Soybean Oil
12:0	0.13	ND
14:0	7.66	ND
14:1	0.09	ND
15:0	0.53	ND
16:0	16.02	11.09
16:1	13.53	0.10
17:0	0.54	0.17
17:1	1.60	0.05
18:0	5.23	5.05
18:1n-9t	10.28	ND
18:1n-9c	3.66	20.99
18:2n-6c	1.52	50.13
18:3n-6	0.45	ND
20:0	ND	0.69
18:3n-3 (ALA)	0.78	9.74
20:1n-9	1.04	0.13
21:0	ND	0.19
20:2n-6	0.37	0.32
20:3n-6	0.23	ND
22:0	ND	0.60
20:4n-6/20:3n-3	1.70	0.02
22:1	0.22	0.04
23:0	1.28	0.07
20:5n-3 (EPA)	11.66	ND
22:2n-6	0.14	0.27
24:0	0.058	0.18
24:1	1.63	0.15
22:5n-6	0.36	ND
22:6n-3 (DHA)	16.25	ND

ND: not detected

3.2 Procedures

Male rat pups (n = 5-12 per group, depending on endpoint, and each from a different litter) were subjected to CCI TBI or sham surgery on PND 17. Those used for sensorimotor testing were tested one, seven, 14, 21, and 28 days after surgery for Aims 1 and 2, or days one, four, and seven after surgery for Aim 3 and then euthanized on the final testing day by transcardial perfusion under pentobarbital anesthesia followed by the removal of the brain. A second cohort of rats not used for behavioral testing were euthanized on day one (28 hrs) or day 7 after surgery (Aim 2) or day one or day four after surgery (Aim 3) by decapitation.

Brains from these rats were rapidly removed and the dissected on ice. The frontal cortex was frozen on dry ice for later fatty acid analysis. The injured motor cortex was divided in half, with the rostral half being frozen on dry ice for later zymographic analysis and the caudal half preserved in RNAlater (Life Technologies/Ambion, Gaithersburg, MD) for mRNA analysis.

3.2.1 Controlled Cortical Impact

The CCI was performed as previously described (Russell et al., 2011). Briefly, rats were anesthetized with isoflurane (induction, 3.0%; maintenance, 2.0%) and stabilized in a Cunningham stereotaxic frame (Stoelting, Wood Dale, IN). A 4 x 4 mm craniotomy was performed lateral (right side) to the mid-sagittal suture, centered at: AP = 0, ML = 2.5 from bregma. The impactor device, previously described in detail (Onyszchuk et al., 2007) was outfitted with a 3.0 mm-diameter tip. The impactor tip was centered within the craniotomy and lowered until the tip just contacted the dura over

motor (M1, M2) and sensory (S1FL, S1HL) cortical areas (Sherwood and Timiras, 1970; Paxinos and Watson, 1986). The parameters of the impact were as follows: 3.0 mm depth, 1.5 m/sec strike velocity, 300 msec contact time. The scalp was closed with a 6-0 silk suture and the animal was able to recuperate until locomotion was recovered. The sham surgery consisted of a scalp incision with no craniotomy or impact from the CCI device because sham procedures involving the use of a trephine or drill to produce craniotomy have been shown to cause brain injury distinct from that caused by impact resulting in an experimental confound (Cole et al., 2011). All rats received 0.05 mg/kg of buprenorphine approximately one hour after surgery and again 24 hours after surgery, after day one behavioral testing was completed.

3.3 Sensorimotor Testing

All behavioral testing occurred between 09:00 and 12:00 h in a brightly lit room specifically reserved for rodent behavioral testing. Animals were allowed to acclimate to the behavioral testing facility in their home cages for approximately 15 minutes before beginning testing.

3.3.1 Grid Walk

Rats were placed on an elevated wire grid (46 x 92 cm), with 2.5 x 2.5 cm square holes and allowed to walk for five min while being videotaped from below, as previously described (Onyszchuk et al., 2007). Three five-minute sessions were performed for each rat on each test day, with the rat spending at least five minutes in the home cage between sessions. Videos were later analyzed for total walking time, number of steps

taken, and the number of foot faults for each foot. Foot faults were defined as an instance where the animal attempted to place weight on a foot, which then passed completely through the plane of the wire grid. Foot fault data were normalized to the total time spent walking to account for differences in the degree of locomotion seen in different trials, and are expressed as foot faults per minute of walking.

3.3.2 Automated Gait Analysis

Gait was assessed using a DigiGait™ imaging system (Mouse Specifics, Inc.). Rats were placed in the lighted Plexiglas™ chamber situated on a motorized transparent treadmill. Paw placement was captured from the ventral aspect using a high-speed digital video camera (150 frames/sec, 5000 pixels/cm2) mounted under the treadmill. Five-sec recordings were made of the rats walking between five and 10 cm/sec and were analyzed using DigiGait™ analysis software to analyze more than 25 parameters of gait.

3.3.3 Rotarod

An accelerating mouse-sized rotarod (Med Associates ENV-575M) was used in accordance with the methods of Hamm et al. (1994). Rats were trained daily for three days prior to injury. Rod speed accelerated from 4-40 RPM over the course of five minutes. Six minutes was the maximum time allowed on the rotarod. Rats were tested three times on each testing day, with a minimum of five minutes of rest in the home cage between trials.

3.3.4 Beam Walk

Rats were tested for their ability to traverse a 75 cm-long wooden dowel elevated 30 cm and ending in a dark goal box. Two training sessions were performed on PND 16 and PND 17 prior to surgery, which were sufficient for all rats to meet the pre-injury performance criterion of being able to complete two traverses of the full length of the rod. Beams of increasing diameter were used to accommodate the growth of the rats over the course of testing. Beam sizes were selected based on the criteria that the diameter was sufficiently large that the rat was able to walk the length of the beam, but not large enough that the rat could lie on the beam and crawl. A 15 mm diameter beam was used for PND 16 and 17 pre-surgery training and testing on days one and seven after surgery. An 18 mm diameter beam was used for testing 14 days after surgery, and a 21 mm diameter beam was used for testing 21 and 28 days after surgery. On testing days, rats traversed the beam three times. Test sessions were video-taped. The videos were scored for ipsilateral and contralateral foot slips, time required to reach the goal box, and total number of steps taken by the right (uninjured) hind foot. Data are reported as the percent of contralateral foot slips to control for the decreasing number of steps needed to traverse the beam as the rats grow, and average speed (cm/sec).

3.3.5 Spontaneous Forelimb Elevation (Cylinder) Test

Rats were placed in a glass cylinder (standard laboratory beaker or cylindrical vase [Living Bright, Inc.]) scaled to the size of the rat so that the cylinder diameter was roughly 4 cm greater than the length of the rat from nose to hind quarters. This provided ample room for the rat to turn, but also minimized horizontal exploration of the

cylinder (12 cm diameter for PND 16 pre-injury measurements and measurements one day after surgery, 15 cm for seven days after surgery, and an 18 cm for 14, 21, and 28 days after surgery). Rats were observed for spontaneous rearings during a single 5-min observation session. The number of wall rearings using both left and right, right only, or left only forelimbs were recorded. A measurement performed on the day prior to TBI surgery, was taken to control for pre-injury limb preference. The laterality score was computed as follows: (number of right only - number of left only) / (number of right only + number of left only + number of both together) (Schallert et al., 2000).

3.3.6 Assessment of Motor Activity Using Force-Plate Actometry

Rats were placed in the force-plate actometer chambers (42 x 28 cm, [Steven C. Fowler, University of Kansas]), enclosed in a dark, sound attenuating cabinet. Behavior was recorded for 20-minutes in two-minute time bins. A small wall-mounted fan in each cabinet provided background noise and air circulation. Data were analyzed for total distance traveled, number of low mobility bouts (≥10 sec within a 20-mm radius), and low mobility distance (distance traveled during bouts of low mobility) as previously described by Fowler et al. (2001), and turning bias (see below). To determine turning bias, the center of force coordinates of the rats' movements were referenced to the geometric center of the floor on which the animals moved, and vector algebra was used to calculate angular direction of movements (degrees) relative to the floor geometric center every 0.01 sec. Movements in a counter-clockwise direction (turning to the left) were coded with a positive algebraic sign while movements in the clockwise direction received a negative algebraic sign. These signed values were summed algebraically as

session time advanced to yield the net directional rotations. The method is analogous to using a bidirectional mechanical counter attached to a rat with a tether such that turns in one direction count up (add) and turns in the opposite direction count down (subtract). Zero turning bias, therefore, is reflected by zero counts at the end of the recording session. All data were analyzed for the entire 20-minute session on each testing day after injury. Data for distance traveled, bouts of low mobility, and low mobility distance were further analyzed in two-minute time bins on day one after injury.

3.4 Quantitative Real-Time PCR

Cortical tissue surrounding the site of injury was dissected on ice at the time of euthanasia and preserved in RNAlater (Life Technologies/Ambion, Gaithersburg, MD) at 4^{o}C until total RNA was to be isolated. Total RNA was isolated from the tissue by homogenizing it in 1 mL of Trizol reagent (Life Technologies/Gibco BRL, Gaithersburg, MD) per 100 mg of tissue weight. The RNA was isolated using a Trizol (Life Technologies/Ambion) phenol-chloroform extraction according to the manufacturer's protocol and precipitated with 75% isopropyl alcohol overnight at -20^{o}C. The quality of isolated RNA quality was determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) with adequate quality being an OD 260/280 greater than 1.8. mRNA quality was further verified using a Agilent Bioanalyzer 2011 (Agilent Technologies, Inc., Santa Clara, CA). First strand cDNAs were synthesized using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) per the manufacturer's protocol using a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA). Exon spanning, gene-specific primers (**Table 3-3**) were prepared using

the NCBI's Primer-BLAST (Ye et al., 2012) and purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Primer specificity was determined by the presence of a single peak in the melt curve.

The qPCR reactions were prepared using 10 ng of RNA-equivalent cDNA, 125 nM of each forward and reverse primer, and 1x iQ SYBR Green Supermix in 96 well plates and run on a iCycler iQ real-time PCR system (Bio-Rad, Hercules, CA). The thermal cycle conditions were as follows: 30 sec at 50^{o}C, 8 min 30 sec at 95°C, followed by 95°C for 15 sec then 65°C for 30 sec for 40 cycles, then 95^{o}C for 1 minute, 55^{o}C for 1 minute, and a melt curve beginning at 55^{o}C and increasing 0.5^{o}C every 10 sec until 100^{o}C.

Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data are expressed as fold change in gene expression compared to the reference gene beta-2-microglobulin (*B2m*), which was experimentally determined to be the most stably expressing gene in our experimental model and brain region of interest (Harris et al., 2009).

Table 3-3. qPCR primers.

Gene	Accession No.	Forward	Reverse	Slope	Y-Intercept	R^2	Efficiency
B2m	NM_012512.2	TGCTTGCCATTC AGAAAACTCC	TTGAGGTGGGTG GAACTGAG	-3.1	22.4	1	2.23
Ccl2	NM_031530.1	TGTCTCAGCCAG ATGCAGTTAAT	TCCAGCCGACTC ATTGGGAT	-3.27	27.1	1	2.06
Gfap	NM_017009.2	GCGTCTGGACCA GCTTACTAC	TTTCATCTTGGA GCTTCTGCCT	-3.6	21.5	1	1.78
Mmp2	NM_031054.2	GGAGCTCTATGG GCCCTCCCC	GTGGCCACCAGC AAGGGACC	-3.21	27.9	1	2.05
Mmp9	NM_031055.1	GTGACACCGCTCA CCTTCAC	GCGTGTGCCAGT AGACCATC	-3.25	29.1	1	2.03
Timp1	NM_053819.1	GATATGTCCACA AGTCCCAGAACC	CCACAGCCAGCA CTATAGGTCTTT	-3.43	25.5	1	1.96
Il-6	NM_012589.1	AAGAGACTTCCA GCCAGTTGCC	ACTGGTCTGTTG TGGGTGGTATC	-3.65	35.4	1	1.74
Il-1β	NM_031512.2	CACCTCTCAAGC AGAGCACAG	GGGTTCCATGGT GAAGTCAAC	-3.1	34.8	1	2.23
Tnfα	NM_012675.3	CAAGAGCCCTTG CCCTAA	CAGAGCAATGAC TCCAAAGTA	-3.67	33.4	1	1.72
Bcl-xl	NM_001033670.1	ACGAGCAGTCAG CCAGAACCCT	ACCAGCTCCCGG TTGCTCTGA	-3.16	27.1	1	2.17
Bdnf	NM_012513.3	GCAAAGCCACAA TGTTCCACCAGG	GGCGCAGCCTTC ATGCAACC	-3.81	31.8	1	1.63

3.5 Gelatin Zymography

Injured cortical tissue was dissected on ice and homogenized in lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, pH 8) and centrifuged. MMP-2 and MMP-9 were purified from the supernatant using gelatin Sepharose 4B affinity media (GE Healthcare Life Sciences, Pittsburgh, PA) for one hour at 4^{o}C. MMP-2 and MMP-9 were eluted from the Sepharose 4B using the lysis buffer containing 10% DMSO. Samples were loaded with a zymogram sample buffer (Bio-Rad, Hercules, CA) onto a 7.5% polyacrylamide gel containing 1 mg/mL porcine gelatin. Samples were run in a tris-glycine sodium dodecyl sulfate (SDS) running buffer (25 mM Tris Base, 192 mM Glycine, 0.1% SDS, pH 8.3) at 100V until the dye front reached the bottom of the gel. Gels were rinsed 2X in rinse buffer (50 mM Tris, 5 mM $CaCl_2$, 2.5% Triton X-100, pH 8) for 30 minutes, 1X in incubation buffer (50 mM Tris, 5 mM $CaCl_2$, pH 8) at room temperature and then in fresh incubation buffer overnight at 37^{o}C. Bands were visualized by staining the gels with Coomassie brilliant blue stain (2.5 mg/mL) for several hours. After destaining, gels were digitized and bands analyzed for color density using NIH ImageJ software (Abramoff et al., 2004). Band density is expressed as a percentage of the density of the positive controls, human recombinant MMP-2 and MMP-9 (Anaspec, Freemont, CA).

3.6 Histology and Immunohistochemistry

Twenty-eight days after surgery (Aim 2) or seven days after surgery (Aim 3), rats were deeply anesthetized with pentobarbital and transcardially perfused with cold 1X phosphate buffered saline (PBS) followed by 4% phosphate buffered formaldehyde

(PBF, pH. 7.4). The brains were removed and post-fixed in 4% PBF for several days then cryoprotected in 30% sucrose in PBS for three days and stored at -80^{o}C until sectioning.

3.6.1 Lesion Volume

Frozen sections were cut at 50 µm through the site of impact collecting every tenth section. Sections were mounted on gelatin-subbed slides and dehydrated in graded ethanol and stained with cresyl violet before being coverslipped. Macro-level images digitized images of each section and ImageJ (Rasband, 1997-2012) were used to determine the area of intact tissue of the ipsilateral and contralateral hemispheres . The total tissue loss was calculated using the following equations modified from (Coggeshall, 1992): (contralateral tissue area – ipsilateral intact tissue area) * section thickness * distance between sections = Subvolume. Total lesion volume =Σ Subvolume ($Section_1 + Section_2 + \ldots Section_n$).

3.6.2 IgG Immunohistochemistry

Frozen sections were cut at 30 µm through the site of impact (+2.0 bregma to -2.0 bregma), or the corresponding area in shams, collecting every tenth section. Sections were mounted on charged slides prior to staining.

Frozen sections were allowed to equilibrate to room temperature for 15 minutes then hydrated in PBS before being stained for IgG using a Vectastain Rat IgG ABC Kit (Vector Labs, Burlingame, CA) according to the manufacturer's protocol. All solutions and dilutions were prepared according to the manufacturer's instructions. Briefly,

mounted sections were air-dried for 15 minutes then rinsed with PBS for 15 and incubated in 0.3% H_2O_2 for 30 minutes to block endogenous peroxide activity. Next, sections were blocked in diluted normal serum for 30 minutes, then 2 x 5 minute rinse in PBS, and then a 1-hour incubation with a secondary biotinylated anti-rat IgG antibody. Sections were rinsed for 5 minutes in PBS before a 30-minute incubation with the ABC reagent (streptavidin-horse radish peroxidase) followed by 2-10 minute incubation with the prepared diaminobenzidine tetrahydrochloride reagent (Vector Labs, Burlingame, CA) to visualize immunoreactivity. Sections were rinsed in water for 5 minute before being dehydrated through a graded alcohol baths and then coverslipped.

ImageJ (Rasband, 1997-2012) was used to determine the ipsilateral cortical IgG density and total area of IgG staining (both hemispheres) from macro-level digitized grayscale images of each section. The total area of IgG staining was calculated from IgG staining in the entirety of both hemispheres. A baseline grayscale threshold was set at 75 based on the background level of nonspecific staining in sham sections. Any areas darker than the threshold were considered positive for IgG staining. The volume of IgG staining was calculated using the following equation: IgG area * section thickness * distance between sections = Subvolume. Total IgG Volume = Σ Subvolume ($section_1$ + $section_2$ + …$section_n$).

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3.7 Brain Total Phospholipid Fatty Acid Composition

Brain total phospholipid fatty acid composition was analyzed as previously described (Levant et al., 2006c). Briefly, phospholipids were extracted from frontal cortex and isolated by thin layer chromatography. The phospholipids were then

transmethylated with boron trifluoride methanol (Sigma, St. Louis, MO) to produce fatty acid methyl esters. Individual fatty acid methyl esters were analyzed using a Varian 3400 gas chromatograph with an SP-2330 capillary column (30 m, Supelco, Inc., Belfonte, PA), using helium as the carrier gas. Peaks were identified by comparing to authentic standards (PUFA 1 and 2, and Supelco 37, Supelco, Inc. and 22:5n-6, Nu-Chek Prep, Elysian, MN) and corrected for response factors. Individual fatty acids were expressed as weight percent of total fatty acids on the basis of peak area.

3.8 Statistical Analysis

All data are expressed at the mean ± SEM.

For Aim 1, data from tests found suitable for use with the rat model of juvenile TBI were analyzed for effects of injury (TBI or sham-injured) and sex (male or female) by repeated measures analysis of variance (ANOVA) with factors of TBI, sex, and day after injury (1-28 days after injury, repeated measure). Force-plate actometry data were also analyzed for effects across the observation session by 3-way ANOVA with factors TBI, sex, and time bin (repeated measure). Post-hoc comparisons were made using one-way ANOVA and the Fisher's Least Significant Difference (Fisher's LSD) test. Differences were considered significant if $P < 0.05$.

For Aim 2, normally distributed data were analyzed for effects of injury (TBI or sham-injured) and diet (Control or Deficient) by repeated measures ANOVA with factors of TBI, diet, and day after injury (1-28 days after injury) (SYSTAT, v.12). Time after injury was analyzed as repeated measure for the sensorimotor function studies. Outliers identified by SYSTAT were discarded from subsequent analyses. Post-hoc

comparisons were made using one-way ANOVA and the Fisher's Least Significant Difference test. In one case of the real-time PCR analysis (*Ccl2*, 1st litter), data were not normally distributed and instead were analyzed by the Kruskal-Wallis nonparametric ANOVA with post-hoc comparisons made using Dunn's Multiple Comparisons test. A significant difference was assumed if $P < 0.05$. Because the experimental design required the production pups from 1st and 2nd litters from the same dam, testing of 1st and 2nd litter pups was performed as separate cohorts. Consequently, with the exception of the brain fatty acid data, comparison of the effects in the 1st and 2nd litter is limited to qualitative comparisons.

