FLUID PERCUSSION INJURY IN THE RAT AS AN ANIMAL MODEL OF CONCUSSION: CUMULATIVE EFFECTS OF REPEATED CONCUSSION AND ITS TREATMENT BY ANTI-CD11D ANTIBODY

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Abstract

Traumatic brain injury is a global health concern with limited treatment options currently available. Concussion is the most common form of traumatic brain injury, and although a single concussion rarely results in long-term neurological dysfunction, repeated concussion can result in cumulative damage and chronic neurodegenerative disease. However, little is known about the factors and mechanisms of concussion involved in these detrimental effects. Animal models provide a means to examine the factors and mechanisms involved in traumatic brain injury, as well as potential treatments, in experiments that cannot be conducted using human participants. In the present thesis a fluid percussion model of traumatic brain injury was used to study single and repeated concussion in adult male Long-Evans rats. Anti-CD11d integrin antibody, a novel compound that reduces neuroinflammation by targeting the infiltration of peripheral leukocytes into the brain after traumatic brain injury, was evaluated as a potential treatment for concussion.

In Study 1 a single mild lateral fluid percussion injury (1.0-1.5 atm) caused short-term (24 hrs) behavioral impairments and neuropathological alterations indicative of neuroinflammation and axonal injury. In Study 2, three or five mild lateral fluid percussion injuries given at 5-day intervals caused cumulative short-term (24 hrs) and long-term (8 weeks) behavioral impairments and neuropathological alterations indicative of neuroinflammation and cortical loss. These results appear to validate the use of single and repeated mild lateral fluid percussion injuries to model important aspects of human concussion. In Study 3 anti-CD11d antibody administered after a single moderate lateral fluid percussion injury (2.5 - 3.0 atm) reduced cognitive, emotional, and motor

impairments, and also reduced neuroinflammation and neuronal loss relative to injured rats treated with a control antibody. In Study 4 anti-CD11d antibody administered after each of three repeated mild lateral fluid percussion injuries similarly reduced cognitive, emotional, and motor impairments, and neuroinflammation and cortical loss relative to injured rats treated with a control antibody. These novel findings suggest the involvement of infiltrating peripheral leukocytes and neuroinflammation in both single and repeated concussion, and they support the further investigation of anti-CD11d antibody as a potential treatment for concussion.

Keywords: traumatic brain injury, mild traumatic brain injury, concussion, repeated concussion, animal model, fluid percussion injury, behavioral neuroscience, neuroinflammation, axonal injury, anti-CD11d integrin antibody

Co-Authorship

All of the contents in the current thesis were carried out in collaboration with Dr. Donald Peter Cain. Sandy Shultz completed all craniotomy surgeries and fluid percussion injuries. Sandy Shultz, Francis Boon, Jeffrey Hepburn, and Charlotte Chiu carried out behavioral testing. Sandy Shultz, Feng Bao, and Francis Boon administered antibody treatments in Studies 3 and 4. Immunohistochemical staining in Study 1 was completed in Dr. Derrick MacFabe's laboratory by Roy Taylor, and quantification of immunohistochemical markers was completed by Sandy Shultz and Kelly Foley. Immunohistochemical staining for Studies 2, 3, and 4 was completed in Dr. Arthur Brown's laboratory by Dr. Feng Bao and Vanessa Omana. Sandy Shultz completed the quantitative and semi-qualitative neuropathological analyses in Studies 2 and 4. Esther Ernst completed the immunohistochemical analysis in Study 3. For the purpose of publication, more detailed neuropathological analyses for Studies 2, 3, and 4 are currently ongoing.

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List of Abbreviations

μm: micrometer

ANOVA: analysis of variance

APP: amyloid precursor protein

atm: atmosphere

C: Celsius

CD68: cluster of differentiation 68

cm: centimeters

Fig: figure

g: gram

GFAP: glial fibrillary acidic protein

hr: hour

ip: intraperitoneal

kg: kilogram

L: liter

LR: long recovery

m: meter

M: mean

mg: milligram

min: minute

ml: milliliter

mm: millimeter

n: number

p: significance

PBS: phosphate-buffered saline

sec: second

SEM: standard error of the mean

SR: short recovery

W: watt

Chapter 1

1. General introduction

Traumatic brain injury, which is defined as a physical injury to brain tissue that temporarily or permanently impairs brain function (Parikh et al., 2007), is a global health concern and a growing socioeconomic problem. Traumatic brain injury is the leading cause of mortality and disability among individuals under the age of 45 (Rao and Lyketsos, 2000), with young adult males accounting for approximately 75% of cases (Langlois et al., 2006; Maas et al., 2008). Due to the complex pathophysiology associated with traumatic brain injury, there is currently no effective pharmaceutical treatment available for widespread clinical use (Atif et al., 2009). Consequently, individuals who suffer debilitating traumatic brain injury often require lifelong medical care and support (Maas et al., 2008). In North America alone, the financial burden of traumatic brain injury has been estimated at over $60 billion annually (Maas et al., 2008).