For Aim 3, normally distributed data were analyzed for effects of injury (TBI or sham-injured) and oil (Fish or Vegetable) by repeated measures ANOVA with factors of TBI, oil, and day after injury (1-28 days after injury) (Systat, v.12). Time after injury was analyzed as repeated measure for the sensorimotor function studies. Outliers identified by Systat were discarded from subsequent analyses. Post-hoc comparisons were made using 1-way ANOVA and the Fisher's Least Significant Difference test. In cases of non-normal distribution, data were analyzed by the Kruskal-Wallis nonparametric ANOVA with post-hoc comparisons made using Dunn's Multiple Comparisons test. Differences were considered significant if $P < 0.05$.

CHAPTER FOUR

ANALYSIS OF SENSORIMOTOR TESTS AND ASSESSMENT OF SEX DIFFERENCES IN SENSORIMOTOR FUNCTION AFTER TBI

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4.1 Abstract

Modeling juvenile TBI in rodents presents several unique challenges compared to adult TBI. One challenge is selecting appropriate sensorimotor behavioral tasks that enable the assessment of the extent of injury and recovery over time in developing animals. To address this challenge, we performed a comparison of common sensorimotor tests in Long-Evans rats of various sizes and developmental stages (PND 16-45, 35-190 g). Tests were compared and selected for their developmental appropriateness, scalability for growth, pre-training requirements, and throughput capability. Sex differences in response to TBI were also assessed. Grid walk, DigiGait™, rotarod, beam walk, spontaneous forelimb elevation test, and measurement of motor activity using the force-plate actometer were evaluated. Grid walk, gait analysis, and rotarod failed to meet one or more of the evaluation criteria. Beam walk, spontaneous forelimb elevation test, and measurement of motor activity using the force-plate actometer satisfied all criteria and were capable of detecting motor abnormalities in rats subjected to CCI on PND 17. No sex differences were detected in the acute effects of TBI or functional recovery during the 28 days after injury using these tests. These findings demonstrate the utility of these tests for the evaluation of sensorimotor function in studies using rat models of pediatric TBI, and suggest that pre-pubertal males and females respond similarly to TBI with respect to sensorimotor outcomes.

4.2 Introduction

Testing the effects of juvenile brain injuries in animal models, particularly assessing recovery of function after injury, presents several challenges that are not encountered in adult models. For example, age-matched controls must be used to account for developmental changes over the course of testing (Prins and Hovda, 2003). Also, many common methods to measure recovery in adult TBI models are unsuitable for use in a juvenile model due to their inability to scale for the rapid growth of the rats during the juvenile and adolescent periods and the necessity for extensive training prior to injury when the rats are quite immature. To address these challenges of studying functional outcomes in an animal model of juvenile TBI, we tested the suitability of six sensorimotor behavioral tests. The animal model is a CCI injury in 17-day old rats, a developmental time point that approximates the toddler period in humans with regard to motor function and used in a number of previous studies (Altman and Sudarshan, 1975; Westerga and Gramsbergen, 1990; Adelson, 1999; Prins and Hovda, 2003) The tests examined included the grid walk, accelerating rotarod, beam walk, and the spontaneous forelimb elevation test, which have been used in a variety of TBI studies in adult rodents (e.g., (Hamm et al., 1994; Hamm, 2001; Baskin et al., 2003; Onyszchuk et al., 2007; Chen et al., 2008). We also examined the automated DigiGait™ analysis system, which has been used to assess motor deficits after SCI (Ek et al., 2010; Springer et al., 2010), and the force-plate actometer, a sophisticated device typically used to measure locomotion and related behaviors (Fowler et al., 2001). Tests were evaluated based on their pre-training requirements, ability to scale for the growth of the rat over time, and whether the task was developmentally appropriate for 17-45-day old rats. Tests were

also assessed for throughput capacity. Three tests met our criteria and detected sensorimotor deficits in a contusion model of TBI using juvenile rats. Furthermore, juvenile male and female rats exhibit similar sensorimotor deficits following TBI.

4.3 Brief Procedures

All sensorimotor behavioral tests assessed are described in detail in Chapter 3 (Materials and Methods). With the anticipation that the sensorimotor tests would be used to assess the effects of a TBI occurring on PND 17 for at least 28 days after injury, the initial evaluation of the sensorimotor tests examined the use of each procedure with rats ranging in age from as early as PND 14 (allowing for as many as three days of pre-training, depending on the test) through PND 45. Tests were evaluated based on four criteria: the necessity for pre-training to learn the task before injury, ability to scale the test to accommodate animals of varying sizes, whether the task was developmentally appropriate for rats at all of the relevant ages, and the throughput capacity including the labor required to analyze the data. Scalability was determined empirically by testing the ability of rats of each age group to perform each test on each available size of apparatus.

4.4 Results

4.4.1 Evaluation of sensorimotor tests for use in a juvenile rat model of TBI

Six tests of sensorimotor function were evaluated for their developmental appropriateness, scalability for growth, pre-training requirements, and throughput capability. Results are summarized in **Table 4-1**.

The accelerating rotarod had relatively high throughput, due to having short trial durations and automated data collection. However, the test required a minimum of three days of training prior to injury. In the juvenile rat model, this necessitated that training begin on PND 14. At this point in development, which is roughly when the pup's eyes open, the rats proved to be insufficiently coordinated to learn this task. Scalability also proved to be a problem. Notably, commercial accelerating rotarods are available only in two sizes designed for adult mice or adult rats. The lack of intermediate sizes makes it difficult to accommodate the growth of juvenile rats over the course of testing without also changing the task for rats of different ages. Thus, the rotarod test was also eliminated for use with the juvenile TBI model.

The beam walk test required only minimal pre-training prior to injury in order for the rats learn to traverse the beam. Two brief training sessions prior to injury, requiring about 15 minutes per rat, were adequate for the pups to meet the criterion of being able to traverse the full length of the beam, and the second session could be done on the day of injury prior to surgery. This test was easily scalable for rats of different sizes by using beams with increasingly larger diameters. Rats as young as PND 16 were capable of completing the test. Time required to test each animal was minimal; thus, experimental throughput was acceptable. Analysis of recordings was comparatively labor intensive, but was less so than for the grid walk test. Thus, the beam walk met all criteria for use with the PND 17 rat model of TBI and was further evaluated for its ability to detect deficits after injury.

The spontaneous forelimb elevation test, or the cylinder test, required no pre-training, though a test session prior to injury is required to determine if any baseline limb

preference exists. This test was easily scalable by using cylinders of varying diameters and wall heights. In addition, the test was readily performed by rats of any of the ages tested. Throughput was relatively high. Thus, the spontaneous forelimb elevation test met all the criteria for use with the PND 17 rat model of TBI and was further evaluated for its ability to detect deficits after injury.

Assessment of motor activity using the force-plate actometer required no pre-training prior to injury, had no developmental limitations, and had a very high throughput due to automated data collection. The actometer chamber accommodated rats of all of the ages of interest, though the relationship between the size of the rat and the size of the chamber changed as the rat grows. Accordingly, the force-plate actometer was also assessed for its ability to detect a deficit after injury.

Table 4-1. Evaluation of tests of rodent sensorimotor function for suitability for use with a juvenile rat model of TBI.

Test	Minimal/No Pre-Training	Fully Scalable	Develop. Approp.[2]	Adequate Throughput	Detects TBI Deficits
Grid walk	Yes	No[1]	Yes	No	--
DigiGait™	No	Yes	Yes	No	--
Rotarod	No	No[1]	No	Yes	--
Spont. forelimb	Yes	Yes	Yes	Yes	Yes
Beam walk	Yes	Yes	Yes	Yes	Yes
Force-plate actometer	Yes	Yes	Yes	Yes	Yes

[1]Large (rat size) and small (mouse size) only

[2]Can be performed by 14-16-day old rats for training or baseline measurements as required by the specific test.

4.4.2. Effects of TBI in Juvenile Rats and Assessment of Sex Differences

4.4.2.1 *Beam Walk*

With respect to percent of contralateral foot slips (**Figure 4-1A**), three-way ANOVA indicated a main effects of injury ($F_{1,48} = 89.35$, $P < 0.001$) and day after TBI ($F_{4,192} = 27.72$, $P < 0.001$), and an interaction of injury with day after injury ($F_{4,192} = 5.29$, $P < 0.001$). Post- hoc analysis indicated that rats with TBI exhibited an increased percentage of contralateral foot slips while traversing the beam compared to sham-injured rats on each day of testing. In addition, while all rats showed improvement in

performance between PND 17 and PND 45, those with TBI exhibited a more pronounced improvement in the first 14 days after injury. There was no main effect of sex, or interaction of sex with the other parameters.

Traverse speed exhibited main effects of injury ($F_{1,48} = 5.01$, $P < 0.05$) and day after injury ($F_{4,192} = 46.63$, $P < 0.001$), but no interaction of injury with day after injury (**Figure 4-1B**). This indicated that rats with TBI traversed the beam more slowly than sham-injured rats and that all rats showed improvement in performance across test sessions. There was no main effect of sex, or interaction of sex with the other parameters.

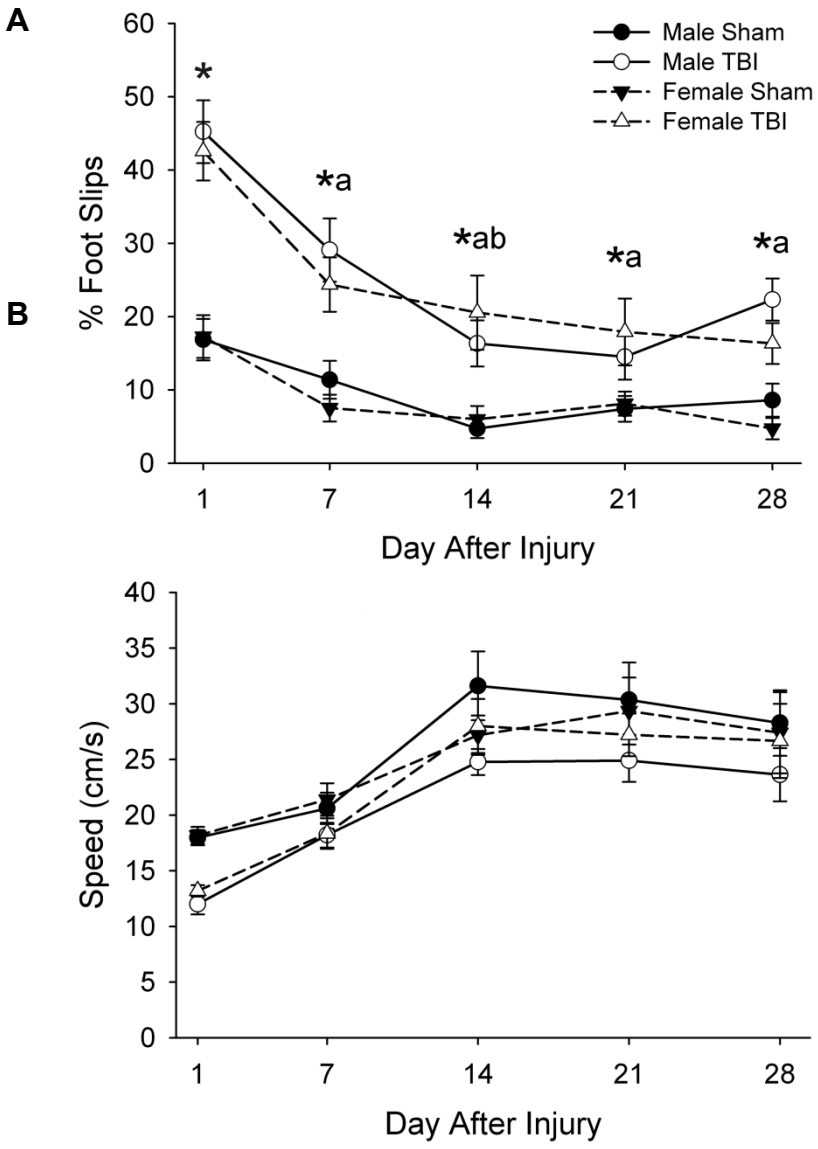


Figure 4-1. Effects of TBI in male and female juvenile rats in the beam walk test on percent foot slips (A) and traverse speed (B). Data are presented as the group means ± SEM (n = 12-15 per group). Traverse speed exhibited only main effects of injury ($P < 0.05$) and day after injury ($P < 0.01$). *TBI different from Sham, same day and for males and females combined ($P < 0.05$). [a]Different from day one, same injury

and for males and females combined ($P < 0.01$). [b]Different from day seven, same injury and for males and females combined ($P < 0.05$) by ANOVA and Fisher's LSD test.

4.4.2.2 *Spontaneous Forelimb Elevation Test*

No differences in laterality were detected prior to injury. After injury, three-way ANOVA revealed only a main effect of injury ($F_{1,50} = 28.05$, $P < 0.001$) indicating that rats with TBI exhibited greater tendency to use the forelimb ipsilateral to the injury compared to sham-injured rats on all days after injury (**Figure 4-2**). There was no effect of sex, or interaction of sex with the other parameters.

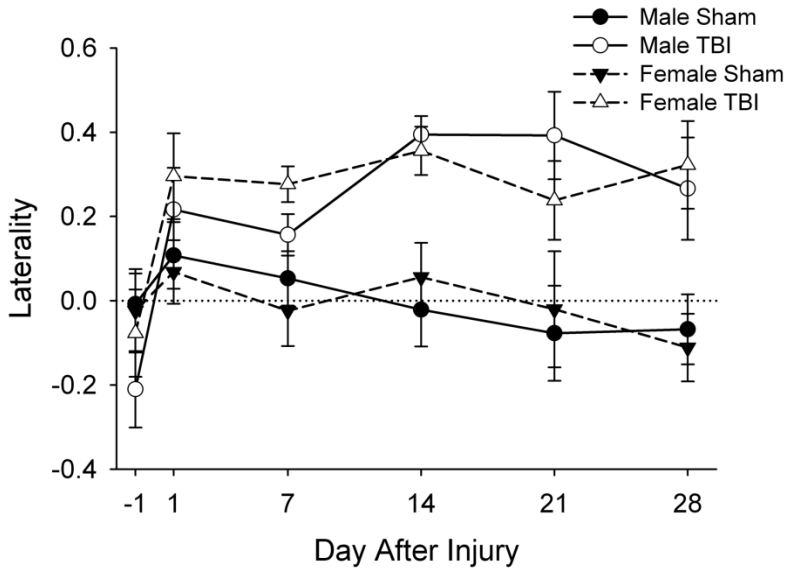


Figure 4-2. Effects of TBI in male and female juvenile rats in the spontaneous forelimb elevation test. Data are presented as the group means ± SEM (n = 12-15 per group). No differences in laterality were detected prior to injury. After injury, three-way

ANOVA revealed only a significant main effect of injury ($P < 0.001$) indicating a greater laterality in rats with TBI rats at all time points.

4.4.2.3 *Assessment of Motor Activity Using Force-Plate Actometry*

Distance traveled exhibited a main effect of day after injury ($F_{4,200} = 40.34$, $P < 0.001$) such that rats exhibited less locomotor activity with increasing age (**Figure 4-3A**). There were no main effects of injury or sex. An interaction of day with sex was detected ($F_{4,200} = 2.92$, $P < 0.05$), but proved non-significant after post-hoc analysis.

Bouts of low mobility (≥10 sec spent within a 20-mm radius) (**Figure 4-3B**), and low mobility distance (the distance traveled during bouts of low mobility) (**Figure 4-3C**) exhibited significant main effects of injury ($F_{1,50} = 13.48$, $P < 0.001$ and $F_{1,50} = 22.50$, $P < 0.001$, respectively), day ($F_{4,200} = 51.33$, $P < 0.001$ and $F_{4,200} = 165.37$, $P < 0.001$, respectively) and interactions of injury with day after injury ($F_{4,200} = 9.16$, $P < 0.001$ and $F_{4,200} = 24.97$, $P < 0.001$, respectively). Post -hoc analysis indicated that rats with TBI had fewer low mobility bouts on days one, seven, and 14 after injury and lower low mobility distance on day one after injury than in sham-injured rats ($P < 0.05$). There was no main effect of sex on any of these parameters. There was an interaction of sex with day after injury for bouts of low mobility ($F_{4,200} = 3.28$, $P < 0.05$) such that females had fewer bouts than males on day 21 after injury ($P < 0.05$). There were no other interactions of sex with injury for either parameter or with day after injury for low mobility distance.

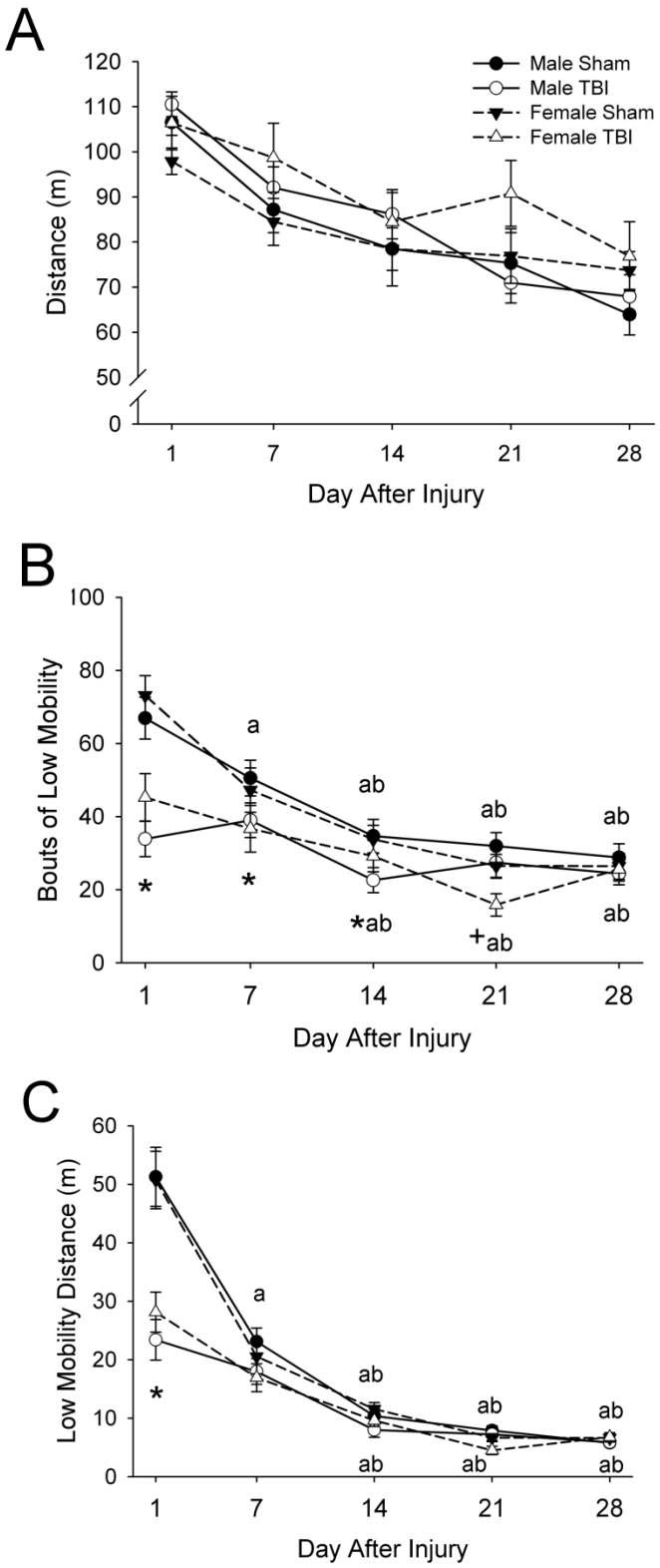
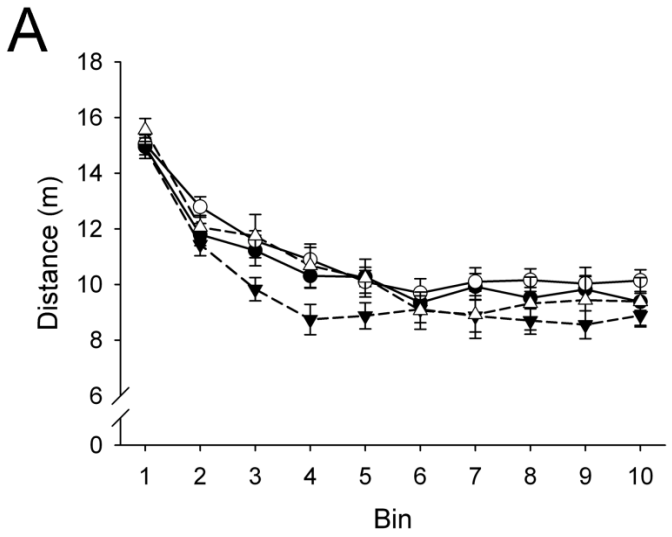