Cerebral concussion, or mild traumatic brain injury, accounts for roughly 80% of traumatic brain injury cases (Anderson et al., 2006). Despite the high incidence of concussion, there is currently a poor understanding of what occurs in the brain following this injury (Cantu, 2009). As a result, there is much debate surrounding the medical management and treatment of concussion. Medical concern is increased for individuals, such as athletes, who are at risk of suffering multiple concussions (Cantu, 2009; Maroon et al., 2000). Growing clinical evidence indicates that repeated concussion can result in long-term neurological damage and possibly neurodegenerative disease (McKee et al., 2009). However, little is known regarding the factors and mechanisms contributing to the cumulative and chronic nature of repeated concussion (McKee et al., 2009). Given the

lack of understanding, high incidence rates, and growing costs associated with traumatic brain injury, it is imperative that research is carried out that potentially addresses these issues.

Animal models of human neurological disorders, such as traumatic brain injury, allow for experimentation that cannot be conducted with human patients, and provide a means to examine factors involved in these disorders as well as their potential therapies. The fluid percussion injury technique is a commonly used and well-characterized animal model of traumatic brain injury (Thompson et al., 2005). In the present dissertation, a rat model of fluid percussion injury was used to study the effects of single and repeated concussion. A novel pharmacological treatment targeting the neuroinflammatory response was also applied and assessed using fluid percussion injury with the rat. The General Introduction will begin by discussing the classification, behavioral and biological features, and treatments associated with human traumatic brain injury, followed by a section focused primarily on concussion. This background will serve to provide insight on traumatic brain injury as well as the current issues surrounding these injuries. This will be followed by discussion of the importance of animal models in the study of traumatic brain injury, a description of techniques available for experimental traumatic brain injury in the rat, and rationale for the use of fluid percussion injury to model and study concussion. The final section will provide a brief description of the studies to be presented in Chapters 2-5.

1.1. Traumatic brain injury in humans

1.1.1. Classification

The classification of traumatic brain injury is commonly based on the clinical severity of the injury, ranging from mild to severe (Maas et al., 2008). The Glasgow coma scale has become the universally accepted severity classification system for traumatic brain injury (Lollis et al., 2008; Maas et al., 2008; Young, 2009). The Glasgow coma scale is comprised of three component tests: the Eyes, Motor, and Verbal scales. The Eyes scale ranges from 1 (no response) to 4 (spontaneous eye movement). The Motor scale ranges from 1 (no response) to 6 (obeys commands). The Verbal scale ranges from 1 (no response) to 5 (oriented and converses normally). The summation of these scales allows for a total Glasgow coma scale score ranging from 3-15. Based on these scores, the Glasgow coma scale classifies traumatic brain injury cases as mild (14-15), moderate (9-13), or severe (3-8; Lollis et al., 2008; Maas et al., 2008; Young, 2009). It is estimated that approximately 80% of traumatic brain injuries fall within the mild category (Anderson et al., 2006; Jennett, 1996).

In addition to clinical severity, traumatic brain injury has also been traditionally classified based on the mechanism of injury (Maas et al., 2008; Parikh et al., 2007). Mechanism-based classification usually categorizes traumatic brain injury as either closed or open head injuries (Maas et al., 2008; Parikh et al., 2007). Closed head injury, also called blunt or non-penetrating brain injury, does not involve a breach of the brain's dura mater; however, skull fractures may occur (Maas et al., 2008; Parikh et al., 2007). Closed head injury is the most common type of traumatic brain injury in the general population, and is typically caused by motor vehicle accidents, sports injuries, and physical assault (Maas et al., 2008; Parikh et al., 2007). In contrast, open head injury involves the penetration of the scalp, skull, meninges, and often brain tissue itself. Such

penetration injuries are more common amongst military personnel, and are usually caused by foreign objects such as bullets (Maas et al., 2008; Parikh et al., 2007). While penetrating traumatic brain injury poses a significant medical problem, with increased incidence in times of war, these injuries are beyond the scope of the current thesis. All remaining discussion will pertain to closed head traumatic brain injury unless stated otherwise.

1.1.2. Behavioral and biological features of traumatic brain injury

Depending on the severity and brain structures affected, various signs and symptoms may appear within the seconds to weeks following traumatic brain injury (Anderson et al., 2006; Parikh et al., 2007; Rao and Lyketsos, 2000). These might include a loss of consciousness, headache, vomiting or nausea, convulsions or seizures, dilation of one or both pupils, cranial nerve palsy, clear fluid draining from ears or nose, loss of bladder or bowel control, slurred speech, confusion, dizziness, sensory problems, sleeping abnormalities, memory loss, cognitive impairments, agitation, irritability, combativeness, disinhibition, impulsivity, anxiety, depression, mood swings, motor problems, and other unusual behaviors like paranoia or mania (Anderson et al., 2006; Parikh et al., 2007; Rao and Lyketsos, 2000).