A
Male Sham
Male TBI
Female Sham
Female TBI
Distance (m)
120
110
100
90
80
70
60
50
0
1
7
14
21
28
Day After Injury
B
Bouts of Low Mobility
100
80
60
40
20
0
a
ab
ab
ab
*
*
*ab
+ab
ab
1
7
14
21
28
Day After Injury
C
Low Mobility Distance (m)
60
50
40
30
20
10
0
a
ab
ab
ab
ab
*
ab
ab
ab
1
7
14
21
28
Day After Injury

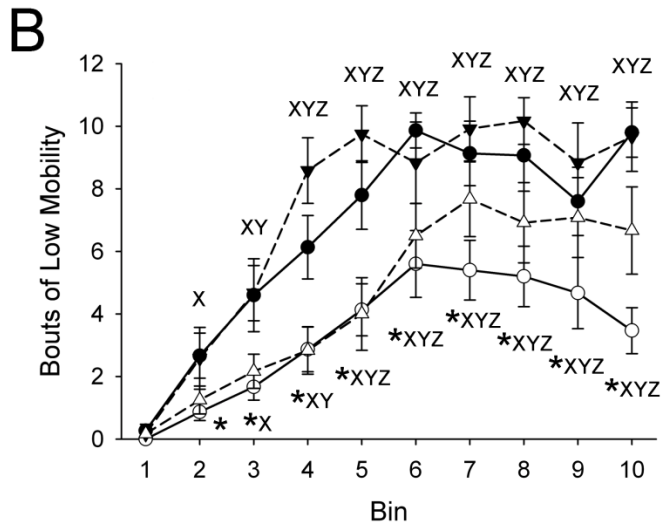
Figure 4-3. Effects of TBI in male and female juvenile rats on distance traveled (A), bouts of low mobility (B), and low mobility distance (C) assessed using the force-plate actometer. Data are presented as the group means ± SEM (n = 12-15 per group) for the entire 20-minute observation period on days one, seven, 14, 21, and 28 after injury. Low mobility bouts are defined as ≥10 sec spent within a 20-mm radius. Low mobility distance is the distance traveled during bouts of low mobility. Distance traveled (A) exhibited only a main effect of day after injury ($P < 0.001$). *TBI different from Sham, same day and for males and females combined ($P < 0.05$). [a]Different from day one, same injury and for males and females combined ($P < 0.05$). [b]Different from day seven, same injury and for males and females combined ($P < 0.05$). [+]Female different from male, same day and for TBI and sham-injured combined ($P < 0.05$) by ANOVA and Fisher's LSD test.

The acute effects of TBI on day one after injury were further analyzed for effects on distance traveled, low mobility bouts, and low mobility distance across the 20-minute observation period in two-minute time bins (**Figure 4-4**). For distance traveled, there was a main effect only of time bin ($F_{9,450} = 89.60$, $P < 0.001$), consistent with the habituation of the rats to the actometer chamber. Bouts of low mobility exhibited main effects of injury ($F_{1,50} = 29.31$, $P < 0.001$) and time bin ($F_{9,450} = 51.49$, $P < 0.001$), and an interaction of injury with time bin ($F_{9,450} = 3.49$, $P < 0.001$). Post-hoc analysis indicated that although the number of low mob bouts increased across time bins in both TBI and sham-injured rats, the number of bouts of low mobility was lower in rats with TBI during

time bins two through 10 ($P < 0.05$). Low mobility distance exhibited main effects of injury ($F_{1,50} = 33.66$, $P < 0.001$) and time bin ($F_{9,450} = 47.56$, $P < 0.001$), and an interaction of injury with time bin ($F_{9,450} = 4.58$, $P < 0.001$). Post-hoc analysis indicated that low mobility distance increased across time bins in all groups, but the increase was smaller in rats with TBI than in sham-injured rats. There were no effects of sex, or interaction of sex with the other parameters.

Turning bias (**Figure 4-5**) exhibited a significant main effect of sex ($F_{1,50} = 6.96$, $P < 0.05$) such that males exhibited a tendency to turn to the left, whereas females did not exhibit a significant turning bias. Turning bias also exhibited a significant main effect of injury ($F_{1,50} = 19.28$, $P < 0.001$) such that rats with TBI exhibited greater tendency to turn towards the contralateral side compared to sham-injured rats, and an interaction of injury with day after injury ($F_{4,200} = 2.44$, $P < 0.05$). There were no interactions of sex with injury, sex with day after injury, or sex with injury with day after injury. Post-hoc analysis indicated that turning bias was greater in female rats with TBI on days one and 21 ($P < 0.05$). A similar pattern was observed after TBI in male rats although the increase in turning bias on day 21 after injury was not quite significant ($P = 0.054$).


A
Distance (m)
18
16
14
12
10
8
6
0
1 2 3 4 5 6 7 8 9 10
Bin


B
Bouts of Low Mobility
12
10
8
6
4
2
0
X
XY
XYZ
*X
*XY
*XYZ
1 2 3 4 5 6 7 8 9 10
Bin

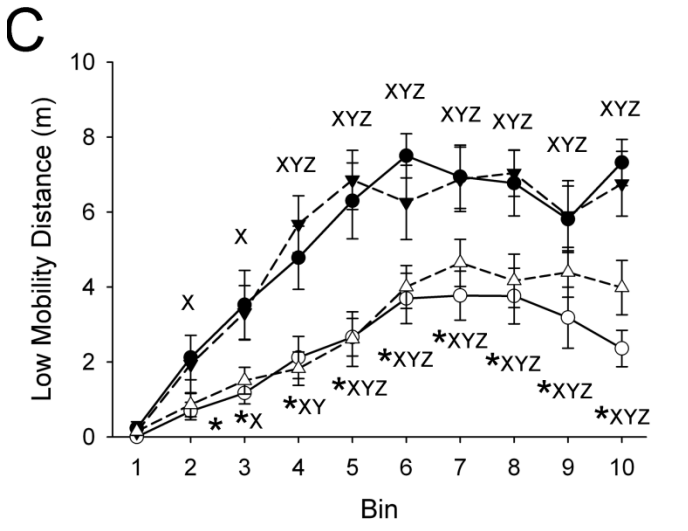

C
Low Mobility Distance (m)
10
8
6
4
2
0
X
XYZ
*X
*XY
*XYZ
1 2 3 4 5 6 7 8 9 10
Bin

Figure 4-4. Effects of TBI in male and female juvenile rats on distance traveled (A), bouts of low mobility (B), and low mobility distance (C) assessed using the force-plate actometer on day one after injury. Data are presented as the group means ± SEM (n = 12-15 per group) and are cumulative activity across the 20-minute observation period is presented in two-minute time bins. Low mobility bouts are defined as ≥10 sec spent within a 20-mm radius. Low mobility distance is the distance traveled during bouts of low mobility. Distance traveled (A) exhibited only a main effect of time bin ($P < 0.01$). *TBI different from Sham, same time bin and for males and females combined ($P < 0.05$). ^{X}Different from time bin 1, same injury and for males and females combined ($P < 0.05$). ^{Y}Different from time bin 2, same injury and for males and females combined ($P < 0.05$). ^{Z}Different from time bin 3, same injury and for males and females combined ($P < 0.05$) by ANOVA and Fisher's LSD test.

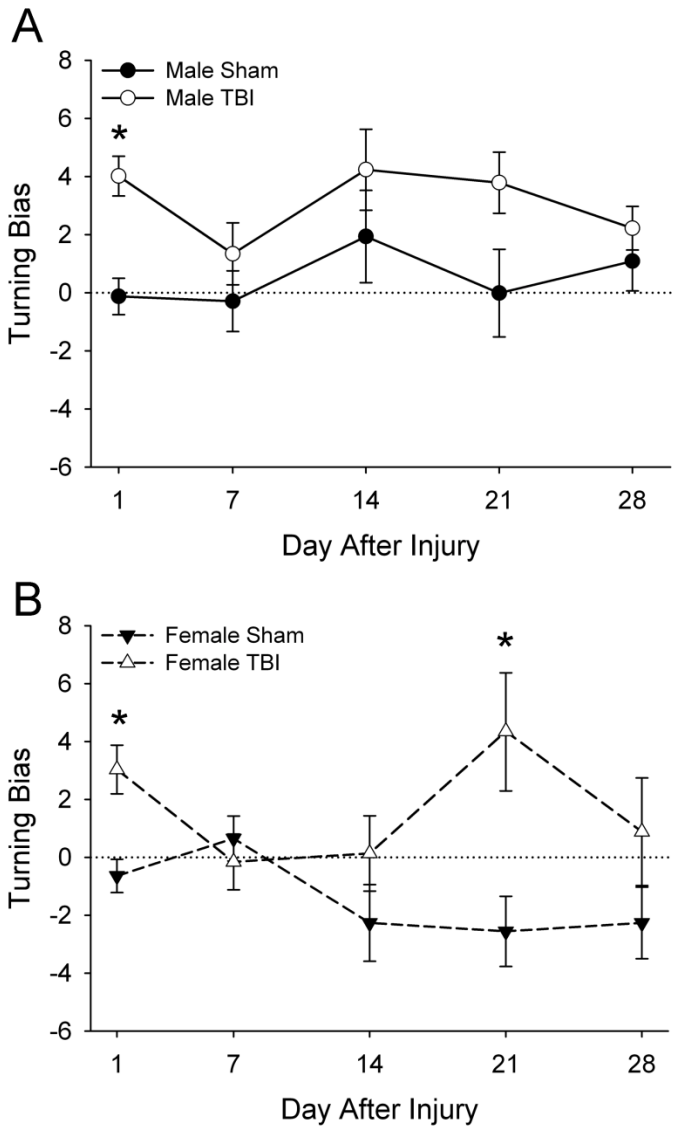


Figure 4-5. Effects of TBI in male and female juvenile rats on turning bias assessed using the force-plate actometer. Data are presented as the group means ± SEM (n = 12-15 per group). Turning bias exhibited a significant main effect of sex ($P < 0.05$) such that males (A) males exhibited a tendency to turn to the left, whereas females (B) did not exhibit a significant turning bias. *TBI different from sham-injured ($P < 0.05$) by ANOVA and Fisher's LSD test.

4.4.2.4 *Effects on Body Weight*

Body weight exhibited main effects of sex ($F_{1,50} = 71.43$, $P < 0.001$), day after injury ($F_{4,200} = 6892.67$, $P < 0.001$), and an interaction of sex with day after injury ($F_{4,200} = 121.04$, $P < 0.001$) by 3-way ANOVA such that males were heavier than females and 14, 21, and 28 days after injury ($P < 0.001$) (**Figure 4-6**). There was no main effect of injury, nor any other interactions. These results indicate that both male and female, and TBI and sham-injured rats maintained regular weight gain and growth throughout the study.

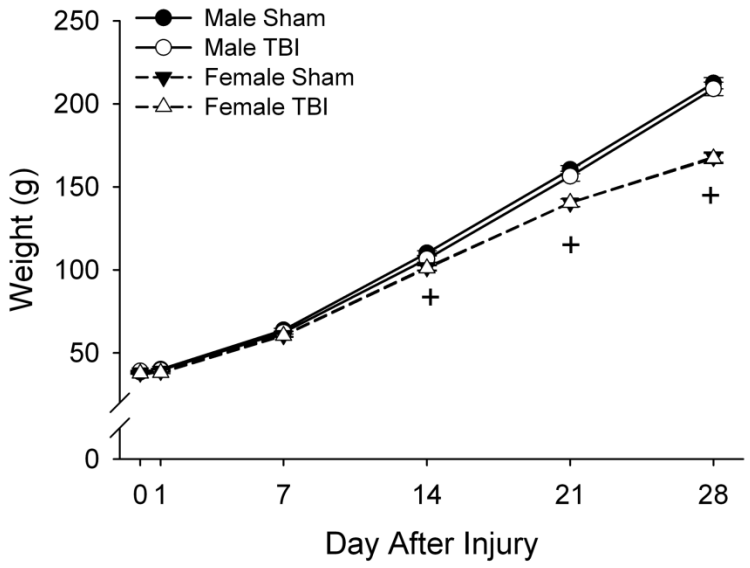


Figure 4-6. Effects of TBI in male and female juvenile rats on body weight. Data are presented as the group means ± SEM (error bars are smaller than symbols) (n = 12-15 per group). There were no significant differences in weight as a result of TBI. Error bars that are not visible are smaller than the symbols. $^{+}$Female different from male for TBI and sham-injured combined ($P < 0.001$) by ANOVA and Fisher's LSD test.

4.5 Discussion

4.5.1 Identification of Sensorimotor Tests for use in Juvenile Rats

This study assessed the suitability of several behavioral tests for the detection of sensorimotor deficits the day after TBI in 17-day old juvenile rats, as well as during the short-to-midterm recovery period. A number of changes in locomotor development occur during this period (PND 17 to PND 45). Although adult-like walking patterns begin to emerge around PND 15, substantial hindlimb coordination does not develop until approximately PND 20 (Altman and Sudarshan, 1975; Westerga and Gramsbergen, 1990). Visual function, another contributor to sensorimotor function, also changes substantially during this period of development. Pups do not open their eyes until about PND 14, and visual acuity does not fully mature until approximately PND 45 (Fagiolini et al., 1994). The immaturity of the motor and visual systems at PND 17 (and even earlier if the tests require pre-training), combined with the dramatic changes in neurodevelopment between PND 17 and adulthood and the rats' rapid growth, underscore the critical need to identify appropriate sensorimotor tests for use in the developing rat.

Several types of behavioral assessments have been used with juvenile rodent models of TBI. Most typically, the Morris water maze has been used to assess cognitive function after TBI in 17-day old rats (Adelson et al., 1997; Prins and Hovda, 1998; Adelson et al., 2000; Fineman et al., 2000; Hickey et al., 2007; Raghupathi and Huh, 2007; Huh et al., 2008; Appelberg et al., 2009; Ochalski et al., 2010). Motor and vestibular function have also been assessed using methods such as the beam balance,

inclined plane, and grip tests (Adelson et al., 2000). These tests, however, have limited ability to detect sided deficits, which would result after a unilateral TBI.

Sensorimotor testing in adult rodent models of TBI and other neural injuries is common (for review see Fujimoto et al. (2004), and has been done using a variety of tests such as the grid walk, accelerating rotarod, DigiGait™, beam walk, and the spontaneous forelimb elevation test. Although these tests assess the motor deficits of interest after TBI, the present data demonstrate that at least some of them proved unsuitable, or not feasible, for use with young juvenile rats. Notably, the rotarod, a well-established test of motor function first used to describe motor deficits in adult rats after TBI by Hamm and colleagues (1994), proved to be unsuitable because of inadequate scalability of the instruments and because the youngest rats of interest in our studies were not developmentally capable of performing the test. The grid walk test, which has been used to detect limb deficits in adult and neonatal rat and adult mouse SCI models (Kunkel-Bagden et al., 1992; Pajoohesh-Ganji et al., 2010; Pitzer et al., 2010) and adult mouse TBI models (Baskin et al., 2003; Onyszchuk et al., 2007; Onyszchuk et al., 2008), could potentially be used with developing rats, but would require the fabrication of multiple appropriately-scaled apparatuses. Furthermore analysis of the grid walk recordings was very labor intensive. Likewise, DigiGait™, which has been used to detect variances in gait after SCI (Ek et al., 2010; Springer et al., 2010), suffered from issues relating to development of the rats and experimental throughput. Thus, these tests were disfavored for use with the 17-day old juvenile rat TBI model.

In contrast, several tests proved appropriate and expedient for sensorimotor testing in juvenile rats. The spontaneous forelimb elevation test, beam walk, and

measurement of motor activity using the force-plate actometer provided useful data in 17-day old rats. The spontaneous forelimb elevation test, which detects CNS injury-related forelimb deficits in multiple rodent models of neurologic injury (Schallert et al., 2000; Baskin et al., 2003; Li et al., 2004; Bretzner et al., 2008; Vandeputte et al., 2010), was easily modified to accommodate growing rats by using cylinders of varying sizes. Likewise the beam walk test, which detects hindlimb deficit after TBI and other neural injuries in adolescent and adult rats (e.g., (Wagner et al., 2007; Appelberg et al., 2009; Kalonia et al., 2010; Scafidi et al., 2010; Sgado et al., 2010), proved achievable by rats as young as PND 16, thus allowing for the pre-training necessary for this test prior to injury on PND 17. The force-plate actometer, which has not been used in TBI but has been used to detect altered motor function in a mouse model of Huntington's disease (Fowler et al., 2009), was suitable for all ages and sizes of rats. In agreement with previous studies (e.g. (Spear and Brake, 1983; Levant et al., 2010), distance traveled in the actometer decreased with age. Although it is not certain whether this change in activity reflects neurodevelopmental maturation of the rats, the change in the size relationship between the rat and the actometer chamber, or both, this procedure is clearly capable of assessing behavior in rats of all the ages of interest in this study. In contrast to the tests previously used in juvenile TBI (see above), the spontaneous forelimb elevation, beam walk, and force-plate actometer tests have the additional advantages of detecting deficits preferentially affecting one side of the body that result from a unilateral CCI injury. In addition, combined use of the spontaneous forelimb elevation and beam walk tests enables separate resolution of fore- and hind-limb

deficits. Furthermore, the tests have sufficient throughput capabilities to render them suitable for testing relative large numbers of subjects.

4.5.2 Effects of TBI on Sensorimotor Function in the Juvenile Rat Model

All tests deemed suitable for use in a CCI model of TBI in 17-day old rats detected sensorimotor deficits after injury. While these deficits are likely due to the effect of the CCI-injury, the present experimental design does not allow for the differentiation of the contributions of the craniotomy and CCI to this effect.

Forelimb deficits assessed by the spontaneous forelimb elevation test were first observed on the day after injury and persisted for at least 28 days after injury (**Figure 4-2**). The persistent deficit in forelimb function after TBI is likely indicative of poorer long-term outcome. The persistence of this deficit also suggests that this test may be useful in assessing interventions to improve outcomes of TBI. The detection of both initial and persistent functional impairment may also prove useful in discerning the mitigation of the various pathological processes initiated by TBI, such as acute neuroinflammation or BBB disruption, which resolve relatively quickly after injury (Adelson et al., 1998; Bolton and Perry, 1998; Gaetz, 2004; Walker et al., 2009), as well as long-term sequelae of TBI such as apoptosis and glial scar formation (Walker et al., 2009).

The beam walk also detected both acute functional impairment and persistent deficits after TBI (**Figure 4-1A**), indicating that this test should be useful in assessing therapeutic interventions for TBI. The improvement in percent foot slips in this test between days one and 14 after injury was greater in the rats with TBI than in sham-injured rats. This indicates some recovery of function after injury in addition to

maturation-associated improvements in limb coordination and/or the effects of practice with repeated testing that were also observed in the sham-injured group. The speed with which the rats traversed the beam also revealed effects of TBI, but only on day one after injury (**Figure 4-1B**). Thus, traverse speed may be useful for comparing the initial effects of injuries of varying severity or the effects of interventions that affect the initial inflammatory response.

The sensorimotor functions assessed by force-plate actometry also clearly detected effects of TBI. Rats with TBI exhibited an increased turning bias toward the left, away from the injured hemisphere (**Figure 4-5**) consistent with the fore- and hind-limb deficits observed in the spontaneous forelimb elevation and beam walk tests. This effect was observed one day after injury, and also at some of the later time points; however, the detection of TBI-induced turning bias was not entirely consistent over time. Accordingly, this parameter should prove useful in detecting acute effects of TBI, though additional evaluation is required to determine its utility for the assessment of recovery after TBI. In addition, bouts of low mobility and low mobility distance, which assess the number of instances that the rats spend time moving confined within a small area (20 mm radius) and the amount of movement within the 20-mm radius, were altered after TBI, but this effect was observed only on the day after injury. Thus, these low mobility parameters may be most useful for assessing the acute effects of TBI, or may prove effective for detecting the effects of factors that may worsen TBI outcomes. Interestingly, rats with TBI exhibited fewer bouts of low mobility (**Figure 4-3B**) and decreased low mobility distance (**Figure 4-3C**) indicating that they make fewer small movements than sham-injured rats when not locomoting. Despite making fewer small

movements, analysis of distance traveled, bouts of low mobility, and low mobility distance across the observation period on day one after injury indicated that the pattern of behavior across the test session was generally similar between TBI and sham-injured rats (**Figure 4-4**). The low mobility parameters are typically used as an index of stereotyped behavior (Fowler et al., 2001), but can also be used to measure other types of movements such as grooming. It is likely that this decrease in low mobility activity represents a transient decrease in grooming, which has been reported previously in adult rats with contusion injuries (Grossman et al., 2011).

4.5.3 Sex Difference in Response to TBI in Juvenile Rats

Sex differences in response to TBI are found in both humans and in adult rodents. Some studies suggest that females have less-adverse responses to TBI than do males, most likely due to the neuroprotective effects of estrogen and progesterone (Roof and Hall, 2000; Siegel et al., 2010), which decrease intracranial pressure and cerebral perfusion pressure after TBI (Shahrokhi et al., 2010), although poorer outcomes in women of childbearing age have also been reported (Bazarian et al., 2010). In addition to these effects attributed to sex hormones, sex differences exist prior to puberty as a result of genes encoded on the sex chromosomes that have sex-specific effects on the brain, selective gene inactivation, and sex-biased expression of genes expressed on other chromosomes (Arnold, 2009; Mank, 2009). Consistent with these pre-pubertal differences, some sex differences in response to neural injury have been reported in juvenile rodents. For example, in a neonatal hypoxia-ischemia model, females exhibited a caspase-3 mediated mechanism of apoptosis whereas males

exhibited a caspase-independent mechanism of cell death (Zhu et al., 2006). These findings suggest that both adult and juvenile rodents exhibit sex differences in cellular response to neural injury; however, the full extent of differences prior to puberty including differences in sensorimotor function after TBI is unknown.

Because of the potential for sex differences in the juvenile rat model, the effects of sex on sensorimotor response to TBI were assessed. In this study, male and female juvenile rats exhibited generally similar behavior in the sensorimotor tests irrespective of injury, with two notable exceptions. First, females had fewer low mobility bouts at the 21 day time point after injury (PND 38) (**Figure 4-3B**), which may be attributable to puberty-associated changes. This effect is consistent with previous observations that locomotor activity is similar in male and female rats until late adolescence (PND 52-54) when females begin to exhibit higher levels of activity that varies with the estrous cycle (Finger, 1969; Lynn and Brown, 2009). In addition, a main effect of sex was observed for turning bias (**Figure 4-5**), where males had greater baseline turning bias than females. Despite this sex difference in baseline turning bias, both male and female rats responded similarly after TBI with respect to turning bias, as well as in all of the other tests used in this study. This supports the hypothesis that the sex differences in TBI outcomes observed in adults are due to post-pubertal secretion of sex hormones.

4.6 Conclusion

We have identified sensorimotor behavioral tests for use in assessing effects shortly after injury and during the short-to midterm recovery period in a PND 17 rat model of TBI. The beam walk and the spontaneous forelimb elevation tests are easily

scalable for growth over time, have a relatively high throughput, are appropriate for rats of any developmental stage, and require limited or no pre-training. Both tests detected acute and persistent functional deficits indicating that they may be useful in identifying interventions to improve outcomes after TBI. The force-plate actometer also detected acute functional deficits after TBI; however the utility of this test and its ability to detect long-term deficits require further evaluation. The lack of sex differences in outcomes in juvenile rats after TBI suggests that outcomes in pre-pubertal males and females may be similar, at least with respect to sensorimotor function.

CHAPTER FIVE

LOW BRAIN DHA CONTENT WORSENS SENSORIMOTOR OUTCOMES AFTER TBI AND DECREASES TBI-INDUCED TIMP1 GENE EXPRESSION IN JUVENILE RATS

5.1 Abstract

Children under five years of age are at high risk for sustaining TBI and tend to have poorer outcomes than adults. N-3 (omega-3) polyunsaturated fatty acids, of which DHA is the major species in brain, accumulate in the brain during late gestation and early childhood, and have multiple neuroprotective and anti-inflammatory activities. Low dietary N-3 fatty acid content results in decreased DHA accumulation in the developing brain. This study examined the effects of dietary modulation of brain DHA content on sensorimotor and molecular outcomes after TBI in a juvenile rat model. Long-Evans rats raised from conception on diets containing adequate n-3 fatty acids (Control) or low in n-3 fatty acids (Deficient), resulting in decreases in brain DHA of 25% and 54%, respectively, were subjected to a CCI or sham surgery on PND 17. Rats with decreased brain DHA levels had poorer sensorimotor outcomes, as assessed with force-plate actometry and the spontaneous forelimb elevation (cylinder) test, after TBI. *Ccl2*, *Gfap*, and *Mmp9* mRNA levels, and MMP-2 and -9 enzymatic activities were increased after TBI regardless of brain DHA level. Lesion volume was also not affected by brain DHA level. In contrast, TBI-induced *Timp1* was lower in rats fed the Deficient diet and was correlated with brain DHA level. These data suggest that decreased brain DHA content contributes to poorer outcomes after TBI through a mechanism involving modulation of *Timp1* gene expression.

5.2 Introduction

Traumatic brain injury (TBI) is one of the leading causes of acquired disability and death in children under five years of age (Faul et al., 2010). Children in this age group tend to have poorer outcomes than adults after sustaining a severe TBI. Adverse outcomes can include sensorimotor deficits, difficulties with long-term memory, attention, language, problem solving, and managing stress and emotions, as well as increased risk for epilepsy and aging-related diseases such as Alzheimer's and Parkinson's (Ylvisaker et al., 2001; NIoNDaS, 2002; Davis and Dean, 2010; Anderson et al., 2011). Worsened outcomes in children are likely due to the unique features of the pediatric population that make the pathophysiology of juvenile TBI different from that of adults; for example, young children have greater brain plasticity, less white matter myelination, higher brain water content, and reduced skull rigidity (Maxwell, 2012; Pinto et al., 2012a; Pinto et al., 2012b). Accordingly, it is imperative that juvenile TBI be studied independently of adult TBI using an age-specific model (Prins and Hovda, 2003).

The n-3 long chain- polyunsaturated fatty acids (LC-PUFA) are a major component of neuronal membranes and accumulate in the brain through early childhood. LC-PUFAs influence cellular function by forming the micro-environment around membrane-bound proteins, modifying lipid rafts (Salem et al., 2001a; Shaikh, 2012) and modulating gene expression through activation of transcription factors (e.g., PPAR and RXR) (Khan and Vanden Heuvel, 2003). Additionally, LC-PUFAs are precursors for lipid-derived signaling molecules such as leukotrienes, prostaglandins, and the more recently discovered anti-inflammatory and pro-resolving lipoxins,

resolvins, maresins and protectins (Serhan et al., 2008a; Serhan et al., 2008b; Serhan et al., 2009).

DHA is derived from the essential fatty acid ALA and represents approximately 15% of total lipids in the brain (Sinclair, 1975). DHA accumulates in the brain during late gestation and early childhood, and is supplied by the mother to the fetus *in utero* and via breast milk after birth (Clandinin et al., 1980b; Clandinin et al., 1980a). DHA accumulation in the brain is a function of the quantity of n-3 LC-PUFAs in the diet, which is notably low in the Western diet (Simopoulos, 2011). Although DHA deficiency does not result in gross developmental pathology (Gordon, 1997), adequate DHA accumulation is essential for optimal brain and visual development and function (Salem et al., 2001b; McNamara and Carlson, 2006).

N-3 LC-PUFAs and their metabolites have anti-inflammatory effects in neural and non-neural tissues (Orr et al., 2012). In animal models, administration of DHA or consuming a diet high in n-3 LC-PUFAs, has been beneficial in various types of neuronal injuries including TBI (Javierre et al., 2006; Huang et al., 2007; Wu et al., 2007; Pan et al., 2009; Bailes and Mills, 2010), although a worsened outcome has also been reported (Yang et al., 2007). However, very little is known about what role endogenous brain DHA has in neuroprotection after TBI. CCI, a model of TBI, causes a rapid and sustained increase in free fatty acids in the brain (Homayoun et al., 2000). We can thus hypothesize that populations lacking a full complement of endogenous n-3 LC-PUFAs, such as young children consuming a Western diet, will be likely to have fewer of these fatty acids released after TBI and thus a poorer outcome. Accordingly, we investigated the effects of low dietary n-3 LC-PUFA content, and consequently lower

brain DHA levels, on outcomes of TBI in a juvenile rat model. Severity and persistence of sensorimotor outcomes was assessed, as well as expression of representative cellular mediators involved in the various pathophysiological processes initiated by TBI. Furthermore, by using procedures developed in our previous studies to produce rats with decreases in brain DHA content of varying magnitudes while feeding a single n-3 LC-PUFA-deficient diet (Ozias et al., 2007), we can determine the dose-response effects of brain DHA content on the outcomes of TBI. The results of this study demonstrate worsened sensorimotor outcomes following TBI are associated with lower brain DHA level and that these functional deficits correlate with mRNA levels of *Timp1*, indicating a potential mechanism for decreased brain DHA-induced sensorimotor deficits after TBI.

5.3 Results

5.3.1 Effects of Diets and Breeding Procedures on Brain Phospholipid Fatty Acid Composition

In agreement with previous studies (Ozias et al., 2007), 1st litter rats raised on the Deficient diet had a 25% decrease in brain DHA compared to those raised on the Control diet ($P < 0.01$) (**Figure 5-1**). Brain DHA content of 2nd litter rats raised on the Deficient diet was decreased 54% ($P < 0.001$ v. Control diet, $P < 0.001$ v. 1st litter Deficient diet). This decrease in DHA content was accompanied by compensatory increases in n-6 DPA [22:5(n-6)] and no alteration in AA [20:4(n-6)] content, in agreement with previous studies (Galli et al., 1971).

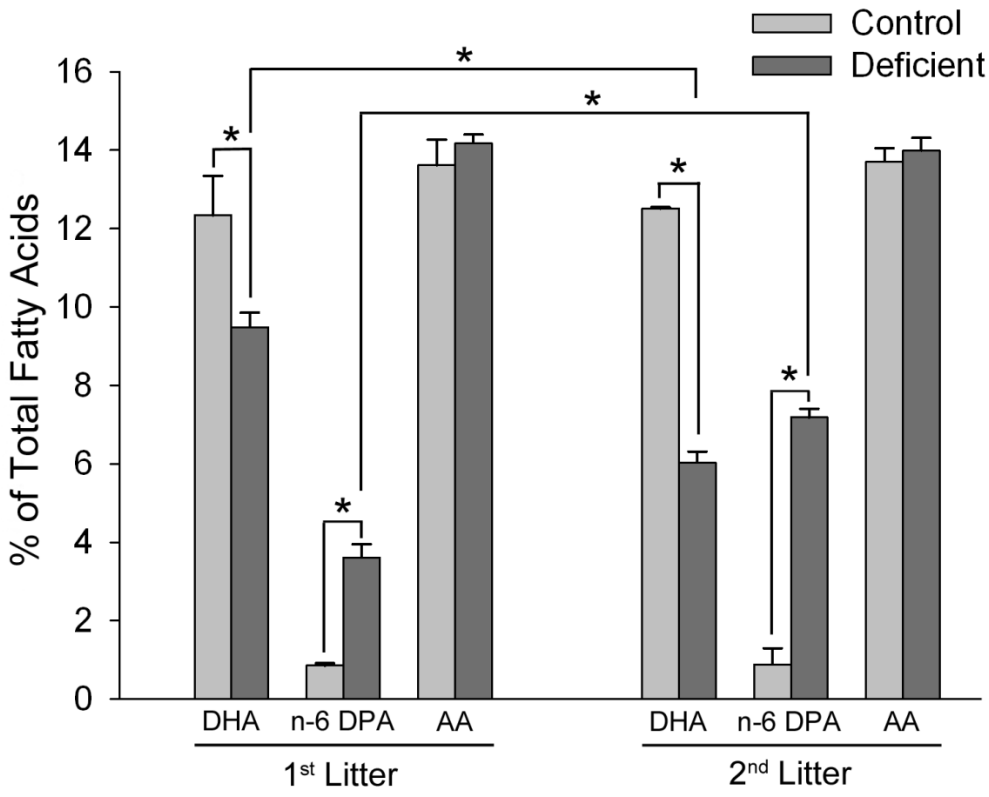


Figure 5-1. Effects of diet and breeding protocols on brain total phospholipid fatty acid composition. Data are presented as the mean ± SEM (1st litter: n = 4-7 per group; 2nd litter: n = 8-9 per group selected at random from the total sample pool). *P < 0.05 by ANOVA and Fisher's LSD test.

5.3.2 Effects of Diets and Breeding Procedures on TBI-Induced Sensorimotor Deficits

TBI resulted in altered locomotor activity on day one after injury as assessed using the force-plate actometer (**Figure 5-2**). TBI-induced locomotor deficits returned to near-sham levels after day one and therefore, data for subsequent test days are not shown.

In rats raised on the Control diet, TBI resulted in a decrease in the number of low mobility bouts ($P < 0.05$) in agreement with our previous findings and/or decreased low mobility distance (Russell et al., 2011) ($P < 0.05$) but no difference in total distance traveled. Both 1st and 2nd litter rats raised on the Deficient diet exhibited decreases in both bouts of low mobility ($P < 0.05$), low mobility distance ($P < 0.05$) and increased total distance traveled ($P < 0.05$) after TBI.

In the spontaneous forelimb elevation (cylinder) test, all injured rats exhibited a preference for using the ipsilateral limb that persisted throughout the 28-day testing period ($P < 0.01$) (**Figure 5-3**). The effects of TBI on forelimb preference in 1st litter rats raised on the Deficient diet were not different from those raised in the Control diet. However, in the 2nd litter, injured rats raised on the Deficient diet had greater sustained preference for the forelimb ipsilateral to the injury than injured rats fed the Control diet ($P < 0.05$).

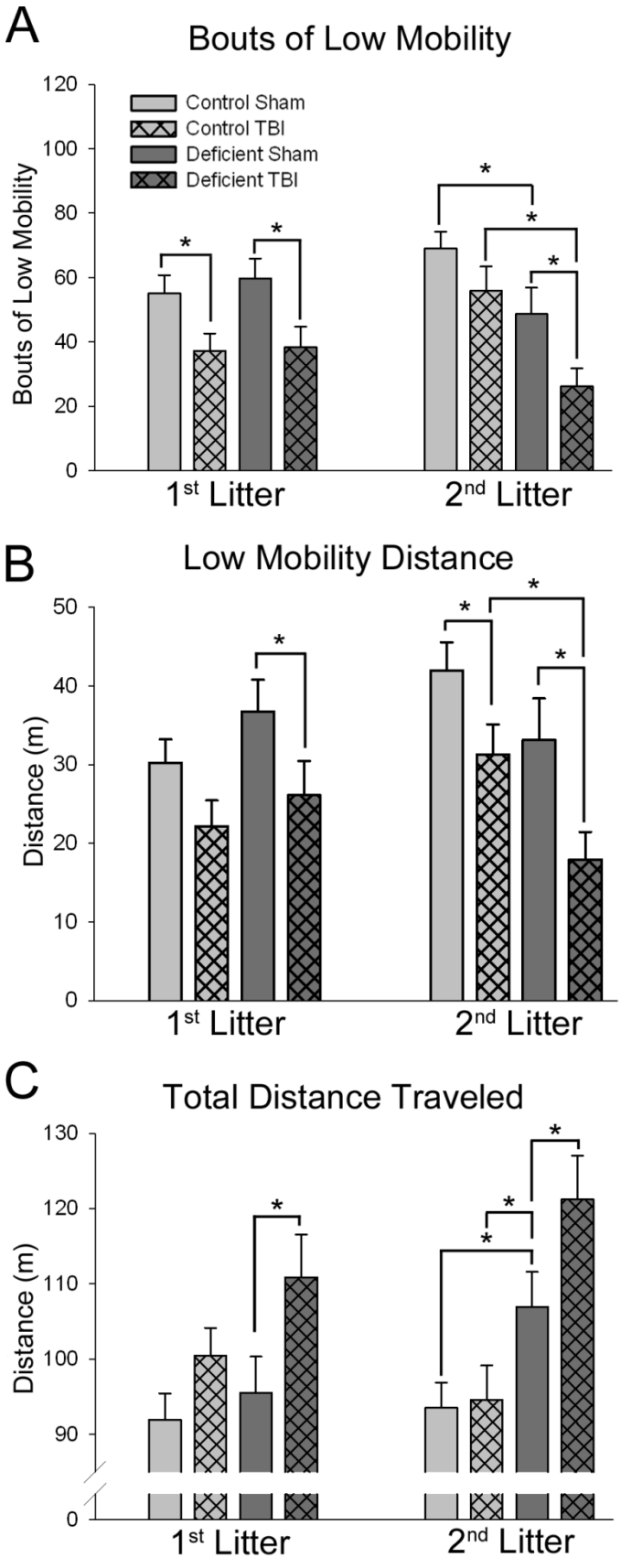

A
Bouts of Low Mobility
Control Sham
Control TBI
Deficient Sham
Deficient TBI
Bouts of Low Mobility
120
100
80
60
40
20
0
1st Litter
2nd Litter
B
Low Mobility Distance
Distance (m)
50
40
30
20
10
0
1st Litter
2nd Litter
C
Total Distance Traveled
Distance (m)
130
120
110
100
90
0
1st Litter
2nd Litter

Figure 5-2. Effects of TBI on locomotor function in rats with diet- and breeding-induced decreases in brain DHA content. Data are presented as the mean ± SEM (n = 11-12 per group). Bouts of Low Mobility are defined as ≥10 sec spent within a 20-mm radius. Low mobility distance is the distance traveled during bouts of low mobility. All data presented are for the entire 20-minute test session on day one after surgery. *P < 0.05 by ANOVA and Fisher's LSD test.

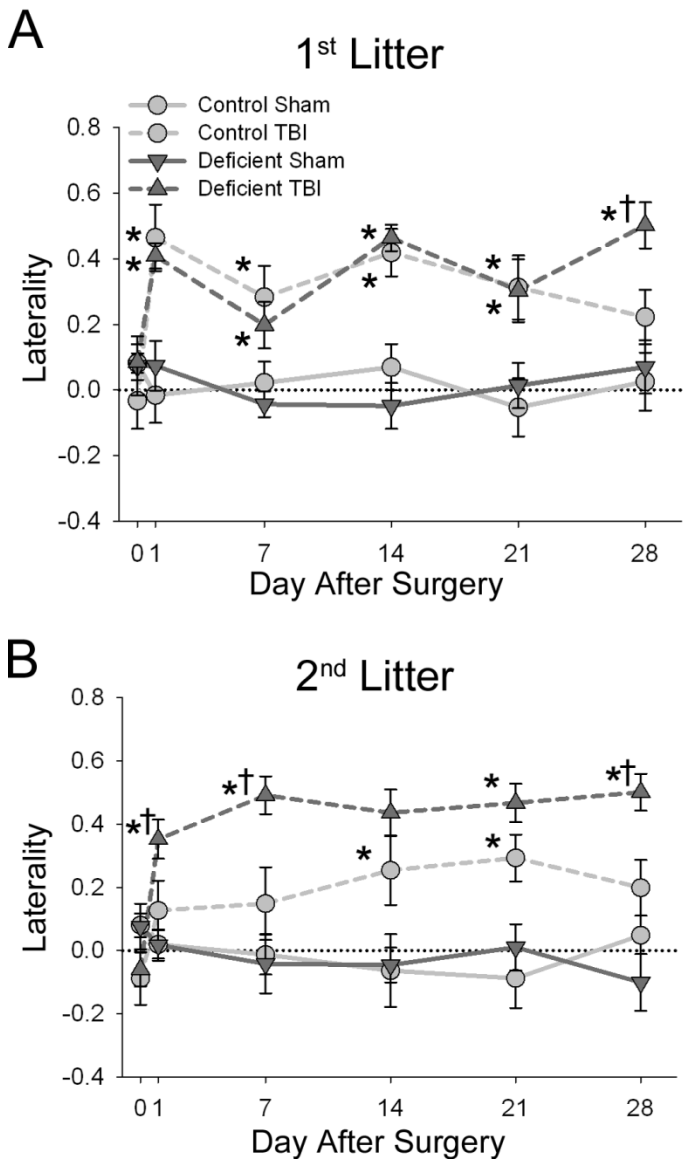


Figure 5-3. Effects of TBI on forelimb preference in rats with diet- and breeding-induced decreases in brain DHA content. Laterality was calculated as: (number of right only - number of left only) / (number of right only + number of left only + number of both together). Data are presented as the mean ± SEM (n = 11-12 per group). *P < 0.05 v. Sham, †P < 0.05 v. Control diet-TBI by ANOVA and Fisher's LSD test.

5.3.3 Effects of Diets and Breeding Procedures on Lesion Volume

TBI caused a significant lesion assessed 28 days after surgery. Lesion volume was not different between rats raised on the Control and Deficient diets for either the 1st or 2nd litters (**Figure 5-4**).

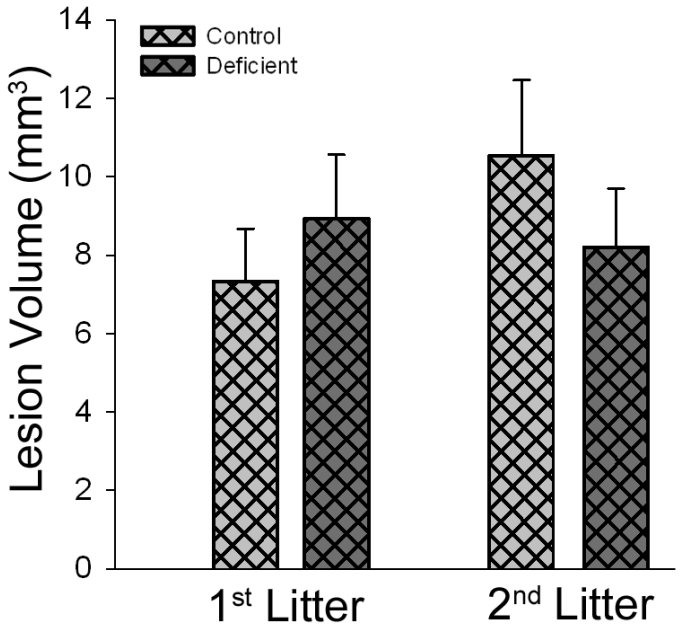


Figure 5-4. Effects of diet- and breeding-induced decreases in brain DHA content on TBI-induced lesion volume 28 days after injury. Data are presented as the mean ± SEM (n = 5-9/ group). No significant differences were indicated by ANOVA.

5.3.4 Effects of Diets and Breeding Procedures on *Ccl2*, *Gfap*, *Mmp*9, *Mmp2*, and *Timp1* mRNA Levels

Levels of mRNA were measured on day one (28 hrs) and day seven after TBI. Significant alterations in expression of these mediators was observed primarily on day one, with mRNA levels returning to, or near, levels observed in sham-injured rats on day seven. Accordingly, data for day one only are presented.

Ccl2 mRNA was increased approximately 4.5-fold on day one after TBI in all injured rats, regardless of diet or litter ($P < 0.001$) (**Figure 5-5A**).

TBI increased *Gfap* mRNA on day one after TBI and average of 10-fold in the 1st litter ($P < 0.001$) and an average of 8-fold in the 2nd litter ($P < 0.001$) (**Figure 5-5B**). Additionally, in the 1st litter, there was an effect of diet, such that injured rats raised on the Deficient diet expressed less *Gfap* than injured rats raised on the Control diet ($P < 0.05$). This diet effect was not seen in the 2nd litter.

Mmp9 mRNA level was increased 9-fold after TBI in all rats on day one after injury ($P < 0.001$). The Deficient diet resulted in lower levels of *Mmp9* mRNA after TBI than those on the Control diet in 1st litter pups ($P < 0.05$), but not in 2nd litter pups (**Figure 5-5C**).

Mmp2 mRNA level was not affected by TBI or diet on day one after injury in either litter (data not shown).

Timp1 mRNA level was affected by TBI in the 1st litter and by both TBI and diet in the 2nd litter (**Figure 5-5D**). In the 1st litter, injured rats fed the Control diet had a 27-fold increase in *Timp1* mRNA level ($P < 0.001$) while those fed the Deficient diet had a 19-fold increase in *Timp1* mRNA level after TBI ($P < 0.001$ v. sham, $P = 0.056$ v. TBI,

Control diet). Second litter pups fed the Control diet exhibited a 24-fold increase of *Timp1* mRNA level ($P < 0.001$ vs. sham), similar to that observed in the 1st litter. In 2nd litter rats fed the Deficient diet, TBI resulted in an increase in *Timp1* mRNA level of only 9-fold ($P < 0.05$ v. Sham, $P < 0.001$ v. TBI, Control diet).

mRNA levels TNFα, IL-1β, and IL-6 were also measured on day one after injury but no effect of TBI or litter was observed (data not shown).

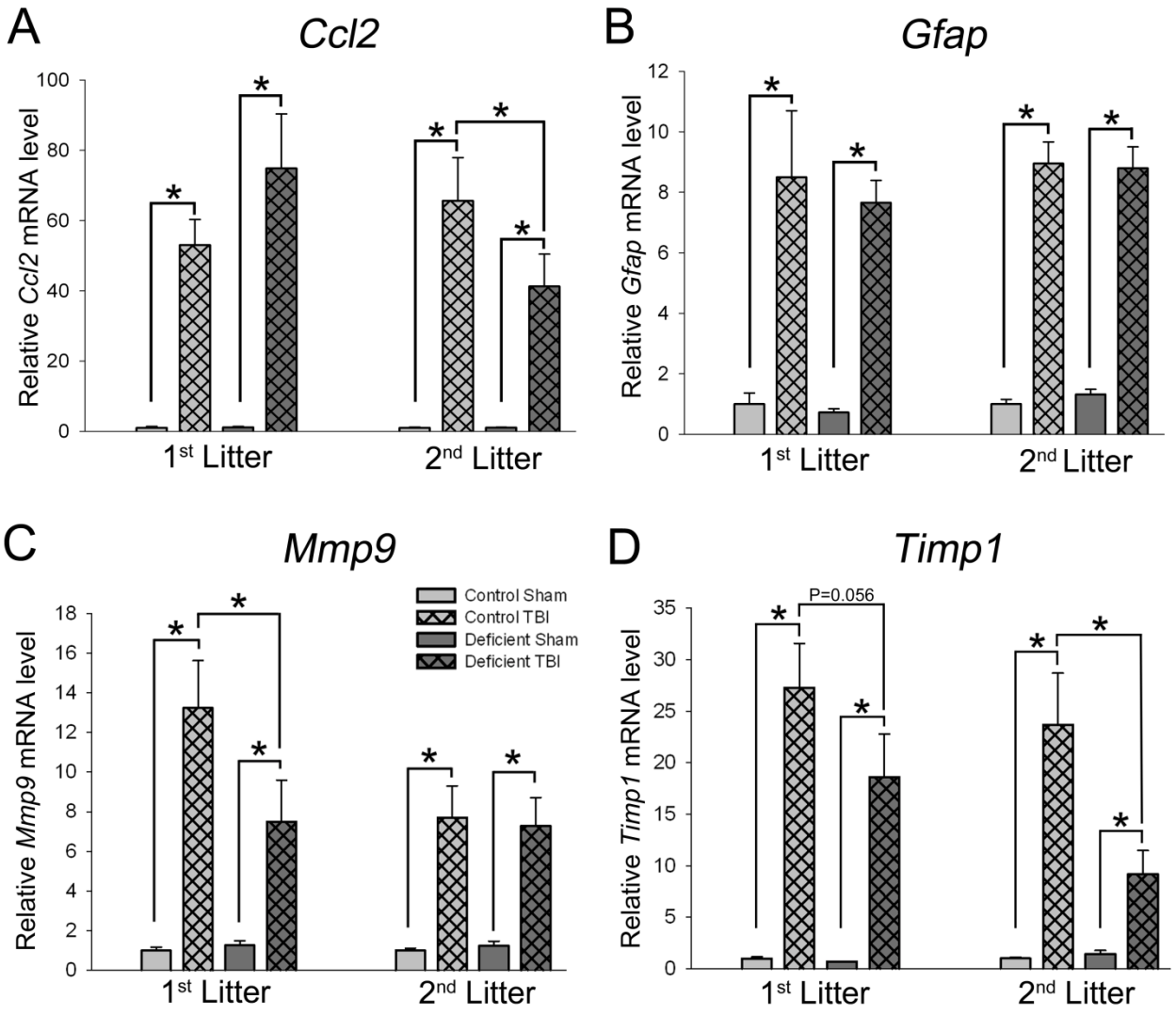


Figure 5-5. Effects of TBI on *Ccl2* (A), *Gfap* (B), *Mmp9* (C), and *Timp1* (D) relative mRNA levels in rats with diet- and breeding-induced decreases in brain DHA content. Data are presented as the mean ± SEM (1st litter: n = 11-13 per group, 2nd litter: n = 7-9 per group). *P < 0.05 by ANOVA and Fisher's LSD test (*Ccl2,* 2nd litter; *Gfap*; *Mmp9*, *Timp1*) or Kruskal-Wallis nonparametric ANOVA and Dunn's Multiple Comparisons test (*Ccl2*, 1st litter).

5.3.5 Effects of Diets and Breeding Procedures on Enzymatic Activity of MMP-2 and MMP-9

Enzymatic activity of MMP-2 was increased roughly 2-fold in 1st and 2nd litter pups on day one after TBI compared to shams ($P < 0.001$), but there was no effect of diet (**Figure 5-6A**). TBI-induced increases in MMP-2 enzymatic activity persisted through day seven after injury in both litters with no effect of diet (data not shown).

MMP-9 enzymatic activity was increased roughly 25-fold on day one after injury in 1st and 2nd litter injured rats (**Figure 5-6B**) regardless of diet treatment ($P < 0.001$, 1st litter; $P < 0.001$, 2nd litter). TBI-induced increases in MMP-9 enzymatic activity returned to near-sham levels on day seven after injury (data not shown).

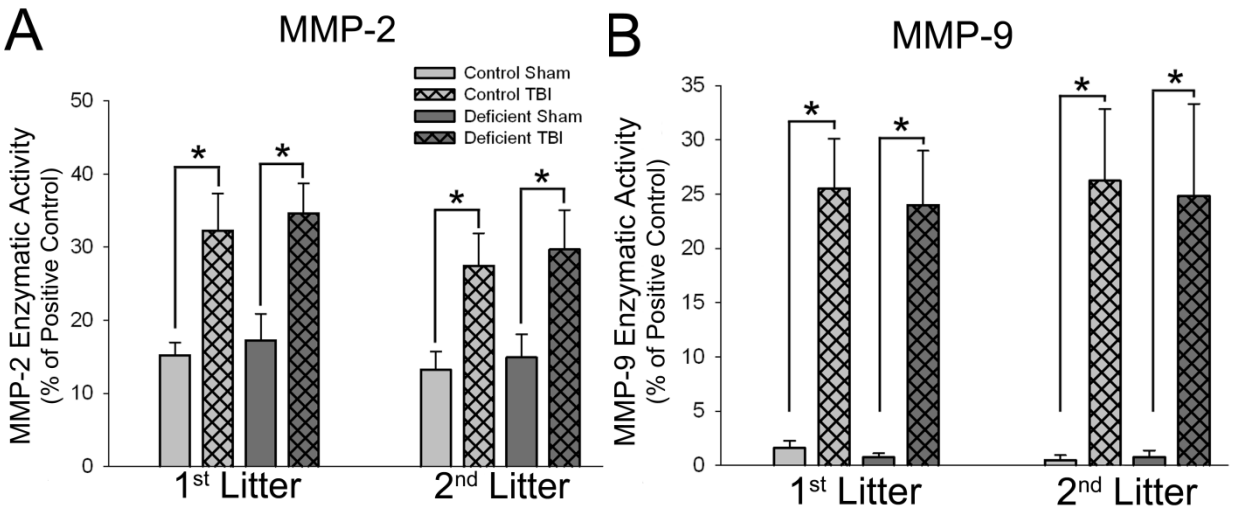


Figure 5-6. Effects of TBI on MMP-2 and MMP-9 enzymatic activities in rats with diet- and breeding-induced decreases in brain DHA content. Data are presented as the mean ± SEM (n = 6-8 per group). *$P < 0.05$ by ANOVA and Fisher's LSD test.

5.4 Discussion

This study examined the effects of an n-3 LC-PUFA-deficient diet and the resulting diet-induced decreases in brain DHA content on sensorimotor and biochemical recovery from TBI in a juvenile model. The use of diet and breeding protocols enabled the assessment of the dose-response effects of brain DHA content on the effects of TBI independently of the effects of dietary n-3 fatty acid content.

5.4.1 Effects of TBI

In concordance with the site of injury in the motor cortex, sensorimotor function was used as the key assessment of functional outcome. Deficits were observed in all groups using sensorimotor tests previously validated for use with this CCI model of juvenile TBI (Russell et al., 2011). These included decreased low mobility movement on day after injury (**Figure 6-2**) consistent with reports of decreased grooming after TBI (Grossman et al., 2011) and a persistent preference for the forelimb ipsilateral to the injury (**Figure 6-3**). TBI also resulted in a notable lesion at 28 days after injury, indicating significant cell loss (**Figure 6-4**).

TBI also increased mRNA levels of several mediators involved in the pathophysiological processes initiated by TBI on day one after injury including *Ccl2*, *Gfap*, *Mmp9*, and *Timp1* mRNA (**Figure 6-5**), as well as MMP-9 and MMP-2 enzymatic activity (**Figure 6-6**). These increases are consistent with the known increases in protein levels for these mediators after TBI and are supportive of the roles they play in inflammation, glial cell activation and degradation of the BBB, respectively, that occur in this time frame after TBI (Werner and Engelhard, 2007). mRNA levels of the

inflammatory mediators TNFα, IL-1β, and IL-6 were also measured on day one after injury but no effect of TBI was observed as one day after injury was beyond the peak of inflammation (data not shown).

5.4.2 Effects of Diet and Breeding Protocols

In agreement with previous studies using these diets to manipulate brain phospholipid fatty acid composition (Ozias et al., 2007), 1st litter and 2nd litter of pups raised on the Deficient diet had decreases in brain DHA content of 25% and 54% DHA, respectively, compared to pups raised on the Control diet (**Figure 5-1**). This graded effect on offspring brain DHA content occurs because consuming the Deficient diet by the dam while gestating and nursing the 1st litter results in depletion of maternal stores of n-3 LC-PUFAs, and thus an even greater failure to deliver DHA to the 2nd litter even though dietary n-3 content is the same for both litters (Levant et al., 2006a; Levant et al., 2007).

In sham injured rats, the deficient diet produced an increase in locomotor activity (**Figure 5-2**) in agreement with previous studies indicating an increase in activity in rats with decreased brain DHA (Levant et al., 2004; Levant et al., 2006b; Vancassel et al., 2007; Levant et al., 2010). However, there were no effects of diet in either 1st or 2nd litter sham rats on any of the other endpoints examined in this study.

5.4.3 Effects of Diet and Breeding Protocols on TBI Outcomes

Functional outcomes after TBI were poorer in rats with decreased brain DHA content. Specifically, injured rats with either a 25% or 54% decrease in brain DHA level exhibited greater distance traveled in the locomotor test, in addition to the fewer bouts of low mobility and decreased low mobility distance also observed after TBI in rats raised on the Control diet. These data indicate that the decreases in low mobility after TBI observed in rats raised on the Deficient diet was of sufficient magnitude that it also resulted in greater total distance traveled compared to those fed the Control diet (**Figure 5-2**). Effects of TBI on locomotor activity were primarily observed on day one after injury (Grossman et al., 2011), suggesting that this test is likely best interpreted as a measure of acute behavioral effects after TBI, rather than of long-term functional outcome. Because this effect was similar after TBI in both litters raised on the Deficient diet, this suggests that augmented early functional response to TBI is the result of the low n-3 LC-PUFA content of the Deficient Diet. Alternatively, this effect may result more specifically from a reduction in the percentage of DHA in brain phospholipids, but may be maximal in the rats with a 25% decrease in brain DHA, and thus additional effect was not observed in rats with a 54% decrease.

In contrast to the effects on locomotor activity, TBI produced persistent deficits in forelimb preference, indicating that this parameter reflects longer-term functional outcome. Rats with a 25% decrease in brain DHA had forelimb deficits similar to those of rats fed the Control diet, whereas rats with a 54% decrease in brain DHA had greater forelimb deficits than their respective controls (**Figure 5-3**). Since the Deficient diet was identical for the 1st and 2nd litters, this suggests that the brain DHA levels, rather than

simply dietary n-3 fatty acid content, are of primary importance in influencing long-term sensorimotor outcomes after TBI.

To assess the cellular mechanisms by which variation in dietary and/or brain DHA content might influence functional outcome after TBI, the effects of the diet and breeding treatments were assessed on representative measures of the various pathobiological processes induced by TBI. Lesion size after TBI was not different between groups (**Figure 5-4**), indicating that differences in neuronal cell loss do not underlie the differences in functional outcomes. Likewise, induction MMP-2 and -9 enzymatic activities after TBI affected by low dietary and/or brain DHA (**Figure 5-6**). Several cellular markers of injury were altered by the diet and breeding protocol, such as mRNA levels of *Gfap* and *Mmp9*; however, the effects were not consistent with the diet treatment between litters, nor did they correlate with brain DHA levels, suggesting that they do not play a primary role in the poorer functional outcomes in rats with lower brain DHA levels (**Figure 5-5**). Thus, these data suggest that changes in these mediators are not primary contributors to the observed differences in outcome.

TBI-induced *Timp1* gene expression, however, was directly related to brain DHA content and correlated with sensorimotor outcomes in the spontaneous forelimb elevation test (**Figure 5-5D**). Specifically, rats with a 25% decrease in brain DHA tended toward expressing less *Timp1* after TBI than rats on the Control diet. Furthermore, although the experimental design allows only qualitative comparison, rats with a 54% decrease in brain DHA expressed even less *Timp1* after TBI than rats with a 25% decrease in brain DHA. This correlation between brain DHA content and *Timp1*

gene expression suggests a possible mechanism for brain DHA-mediated improvement in functional outcomes.

MMP-2 and -9 are proteases that are rapidly induced after TBI and other neuroinflammatory conditions (Candelario-Jalil et al., 2009; Jia et al., 2010). Unlike *Mmp9*, *Mmp2* is constitutively expressed and is primarily regulated at the level of enzyme-activation (Strongin et al., 1995; Gottschall and Deb, 1996), in agreement with mRNA levels measured after TBI. Early after injury, MMPs initiate apoptotic cell death and degrade the extracellular matrix, contributing to opening the BBB and facilitating vasogenic edema (Candelario-Jalil et al., 2009). Consistent with the present findings, peak gene expression and enzymatic activity of MMPs occurs approximately 24 hours after juvenile TBI (Sifringer et al., 2007).

TIMP-1 is one of a family of four endogenous MMP inhibitors (Brew and Nagase, 2010). All four TIMP family members are expressed in the brain (Candelario-Jalil et al., 2009). TIMP-1, which has the broadest substrate specificity, inhibits MMPs in a 1:1 ratio by binding to the MMP active site (Gomis-Ruth et al., 1997). TIMP-1 also has anti-apoptotic and growth factor properties that are independent of its MMP-inhibiting ability (Hayakawa et al., 1992; Gardner and Ghorpade, 2003; Jourquin et al., 2005), and are thought to occur through interactions with cell surface receptors including CD63 (Strongin et al., 1995; Jung et al., 2006). In this study, the decreased induction of *Timp1* gene expression after TBI in rats with either a 25% or 54% decrease in brain DHA, which would be anticipated to lead to decreased levels of TIMP-1 protein, was not associated with altered MMP-2 or MMP-9 enzymatic activity (**Figure 5-6**). This suggests that it is the anti-apoptotic and/or growth factor properties of TIMP-1, rather

than inhibition of MMP enzymatic activity, that may provide neuroprotection after TBI in rats with sufficient levels of brain DHA, and that the decreased *Timp1* gene expression observed in rats with decreased brain DHA is contributing to worsened functional outcomes.

TIMP-1 is mainly regulated at the level of gene transcription, supporting the use of mRNA as a proxy for levels of functional protein. Upstream regions of the *Timp1* gene contain a serum response element that confers *Timp1*'s responsiveness to a variety of agents including cytokines and growth factors (Campbell et al., 1991; Edwards et al., 1992; Bugno et al., 1995; Gardner and Ghorpade, 2003). LC-PUFAs are known to regulate gene expression through binding to several transcription factor response elements, including PPAR, and RXR (Khan and Vanden Heuvel, 2003). Identification of putative LC-PUFA response elements within 1500 base pairs upstream of the mouse *Timp1* gene transcription start site, using JASPAR (Bryne et al., 2008), revealed 6 possible PPAR/RXR binding sites, indicating a potential mechanism by which variation in brain DHA content may influence regulation of *Timp1* gene expression.

5.5 Conclusions

Diet-induced decreases in brain DHA content resulted in worsened sensorimotor outcomes after TBI compared to rats with adequate levels of brain DHA. The poorest long-term function was observed in rats with the greatest decrease in brain DHA, suggesting that brain DHA level, rather than dietary n-3 LC-PUFA content, is of greatest importance in influencing the ultimate outcomes after TBI. *Timp1* mRNA levels after

TBI correlated with brain DHA content, suggesting that lower *Timp1* gene expression, as a result of decreased brain DHA, contributes to poorer sensorimotor outcomes, most likely through its anti-apoptotic and/or growth factor activities. Thus, the present data indicate a novel mechanism by which LC-PUFAs modulate a response to neural injury, in addition to serving as a precursor for many anti-inflammatory molecules (Serhan et al., 2008b). Furthermore, these data suggest that diet regulates brain development and the ability of the brain to respond to injury. Therefore, young children may be provided with greater protection against the deleterious effects of TBI by ensuring optimal accretion and maintenance of brain DHA levels through appropriate nutrition.

CHAPTER SIX

FISH OIL IMPROVES MOTOR FUNCTION, LIMITS BLOOD-BRAIN BARRIER DISRUPTION AND REDUCES MMP9 GENE EXPRESSION AFTER JUVENILE TBI

6.1 Abstract

Treatment of TBI in children presents a number of challenges including age-specific response to injury and differences in pharmacokinetics and pharmacodynamics between young children and adults. This study investigated the effects of a high dose oral fish oil dosing regimen on sensorimotor, BBB, and biochemical outcomes of TBI in a juvenile rat model. Seventeen-day old Long-Evans rats were given a 15 mL/kg fish oil (2.01 g/kg EPA, 1.34 g/kg DHA) or soybean oil dose via oral gavage thirty minutes prior to being subjected to a CCI injury or sham surgery, followed by daily doses seven days. Fish oil treatment resulted in improved hindlimb deficits after TBI as assessed with the beam walk test, decreased IgG infiltration into the ipsilateral and contralateral hemispheres, and decreased TBI-induced expression of the *Mmp9* gene one day after injury. TBI-induced increases in *Gfap* gene expression were also less persistent in rats treated with fish oil. These results indicate that fish oil may improve sensorimotor outcomes after TBI in juveniles by decreasing blood-brain barrier disruption by a mechanism involving decreased expression of the *Mmp9* gene and also modulating a mediator of astroglial activation.

6.2 Introduction

TBI is the leading cause of acquired disability and death in young children. Despite having a high degree of neuroplasticity, young children tend to have poorer outcomes after TBI than adults (Giza and Prins, 2006). Children also have pharmacologic challenges not observed in adults including problems with formulations and issues of dosing, and altered bioavailability, metabolism, and drug response (Conroy et al., 2000; Kearns et al., 2003). Therefore, it is imperative that TBI and potential therapeutics be investigated in an age-appropriate model (Prins and Hovda, 2003).

The long-chain n-3 polyunsaturated fatty acids EPA (20:6n-3) and DHA (22:6n-3), the main constituents in fish oil, are biologically active with many neuroprotective properties. N-3 fatty acids, particularly DHA, are incorporated into the developing brain during late gestation and early post-neonatal life in humans and rats, a time at which children have a high risk for sustaining TBI. When consumed in the diet or via supplementation, n-3 fatty acids are incorporated into the phospholipids that form cell membranes where they can alter the physiochemical and membrane-signaling properties of the cell (Salem et al., 2001a; Shaikh, 2012). DHA, EPA and their metabolites have well-documented anti-excitotoxic (Hogyes et al., 2003), antioxidant (Hossain et al., 1998), anti-apoptotic (Florent et al., 2006; Sinha et al., 2009), and anti-inflammatory properties (Bazan et al., 2005). Additionally, free n-3 fatty acids and membrane-incorporated EPA and DHA, can be metabolized into several families of molecules including NPD1, docosanoids, resolvins, *etc*. These fatty acid-derived molecules have been shown be neuroprotective through their anti-inflammatory and

pro-resolving properties (Serhan et al., 2008b; Bazan, 2009; Serhan et al., 2009). LC-PUFAs can also directly or indirectly modulate gene expression through activation or suppression of cell signaling pathways and transcription factors (e.g., PI3K/Akt, NF-kB, PPAR and RXR) (Khan and Vanden Heuvel, 2003; Akbar et al., 2005; Draper et al., 2011).

Currently, clinical therapies for TBI are very limited. Fish oil, a source of DHA and EPA, is very well tolerated, has no known toxicity or significant adverse side effects, is well absorbed, and readily crosses the BBB. In view of the many potentially beneficial effects on n-3 fatty acids (see above), fish oil is an attractive treatment for conditions like neural injuries, which initiate multiple cascades of responses. The preponderance of studies investigating fish oil, or DHA or EPA alone, in a variety of neural injury models including TBI and spinal cord injuries indicate that these preparations produce beneficial effects (King et al., 2006; Pan et al., 2009; Mills et al., 2011b). Furthermore, in a case report, high dose fish oil supplementation (19.2 g/day) was associated with substantial clinical improvement in a young patient with severe head trauma deemed likely lethal (Lewis et al., 2013). However, to date, all animal studies demonstrating the benefit of LC-PUFAs in neural injuries have been conducted in adults. The effects of LC-PUFA or fish oil dosing have not been investigated in juvenile brain injury. Accordingly, this study investigated the use of high-dose oral fish oil dosing on sensorimotor and biochemical outcomes of TBI in a juvenile rat model. This study will show that fish oil improves functional outcomes after TBI through preservation of the BBB possibly through decreasing *Mmp9* gene expression and faster resolution of *Gfap* gene expression, a marker of astrocytosis and glial scarring.

6.3 Results

6.3.1 Effects on Growth, Development, and Brain Phospholipid Fatty Acid Composition

The oil dosing was well tolerated in all groups. No gross adverse effects were observed.

Overall growth and development of the rats was not affected by TBI or oil treatment. Neither body weight nor rate of growth was significantly altered by injury or oil at any time point (**Figure 6-2**).

Acute oil dosing slightly altered the phospholipid composition of the frontal cortex four days after injury (**Table 6-1**, **Figure 6-1**). Compared to soybean oil treated rats, in shams, fish oil doing reduced the percentage of the monounsaturated fatty acid (MUFA) 24:1 and Other MUFAs by 0.75% ($P < 0.05$) and 1.3% ($P < 0.01$), respectively. Fish oil administration also significantly reduced n-6 LC-PUFA 22:5n6 and Other N-6s by 0.4% ($P < 0.01$) and 0.5% ($P < 0.01$), respectively compared to soybean oil treated shams.

TBI did not change the relative abundance of any individual fatty acids in brain phospholipids. However, regardless of oil treatment, TBI caused a decrease in total n-6s by 2% in both oil treatment groups ($P < 0.01$ vs. sham, same oil) and a decrease in total n-3s by 5% and 2% in soybean oil and fish oil groups, respectively ($P < 0.01$ vs. sham, same oil). The overall decrease in total long chain-polyunsaturated fatty acids was 7% in soybean oil treated rats ($P < 0.01$ vs. sham, same oil) and 4% in fish oil treated rats ($P<0.01$ vs. sham, same oil). The loss of LC-PUFAs was accompanied by a 5% increase in total saturated fatty acids ($P < 0.01$ vs. sham, same oil) in both oil treatment groups. There was no difference in the TBI-induced total LC-PUFA loss or saturated fatty acid (SFA) gain between soybean and fish oil groups.

Table 6-1. **Effects of TBI and fish or soybean oil dosing on frontal cortex fatty acid composition four days after fish oil and soybean oil dosing.** Other saturated fatty acids (SFA): 13:0, 14:0, 15:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0; Other monounsaturated fatty acids (MUFA): 15:1, 16:1, 20:1n-9; Other N-6: 18:2n-6c, 18:3n-6, 20:2n-6, 20:3n-6, 22:2n-6; Other N-3: 18:3n-3, 20:5n-3. Data are presented as the mean ± SEM; n = 6-7 per group. [a]P < 0.05 vs. Sham, Same Oil; [b]P < 0.05 vs. Sham, Different Oil; [c]P < 0.05 vs. TBI, Different Oil.

Fatty Acid (% of Total)	Soybean Oil Sham	Soybean Oil TBI	Fish Oil Sham	Fish Oil TBI
16:0	23.99 ± 0.89	27.27 ± 1.13	25.57 ± 1.21	25.81 ± 0.73
Other SFA	24.42 ± 0.86	25.70 ± 0.91	25.83 ± 1.07	26.66 ± 0.70
18:1n9	9.12 ± 1.66	10.17 ± 1.25	11.41 ± 0.72	10.20 ± 0.53
24:1	4.74 ± 0.24	4.07 ± 0.25[a]	3.99 ± 0.20[b]	3.47 ± 0.19
Other MUFA	2.24 ± 0.12	1.43 ± 0.26[a]	0.95 ± 0.16[b]	1.57 ± 0.35
20:4n6/20:3n3	14.55 ± 0.38	13.60 ± 0.44	15.21 ± 0.76	13.49 ± 0.46[a]
22:5n6	1.99 ± 0.11	1.59 ± 0.09[a]	1.55 ± 0.09[b]	1.34 ± 0.09
Other N-6	2.27 ± 0.08	2.23 ± 0.06	1.81 ± 0.11[b]	1.71 ± 0.02[c]
22:6n3	18.26 ± 0.77	13.79 ± 0.66[a]	18.11 ± 1.12	15.70 ± 0.79
Other N-3	0.08 ± 0.04	0.02 ± 0.01	0.06 ± 0.02	0.05 ± 0.01

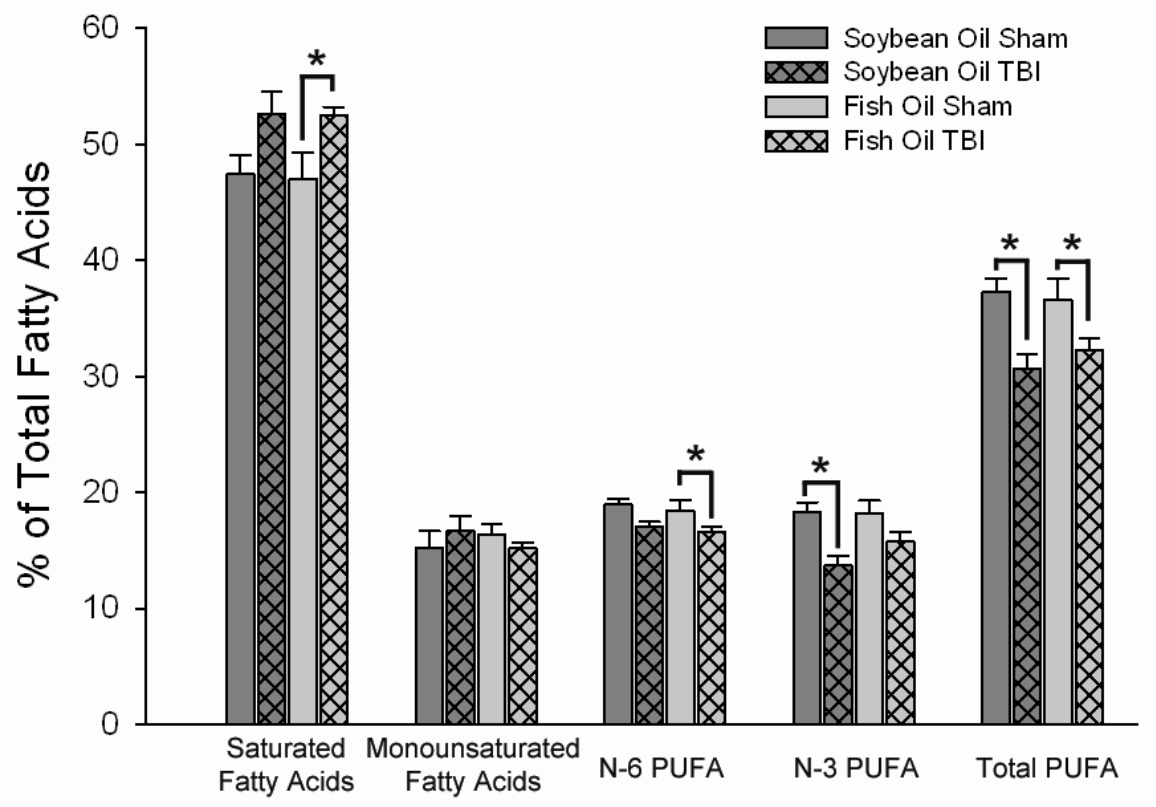


Figure 6-1 Frontal cortex composition of the major brain phospholipid classes. Fatty acid composition was determined 4 days after TBI or sham surgery. Data are presented as the mean ± SEM (n = 7-8 per group). *P < 0.01 vs. Sham, same oil by ANOVA and Fisher's LSD.

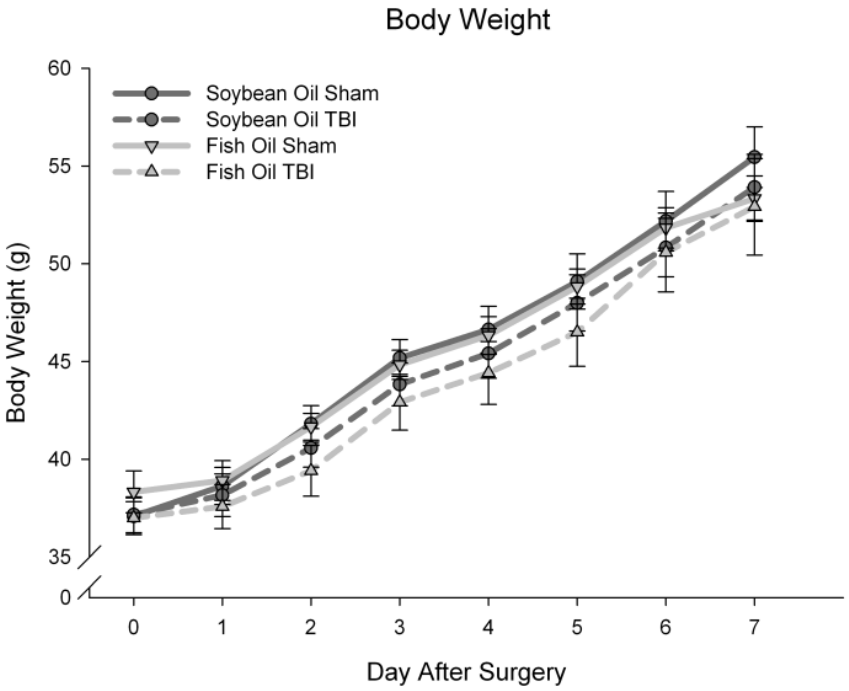


Figure 6-2. Effects of injury and fish oil treatment on body weight. Data are the mean ± SEM (n = 11-12 per group). Rate of weight gain did was not altered by TBI or oil treatments as assessed by repeated-measures ANOVA.

6.3.2 Effects on Sensorimotor Function

Sensorimotor function was altered in all rats that sustained a TBI as indicated by an overall significant increase in the percentage of unilateral beam walk slips (**Figure 6-3**). A two-way ANOVA with factors of oil type and injury indicated a significant main interaction of injury and oil ($P < 0.001$) such that injured rats dosed with fish oil had a lower overall percentage of foot slips on the beam walk than did injured rats receiving soybean oil. Post -hoc analysis indicated that rats treated with fish oil had lower levels of functional deficit days one and seven after injury ($P < 0.05$). In a repeated-measures

ANOVA, both groups showed significant improvement from day one to day seven; however, post-hoc analysis indicated that rats treated with soybean oil, maximal improvement was achieved by day four after injury whereas rats treated with fish oil had a trend towards improvements in performance through day seven.

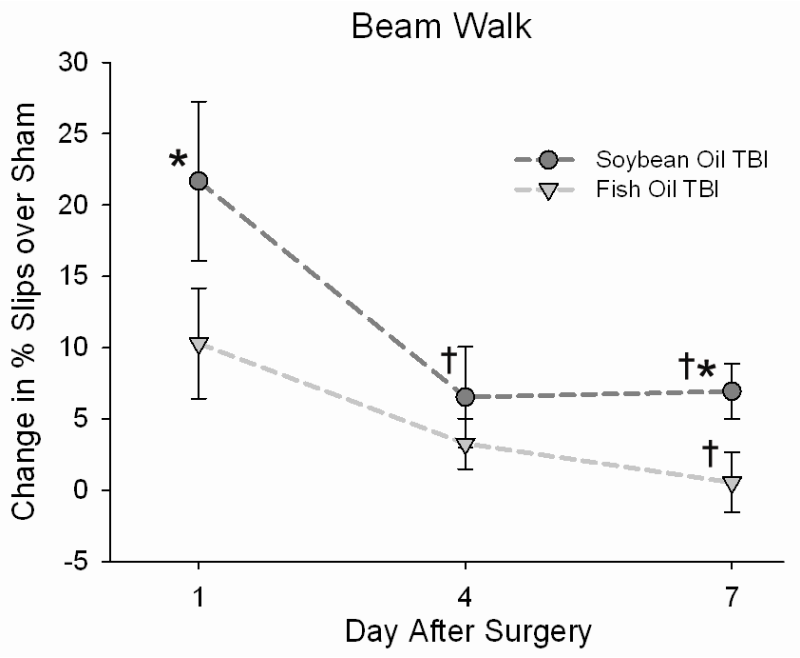
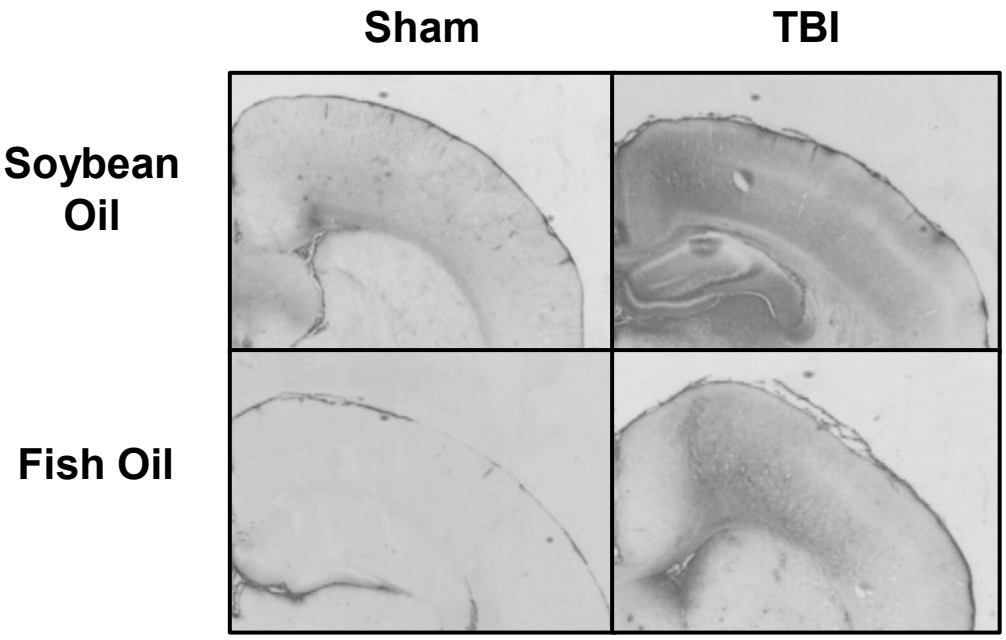


Figure 6-3. Effects of fish oil treatment of TBI-induced hindlimb deficits assessed using the beam walk test. Data are the mean ± SEM (n = 11-12 per group). *P < 0.05 vs. same day, different oil; †P < 0.05 vs. same oil, day one by repeated-measures ANOVA and Fisher's LSD test.

6.3.3 BBB Disruption

TBI induced extensive IgG staining seven days after TBI. There was a significant interaction with the oil treatments such that IgG infiltrated a smaller volume of brain area in rats treated with fish oil than with soybean oil ($P < 0.05$) (**Figure 6-4**). The volume of IgG stained tissue was not different in sham-injured rats treated with either oil (not shown).



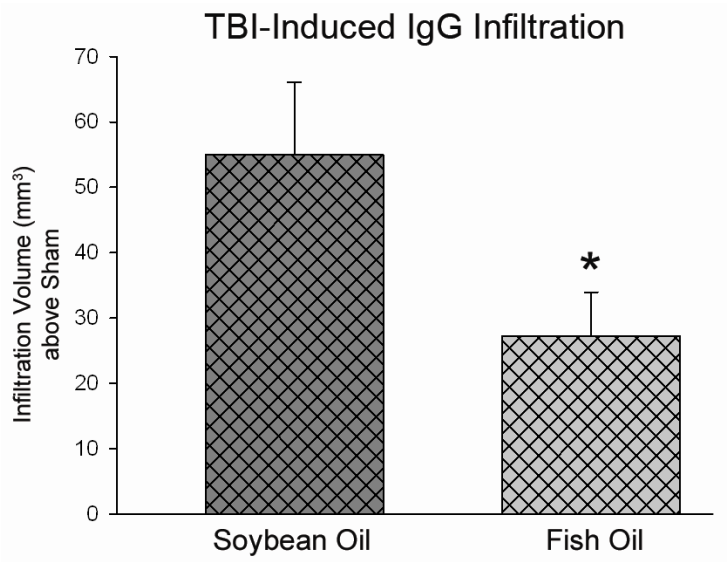


Figure 6-4. Effects of fish oil treatment on TBI-induced IgG Infiltration. The volume of TBI-induced IgG infiltration, visualized by immunocytochemistry, was significantly reduced by fish oil treatment. Data are the mean ± SEM (n = 10-11 per group). *P < 0.05 by Student's-t test.

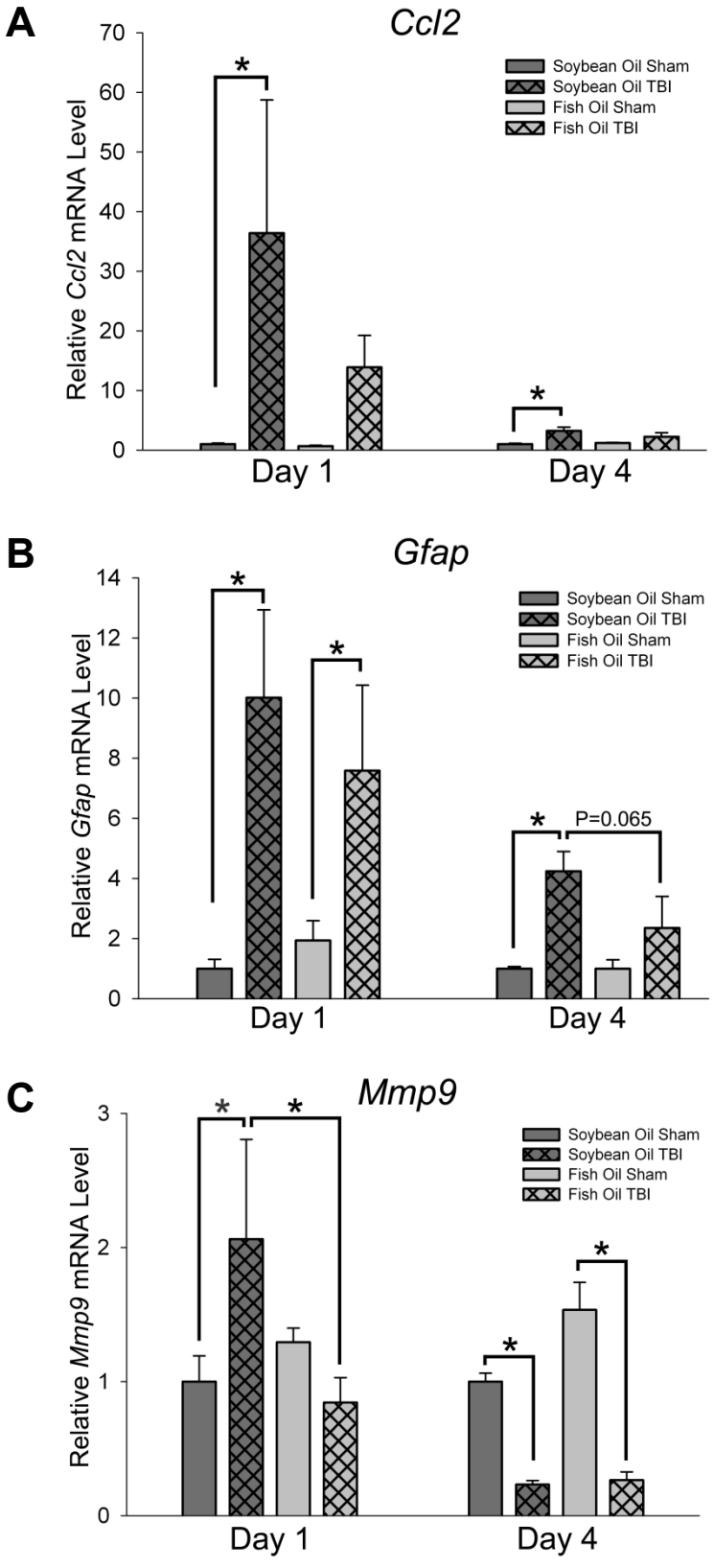

A
Ccl2
Relative Ccl2 mRNA Level
70
60
50
40
30
20
10
0
Soybean Oil Sham
Soybean Oil TBI
Fish Oil Sham
Fish Oil TBI
*
*
Day 1
Day 4
B
Gfap
Relative Gfap mRNA Level
14
12
10
8
6
4
2
0
Soybean Oil Sham
Soybean Oil TBI
Fish Oil Sham
Fish Oil TBI
*
*
*
P=0.065
Day 1
Day 4
C
Mmp9
Relative Mmp9 mRNA Level
3
2
1
0
Soybean Oil Sham
Soybean Oil TBI
Fish Oil Sham
Fish Oil TBI
*
*
*
*
Day 1
Day 4

Figure 6-5. Effects of fish oil on TBI-induced expression of (A) *Ccl2*, (B) *Gfap*, and (C) *Mmp9* mRNA levels. Data are the mean ± SEM (n = 4-7 per group). *P < 0.05 by ANOVA and Fisher's LSD test.

6.4 Discussion

LC-PUFA or fish oil treatment has been beneficial in treating adult neural injuries in several models, including humans (Javierre et al., 2006; King et al., 2006; Pan et al., 2009; Mills et al., 2011a; Shin and Dixon, 2011; Lewis et al., 2013). This study investigated the benefits of fish oil treatment on outcomes of juvenile TBI.

6.4.1 Effects of TBI

In this study, injury to the primary motor cortex produced deficits in sensorimotor function as assessed using the beam walk test (**Figure 6-3**), a sensorimotor test previously validated for use with this CCI model of juvenile TBI (Russell et al. 2011). TBI also increased expression of the *Ccl2* and *Gfap* genes (**Figure 6-5)**, mediators involved in monocyte recruitment and astrocytosis, respectively. These increases are consistent with the known increases in protein levels for these mediators after TBI (Laird et al., 2008; Semple et al., 2010). Also in agreement with previous studies (Aihara et al., 1994; Onyszchuk et al., 2008), TBI caused significant BBB disruption as indicated by infiltration of IgG, a serum protein, into the brain parenchyma. Together, these data indicate that a successful injury specific to the sensorimotor cortex with persisting deficits was achieved.

In addition to the effects of TBI on sensorimotor function, TBI significantly altered the fatty acid composition of the frontal cortex. Four days after injury, TBI resulted in an overall decrease in LC-PUFAs regardless of oil treatment, which was accompanied by an increase saturated fatty acids. Because this study used semi-quantitative methods which assess the fatty acid composition of brain phospholipids, rather that the absolute amounts of each fatty acid, these changes could be due to substitution of saturated fatty acids for LC-PUFA or changes in the absolute amounts of specific fatty acids. However, other studies indicate that TBI causes a rapid, sustained increase of free fatty acids (Homayoun et al., 2000), including DHA (Chris Butt, personal communication). Therefore, it is likely that the cleavage of n-6 and n-3 fatty acids from the membrane, as a result of TBI, is responsible for the observed decrease in the total percentage of membrane LC-PUFAs. When cleaved from the membrane, DHA and EPA can directly or indirectly modulate gene expression through activation or suppression of cell signaling pathways and transcription factors (e.g., PI3K/Akt, NF-kB, PPAR and RXR) (Khan and Vanden Heuvel, 2003; Akbar et al., 2005; Draper et al., 2011) or be metabolized into several neuroprotective, anti-inflammatory, and pro-resolving families of molecules including neuroprotectin D1, docosanoids, resolvins, *etc*. (Serhan et al., 2008b; Serhan et al., 2009).

6.4.2 Effects of Oil Administration in Sham-Injured Rats

Administration of either fish oil or soybean oil in sham-injured rats did not cause any alterations in growth (**Figure 6-2**), behavior (**Figure 6-3**), expression of *Ccl2*, *Gfap*, or *Mmp9* genes (**Figure 6-5**) or IgG infiltration in to the CNS (**Figure 6-4**). This

indicates that soybean oil and fish oil administration was well-tolerated and that neither oil stimulated expression of TBI-induced mediators in the absence of TBI.

With regard to brain fatty acid composition, fish oil administration decreased the percentage of 24:1 and other MUFAs, though total MUFA percentage was not altered. Similarly, fish oil administration decreased the percentage of 22:5n6 and other N-6 LC-PUFAs, but did not affect total LC-PUFAs.

6.4.3 Effects of Fish Oil Administration in TBI Rats

In rats with a CCI injury, fish oil prevented the TBI-induced decrease in brain DHA (**Table 6-1**) that was observed in rats treated with soybean oil. However, the DHA content in injured rats administered soybean oil was not statistically different from injured rats administered fish oil. Furthermore, the decrease in total LC-PUFA as a result of TBI was not altered by fish oil administration. This suggests that fish oil administration can have small changes on individual fatty acids but classes as a whole are not affected.

Fish oil administration also decreased the magnitude and persistence of TBI-induced motor function deficits, reduced the extent of IgG infiltration into the brain parenchyma (**Figure 6-4**), and decreased the persistence *Gfap* gene expression (**Figure 6-5B**). Furthermore, fish oil treatment prevented a TBI-induced increase in *Mmp9* mRNA levels (**Figure 6-5C**), a key mediator in the breakdown of the BBB. Although the endpoints were different from those measured in this study, beneficial effects of n-3 LC-PUFA supplementation have been reported in studies using adult models of TBI (Wu et al., 2004; Wu et al., 2007; Bailes and Mills, 2010; Mills et al.,

2011a; Mills et al., 2011b; Shin and Dixon, 2011; Wu et al., 2011). Thus, although the underlying mechanism(s) remain to be fully characterized, these studies support the use of fish oil as a treatment for TBI in both juveniles and adults.

6.4.4 Potential Mechanisms of Fish Oil-Mediated Neuroprotection

Taken together the present findings suggest that n-3 LC-PUFA in fish oil enhance outcomes after TBI through limiting blood-brain barrier damage after TBI and/or expediting its repair. The BBB is a dynamic, complex structure made up of vascular endothelial cells surrounded by support cells and astrocytic foot processes (Abbott et al., 2010). TBI causes significant astrocytosis, as indicated by increased *Gfap* gene expression (Mucke et al., 1991; Eng and Ghirnikar, 1994) an intermediate filament protein, and the secretion of the gelatinases MMP-2 and -9 (Candelario-Jalil et al., 2009; Jia et al., 2010).

There is a growing body of evidence implicating MMP-9 in the breakdown of the BBB after neural injury in both developmental and adult models (Gasche et al., 1999; Asahi et al., 2001; Shigemori et al., 2006; Sifringer et al., 2007; Svedin et al., 2007). MMP-9 disrupts the BBB by degrading collagen IV and laminin in the basal lamina (Harkness et al., 2000). Additionally, increased MMP activity at the BBB leads to MMP-dependent cleavage of BBB tight junction proteins and causes significant disruption of cell-cell contact (Lohmann et al., 2004). Similar to our results in juvenile TBI, others have reported decreased BBB permeability (Pan et al., 2009) and decreased infarct volume (Belayev et al., 2009; Eady et al., 2012) in adult models of I/R after treatment with DHA, supporting our conclusion of DHA and/or EPAs role in maintenance of the

BBB after injury. DHA and EPA have been shown to decrease MMP-9 protein levels and activity *in vitro* (Shinto et al., 2011), suggesting DHA and EPA may also be modulating MMP-9 *in vivo* and contributing to our observed outcomes.

Mmp9 is regulated at the level of gene transcription, primarily through an NF-κB site in the promoter region (Ogawa et al., 2004) and consistent with the present findings, peak expression of the *Mmp9* gene occurs approximately 24 hours after juvenile TBI (Sifringer et al., 2007). LC-PUFAs are known to regulate NF-κB signaling through binding to and activating PPAR receptors thereby antagonizing the NF-κB signaling pathway (Zuniga et al., 2011) or through directly inhibiting activation of NF-κB independently of PPAR (Novak et al., 2003; Draper et al., 2011). From this we can propose that fish oil is providing BBB protection after TBI by limiting gene expression of *Mmp9* in activated astrocytes through inhibition of NF-κB signaling early after injury.

Modulation of astrocytosis and glial scar formation represents an additional mechanism by which fish oil may improve outcomes after TBI. In support of both a beneficial and harmful role of astrocytosis in neural injury (Laird et al., 2008), DHA treatment increased GFAP staining seven days after I/R in an adult model (Belayev et al., 2009; Eady et al., 2012). The authors suggest this is a protective scarring mechanism to spare adjacent neurons from damage. However, in an adult model of TBI, vitamin B3 treatment reduced *Gfap* gene expression and improved functional outcomes (Hoane et al., 2003). Similarly, in our juvenile model of TBI, DHA treatment improved functional outcomes and decreased *Gfap* gene expression to sham-levels by day four after injury, suggesting the fish oil-induced reduction of astrocytosis is beneficial in this model. These findings, compared to adult models of neural injury,

indicate there may be age, injury model, or time after injury-dependent responses to the effects of DHA on GFAP mRNA and protein levels.

Transcriptional regulation and astrocyte-specific expression of the *Gfap* gene is very complex (Brenner, 1994). Expression of the *Gfap* gene, however, is induced by hormones, growth factors, and cytokines (Laping et al., 1994). DHA, EPA, and their metabolites are known to have anti-inflammatory properties through promoting resolution of inflammation and through decreasing cytokine expression via NF-κB-dependent and independent mechanisms (Mori and Beilin, 2004; Calder, 2007; Kang and Weylandt, 2008). Therefore, we can hypothesize at least one mechanism by which DHA and EPA regulate *Gfap* gene expression via modulation of inflammation and cytokine production.

In addition to the various signaling roles of n-3 LC-PUFA, these molecules also influence cellular function through their role as components of membrane phospholipids. It is possible that neuroprotection afforded by fish oil after TBI may also be due to slight alterations in lipid membrane microenvironments or lipid rafts. Additional studies are needed to fully investigate the TBI-induced changes in membrane fatty acid composition.

6.5 Conclusion

In agreement with reports from studies in adult animals, these results indicate that fish oil can improve TBI outcomes in juvenile animals. There are numerous potential mechanisms by which the DHA and EPA contained in fish oil may contribute to improved TBI outcomes. This study demonstrates that fish oil resulted in improved

functional outcome, in part, by limiting disruption of the BBB by preventing TBI-induced expression *Mmp9* and by modulating expression of *Gfap*, which potentially also affects BBB function and/or astrocytosis and glial scarring. Accordingly, these findings suggest that administration of fish oil may improve outcomes after TBI in children.

CHAPTER SEVEN

DISCUSSION AND FUTURE DIRECTIONS

7.1 Developing a Model of Juvenile TBI

The field of juvenile TBI research is relatively small and lacking the tools to appropriately assess functional recovery. The most widely used behavioral test in juvenile TBI is the Morris Water Maze (MWM). However, the MWM only assesses cognitive impairments and is conventionally used several weeks after the initial injury has occurred. Therefore, while establishing a juvenile TBI model, it was important to establish a battery of behavioral tests that could measure the initial injury acutely as well as the persistence of deficits and long-term recovery.

The first aim established a juvenile TBI model with consistent, measurable deficits, without debilitating injury or mortality. In order to easily assess recovery, it was decided to injury the sensorimotor cortex allowing us to measure motor function as a TBI outcome, something that is easily done in rats. After the model was established, three reliable, easily adaptable sensorimotor tests were identified for use in a rapidly growing model, something that was lacking in the field. Lastly, it was determined that there are no sex differences in sensorimotor response to TBI using the three tests meeting our evaluation criteria allowing for the use of both males and females in TBI studies evaluating behavioral outcomes.

7.2 The Effects of Diet and Brain Fatty Acid Composition on TBI Outcomes

The second aim studied the influence of dietary n-3 fatty acids and brain fatty acid content on recovery from TBI in juvenile rats. The main goal of this study was to determine if dietary n-3 fatty acid content or brain fatty acid composition that has more influence on outcomes of TBI. Knowing whether dietary n-3 fatty acids or brain fatty

acid content has more influence on TBI outcomes would facilitate improved outcomes of TBI. If dietary n-3 fatty acid content has a greater influence, then n-3 supplementation after TBI would provide the most benefit. However, if n-3 brain fatty acid content has greater influence, this would encourage the consumption of a diet high in n-3 fatty acids by pregnant and nursing women as well as neonates and toddlers, during the times at which DHA rapidly accumulates in the developing brain.

In Aim 2, it was determined that rats with diet-induced decreases in brain DHA content had worsened outcomes after TBI. Furthermore, by testing rats with varying decreases in brain DHA content, but fed an identical n-3-deficient diet, I determined that brain fatty acid content, and thus maternal nutrition, has the greatest influence on juvenile TBI outcomes. Rats with the greatest decreases in brain DHA had the worst sensorimotor outcomes after TBI. These rats also had the smallest induction of *Timp1*, an endogenous MMP inhibitor. TIMP-1 has known anti-apoptotic and growth factor properties that are independent of its MMP-inhibiting ability (Hayakawa et al., 1992; Gardner and Ghorpade, 2003; Jourquin et al., 2005), and are thought to occur through interactions with cell surface receptors (Strongin et al., 1995). Because the increase in *Timp1* gene expression was not accompanied by a decrease in MMP2 or -9 message levels or activity, it is likely that it is the anti-apoptotic and growth factor properties of TIMP-1 that are contributing to improved outcomes in our model.

7.3 Implications for Maternal and Early Childhood Nutrition

In Aim 2, it was determined that brain fatty acid composition, not dietary n-3 content, had the greatest effects on TBI outcomes. This result encourages the

consumption of diet high in n-3 fatty acids by pregnant and nursing mothers, as well as toddlers.

DHA and other n-3 LC-PUFAs appear to have very little, if any toxicity. In a toxicologic evaluation of DHA-rich algal oil, an oral dose of 2000 mg/day in pregnant dams did not have any adverse effects on dams or pups before or after birth (Schmitt et al., 2012). Furthermore, pregnant dams consuming a diet high in n-3 fatty acids did not experience any adverse effects relating to reproductive capacity or pup development (Blum et al., 2007). Also, rats experienced no adverse effects when administered DHA in utero and for 90 days at dietary levels resulting in exposures up to 22 or 66 times higher than those expected in infant formulas (Burns et al., 1999).

The current recommended daily intake of DHA during pregnancy and lactation is 300 mg/day (ISSFAL, 2004). A greater DHA and EPA recommended daily intake for in pregnant and nursing mothers and a greater DHA and EPA concentration in infant formulas is called for based on the benefits of DHA on both post-partum maternal mental health (Kendall-Tackett, 2010) and fetal brain development (Carlson, 2009), and now the implications of worsened TBI in juveniles with low brain DHA. Additionally, greater n-3 intake may be even more beneficial in mothers with multiple pregnancies or pregnant with multiples because in these instances the average intake per fetus/child is reduced.

7.4 Potential Mechanisms for LC-PUFA Membrane Composition-Mediated Modulation of *Timp1* Gene Expression

TIMP-1 is an endogenous inhibitor of MMPs whose gene expression is highly induced by many cytokines and hormones. If reduced brain DHA caused worsened TBI through increased inflammation and cytokine activity, we would expect rats with the greatest decreases in brain DHA levels to have the greatest levels of TBI-induced *Timp1* gene expression; however the opposite was true. Therefore, we can propose a mechanism(s) by which *Timp1* is expressed involving altered membrane signaling as a result of decreased brain DHA, either through modulation of lipid raft composition or membrane fluidity or though free fatty acids cleaved from the membrane.

7.4.1 Increased Akt Signaling

One way that endogenous DHA is known to modulate cell signaling is through preferential incorporation into PtdSer in the inner leaflet of the membrane bilayer and promoting PI3K/Akt signaling (Akbar et al., 2005). PI3K/Akt is a well-studied anti-apoptotic pathway overactive in cancer cells (Fresno Vara et al., 2004). Increasing concentrations of DHA and PtdSer in the membrane facilitates the translocation and phosphorylation of Akt. The phosphorylation and activation of Akt suppresses caspase-3 activation and cell death, thus promoting cell survival (Akbar et al., 2005). Conversely, DHA-depleted membranes slow translocation and phosphorylation of Akt (Akbar and Kim, 2002; Akbar et al., 2005). Like endogenous DHA, TIMP-1, in cancer cells, exerts its anti-apoptotic properties through initiating phosphorylation of Akt through activation of the CD63 surface receptor (Lambert et al., 2003; Jung et al., 2006;

Wurtz et al., 2008). Thus, we can speculate based on endogenous DHA's effects on TBI-induced *Timp1* gene expression, demonstrated in Aim 2, and Akt activation, that the two pathways are related. *Timp1* gene transcription may be initiated by membrane DHA mediated-Akt signaling and the anti-apoptotic signal further amplified through TIMP-1-mediated activation of CD63 and Akt.

7.4.2 Transcription Factor Modulation

A second potential mechanism by which DHA might regulate *Timp1* gene expression includes DHA's direct effects on transcription factors. TBI causes a rapid, sustained increase in free fatty acids. LC-PUFAs are known to regulate gene expression through binding to several transcription factor response elements, including PPAR, and RXR (Khan and Vanden Heuvel, 2003). Upstream regions of the *Timp1* gene contain a serum response element that confers *Timp1*'s responsiveness to a variety of agents including cytokines and growth factors (Campbell et al., 1991; Edwards et al., 1992; Bugno et al., 1995; Gardner and Ghorpade, 2003). Identification of putative LC-PUFA response elements within 1500 base pairs upstream of the mouse *Timp1* transcription start site, using JASPAR (Bryne et al., 2008), revealed 6 possible PPAR/RXR binding sites, indicating a second mechanism by which variation in brain DHA content may influence regulation of *Timp1* gene expression.

Very little is known about TIMP-1s involvement in TBI. MMPs have an important role in neurogenesis, neurovascular remodeling, and matrix-trophic signaling in the later stages of recovery from TBI (Falo MC, et al. J Neuroscie Res 2006). Therefore, the best approach to improving TBI outcomes may be through increasing TIMP-1 gene

expression and enzymatic activity and not through inhibiting MMPs. Knowing more about how *Timp1* gene expression is regulated in this context, and influenced by brain fatty acid content, will help us better understand the sequlae of juvenile TBI. Additionally, knowing more about the specific actions of TIMP-1and its target(s) in juvenile TBI will help develop small molecule therapies to improve outcomes, as currently no TIMP-1 mimetics exist.

7.5 Acute Fish Oil Treatment in Juvenile TBI

The third aim examined the effects of acute fish oil administration on behavioral and biochemical recovery from juvenile TBI. Rats treated with fish oil had improved functional outcomes, reduced IgG infiltration into the brain, as well as reduced *Mmp9* gene expression and faster resolution of *Gfap* gene expression. Together, these data suggest that fish oil treatment improves functional outcomes through protection of the BBB through a mechanism that includes faster resolution of gliosis and/or reduced *Mmp9* mRNA levels.

Current clinical therapies for TBI are very limited. Because TBI is a very complex injury, a single therapeutic targeting one post-TBI process is not likely to have much benefit. However, therapies targeting multiple TBI processes may provide more benefit. Fish oil, and specifically DHA, makes for an attractive therapeutic because of the broad range of processes it influences and its low toxicity. Even at the high dose of 15 mL/kg was well tolerated in rats. In fact, high dose fish oil supplementation (19.2 g/day) provided significant benefit in a young patient with severe head trauma deemed likely

lethal (Therapeutic use of omega-3 fatty acids in severe head trauma, Lewis et al. 2013).

7.6 Differential Signaling of Membrane LC-PUFAs and Free Fatty Acids in TBI: MMP-9 as an Example

Brain fatty acid composition and free fatty acids both influence TBI outcomes. However, their influences appear to occur through different mechanisms, though overlapping mechanisms may also exist but were not examined in these studies. Rats with greater brain DHA content had a less severe injury than did those with reduced brain DHA even though both were consuming the same diet, and theoretically, should have the same plasma levels of LC-PUFAs. This suggests that brain fatty acid composition is influencing cellular signaling through alterations in lipid rafts or membrane fluidity. Fish oil dosing improved function and reduced BBB damage in rats with the same brain fatty acid composition suggesting free fatty acids themselves are altering intracellular signaling.

Mmp9 message levels and enzymatic activity, as an example, were increased equally after TBI in rats that sustained a brain injury, regardless of brain fatty acid composition (Aim 2). Fish oil dosing, on the other hand, prevented TBI-induced transcription of the *Mmp9* gene. One possible mechanism for fish oil's effects on *Mmp9* gene expression in injured rats is through fish oil's influence on NF-κB. DHA and EPA both inhibit phosphorylation of IκK, the NF-κB inhibitor (Yang et al., 2013), thereby preventing activation of NF-κB and transcription of NF-κB-regulated genes, including *Mmp9*. There was no effect of brain fatty acid composition on *Mmp9* gene expression.

From that we can conclude that brain fatty acid composition does not directly alter NF-κB signaling, nor do fatty acids cleaved from the membrane, at least not to the extent that high-dose LC-PUFA supplementation does. I would hypothesize that fish oil inhibits transcription of NF-κB-regulated genes in a dose dependent manner, though a study like this has yet to be done.

7.7 Fatty Acids and PPAR

PPARs are a family of three nuclear receptors isoforms (α, β/δ, and γ). They form a heterodimer with the nuclear receptor RXR to play a critical role as lipid sensors and regulators of lipid metabolism. Fatty acids, including DHA and EPA, and eicosanoids are endogenous ligands for PPARs but several exogenous ligands such as the thiazolidinediones (glitazones) and fibrates have been developed to treat type 2 diabetes and hypercholesterolemia.

Recently PPAR agonists have been investigated for the treatment of neural injuries particularly through activation of PPAR-γ. Fenofibrate (Besson et al., 2005) and rosiglitazone (Chen et al., 2007; Hyong et al., 2008; Yi et al., 2008) have demonstrated neuroprotective effects in models of TBI via reducing inflammation, oxidative stress, and apoptosis. PPAR-γ is thought to elicit its anti-inflammatory effects by blocking NF-κB-dependent gene expression as well inhibiting phosphorylation of MAPK and preventing MAPK-dependent pro-inflammatory gene expression (Desreumaux et al., 2001). Additionally, PPAR- γ is able to block transcription of NF-AT, AP-1, and STAT-dependent genes (Lehrke and Lazar, 2005).

However, the molecular mechanism for transcriptional repression by PPARs in response to the binding of ligands is still poorly understood.

Synthetic PPAR ligands have many side effects that make their use less than ideal. The thiazoladine-derived glitazones have side effects including weight gain, peripheral edema, and, as a result, congestive heart failure. Additionally, many glitizars, non-thiazolidine derived PPAR agonists, have failed clinical trials because of serious side effects and/or carcinogenesis-related issues. The demonstrated benefit of PPAR agonists in treating TBI validate potential short-term use of these drugs and justify the need for safer PPAR agonist drugs. The actions of DHA and EPA on PPARs, as well as their other neuroprotective properties and low toxicity, make them an ideal therapeutic alternative.

With regard to the studies in this dissertation, fish oil treatment provided benefit after juvenile TBI by improving functional outcomes, reducing IgG infiltration, preventing TBI-induced expression of the *Mmp9* gene, and faster resolution of *Gfap* gene expression. Though we did not investigate the specific mechanisms of actions of fish oil in these studies, it is possible that fish oil is providing protection in TBI, in part, through agonist effects at PPAR receptors.

7.8 Future Directions

7.8.1 Benefits and Limitations of *Fat-1* Transgenic Mice

In 2004, a transgenic mouse containing the *C. elegans fat-1* gene was created (Kang et al., 2004). The *fat-1* gene, absent in mammals, encodes n-3 fatty acid desaturase that introduces a double bond into n-6 fatty acids at the n-3 position of the

hydrocarbon chain, allowing for the production n-3 fatty acids in the absence of a dietary n-3 supply. Using this transgenic mouse model would eliminate many common confounds associated with nutritional studies. For example, wild-type and transgenic litter mates can be fed the same diet and produce different fatty acid profiles, eliminating the need for multiple differing diets. Also, because litter mates can produce different fatty acid profiles, this eliminates possible maternal influences on outcomes. Another benefit of the *fat-1* transgenic mouse model is that it can be crossed with other transgenic or knockout disease models to produce combined models, allowing for the evaluation of the effects of n-3 fatty acids and or n-6/n-3 ratio on disease development and progression.

While this model has many benefits, it also has some limitations. One significant limitation of this model is that it produces only two levels of n-3 fatty acids: high (*fat-1* transgenic) or low (wild type). A multi-generational or a multi-litter model, as was done in Aim 2, has the benefit of producing of several levels of n-3 fatty acid depletion and thus the examination of a dose-response effect on outcomes. Additionally, while transgenic mice are useful for mechanistic studies, they're not as applicable to human health as are wild type, outbred animals because they've been intentionally genetically manipulated.

7.8.2 A Comprehensive Evaluation of DHA-dosing in Adult and Juvenile Models of TBI

The studies within this dissertation have demonstrated that brain fatty acid content, more so than dietary fatty acid content, influences TBI outcomes in juvenile rats. Additionally, this effect may be due to greater TBI-induced *Timp1* gene expression

in rats with the greatest brain DHA content. Also, dosing rats with fish oil improves outcome and may have specific neuroprotective effects on the BBB. However, it remains to be determined if these effects also occur in adults, and whether brain DHA level at the time of TBI influences the magnitude of benefit produced by n-3 LC-PUFA treatment at the time of injury. Additionally, most studies investigating the use of acute n-3 LC-PUFAs treatment for neural injuries have investigated a single concentration of DHA in adult rodents. Furthermore, these animals have all been fed a standard chow causing them to have high levels of brain DHA. No studies have investigated various doses of DHA in adults or juveniles with various degrees of brain n-3 depletion.

If given an unlimited research budget, based on the current literature, and to provide the most comprehensive study of n-3 fatty acid supplementation in TBI treatment, it would be important to investigate a dose-response effect of acute DHA dosing in adult and juvenile models of TBI in rats raised on a control diet, n-3 deficient diet, and an n-3 enriched diet. Measured outcomes would include sensorimotor deficits using the tests identified in Aim 1, MMP and TIMP expression and activity, and markers of inflammation, excitotoxicity, and apoptosis, all processes identified in this dissertation and in the literature to be improved by DHA. Based on proposed mechanisms of DHA signaling, it would be important to investigate phosphorylation of IκK and Akt, as well. Decreased phosphorylated IκK would support DHA's role in inhibiting activation of NF-κB through inhibition of IκK phosphorylation. Additionally, decreased phosphorylation of Akt would demonstrate membrane DHA's proposed agonistic effect on Akt signaling, specifically translocation and phosphoylation of Akt.

7.8.3 Preferential synthesis of anti-inflammatory DHA-derived lipid mediators after TBI

As described previously, n-3 fatty acids, particularly DHA and EPA, are metabolized into many families of anti-inflammatory, pro-resolving lipid mediators including resolvins and neuroprotectins. Although resolvins have not been investigated in the brain, they decrease neutrophil recruitment, pro-inflammatory cytokines, improve survival, and have many other beneficial effects in mouse models of peritonitis, inflammatory pain, sepsis, and colitis (Spite and Serhan, 2010). NPD1, synthesized from DHA, inhibits leukocyte infiltration and inflammatory gene expression in a model of I/R (Marcheselli et al., 2003). It is not known, however, what role these mediators play in TBI or if they are synthesized from free or membrane-bound fatty acids. Therefore, to better understand whether a long-term n-3 fatty acid-enriched diet or acute n-3 supplementation has greater influence on TBI outcomes, it would be beneficial to know more about the synthesis of these lipid-mediators.

An efficient way to investigate this would be *in vitro* using neuronal cultures or hippocampal slices. An *in vivo* experiment could be proposed, though it would be very costly. In culture, the production of lipid mediators after injury in the presence and absence of [^{13}C]-DHA in the media could easily be investigated. DHA-derived mediators in the cell lysates and media could be identified via HPLC and further analyzed via NMR. Based on the nuclear magnetic spin profile of the different mediators we would be able to determine if dietary fatty acids, membrane-incorporated fatty acids, or both equally, are made into these beneficial lipid-derived anti-inflammatory mediators after injury.

7.8.4 Doxycycline to Improve Outcomes of TBI

Tetracycline antibiotics, particularly doxycycline, have MMP-inhibiting properties independent of their antimicrobial properties. If TIMP-1 is a crucial determinant of TBI outcomes, it would stand to reason that inhibiting MMPs using another molecule would free TIMP-1 to exert its other properties as a growth factor and anti-apoptotic factor. Additionally, besides inhibiting MMPs, sub-microbial doses of doxycycline also exhibits anti-inflammatory and anti-oxidant properties, possibly though inhibition of COX-2 and TNFα converting enzyme. Knowing the role of MMPs, TIMPs, TNFα, and COX-2 in the pathphysiology of TBI, doxycycline, in theory, should improve outcomes from several cellular angles.

Doxycycline does not readily cross an intact BBB or enter CSF (Andersson and Alestig, 1976). However, in TBI, where the BBB is disturbed until at least four days after injury according to Aim 3, and possibly longer, early administration of doxycycline may help improve outcomes after TBI. To confirm this, after injury, rats could be treated with doxycyline, celeoxib, a selective COX-2 antagonist, or etanercept, a selective TNFα-inhibitor. Endpoints measured could include behavior and *Gfap* gene expression to confirm injury, as well levels of TNFα, markers of TNFα-induced inflammation, COX-2 activity, COX-2-produced prostaglandins, and MMP and TIMP gene expression and activities.

DHA and EPA are both substrates for COX-2 in the formation of resolvins (Rv) D1 and E1, respectively. RvD1 and RvE1 have potent *in vitro* and *in vivo* anti-inflammatory properties (Marcheselli et al., 2003; Bannenberg et al., 2005). It is possible that celecoxib may worsen injuries via inhibiting production of RvD1 and RvE1.

However, other potent anti-inflammatory fatty-acid derived mediators are made via other enzymes not affected by celecoxib; for example, formation of NPD1 is initiated by converting DHA to 17S-H(p)DHA by 15-LOX (Marcheselli et al., 2003). In itself, treating rats with TBI with or without celecoxib would demonstrate the contributions of the resolvins versus NPD1 in TBI pathology.

Overall, this study would demonstrate the efficacy, or lack thereof, of doxycycline in the treatment of TBI as well as delineate its primary neuroprotective mechanism(s) of action. This would allow for a better understanding of the pharmacologically-targetable pathophysiological processes occurring after TBI and help identify new, or repurpose old, pharmacologic agents for the treatment of TBI.

7.9 Conclusion

In conclusion, brain injuries, particularly those in children, are tremendously understudied and lack efficacious therapeutics. N-3 LC-PUFAs, which exhibit anti-inflammatory, anti-excitotoxic, and anti-apoptotic properties, are an excellent candidate for treating TBI. Additionally, n-3 fatty acids have no known toxicities, even at high doses, are well absorbed, and have few side effects. Better understanding of how both endogenous and exogenous n-3 fatty acids work in the brain after TBI will lead to better therapeutics and, ultimately, better outcomes for those suffering from TBI.

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