Traumatic brain injury might involve various types of gross or microscopic brain damage depending on the mechanism, forces, and duration of injury (Maas et al., 2008; Parikh et al., 2007; Raghupathi et al., 2000). Despite this heterogeneity, the associated damage of traumatic brain injury is often categorized as resulting from either primary or secondary injuries (Graham et al., 2000; Maas et al., 2008; Marshall, 2000; Schmidt et al., 2005). Primary injuries are induced at the moment of impact, when both linear and/or

rotational mechanical forces are applied to the brain (Graham et al., 2000; Maas et al., 2008; Marshall, 2000). These forces most commonly affect the frontal, parietal, and temporal lobes (Umile et al., 2002), and result in focal and/or diffuse injury patterns (Rao and Lyketsos, 2000). A focal injury pattern typically occurs following a direct blow to the head and may result in contusion, hemorrhage, and ischemic infarct (Rao and Lyketsos, 2000). Diffuse injury patterns are typically a result of the stretching and/or shearing of white-matter tracts due to the differential motion of the brain within the skull (Maas et al., 2008, Rao and Lyketsos, 2000). The severity of this diffuse pattern ranges from a brief disruption and misalignment of axonal neurofilaments to widespread axonal tearing (Rao and Lyketsos, 2000).

Secondary injuries result from processes that are initiated by the primary insult, and may develop over the hours, days, or weeks that follow (Graham et al., 2000; Maas et al., 2008; Marshall, 2000). While different variations of traumatic brain injury may initiate a range of secondary mechanisms, with variable extent and duration, these processes most commonly involve apoptosis, increased excitatory neurotransmitter release, calcium-mediated damage, mitochondrial dysfunction, free radical generation, and a neuroinflammatory response (Graham et al., 2000; Maas et al., 2008).

1.1.3. The neuroinflammatory response in traumatic brain injury

Although all of the above mentioned processes might contribute to brain damage following traumatic brain injury, the neuroinflammatory response may be the most important secondary component, as it is capable of mediating a number of important mechanisms that can occur in the aftermath of traumatic brain injury (Graham et al., 2002; Maas et al., 2008; Schmidt et al., 2005). The neuroinflammatory response is

characterized by the activation of astrocytes and microglia, the release of pro-inflammatory cytokines, chemokines, and adhesion molecules, the opening of the blood-brain barrier, and the migration of leukocytes across the leaky blood-brain barrier (Kadhim et al., 2009; Laird et al., 2008; Lu et al., 2009; Schmidt et al., 2005). This response is initiated within minutes of central nervous system injury and can last for months (Donnelly and Popovich, 2008; Lu et al., 2009; Morganti-Kossman et al., 2007). Neuroinflammation has been shown to have dualistic properties following traumatic brain injury, in that it can serve in both neuroprotective and neurotoxic roles (Ekdahl et al., 2009; Laird et al., 2008; Morganti-Kossman et al., 2007). After traumatic brain injury, neuroinflammation is necessary to clear debris, isolate injured tissue, and potentially mediate regeneration and recovery (Ekdahl et al., 2009; Laird et al., 2008; Morganti-Kossman et al., 2007). However, in excess, the neuroinflammatory response can have negative effects and contribute to secondary brain damage, either directly or indirectly, through mechanisms such as apoptosis, free radical formation, and lipid peroxidation (Bao et al., 2004; Morganti-Kossman et al., 2007; Schmidt et al., 2005).

Astrocytes are glial cells that are involved in many aspects of brain function, including neuroinflammation (Laird et al., 2008). At the onset of traumatic brain injury, astrocytes undergo a morphological change known as reactive astrocytosis that involves cellular hypertrophy, lengthened processes, and increased expression of GFAP. In traumatic brain injury, reactive astrocytes have several neuroprotective properties including the repair of the blood-brain barrier, the signaling of regenerative processes, and the isolation of tissue damage through scar formation (Laird et al., 2008). However, astrocytes may also have damaging properties after traumatic brain injury. Although

astrocyte mediated scarring helps to isolate tissue damage after traumatic brain injury, this scarring may also restrict neural repair and axonal regeneration (Laird et al., 2008). Astrocytes also produce and release a number of pro-inflammatory mediators that may contribute to secondary brain damage through mechanisms such as apoptosis and the recruitment of peripheral leukocytes (Kadhim et al., 2008; Laird et al., 2008).

Microglia are immune cells in the brain that are considered to be the primary mediators in the innate neuroinflammatory response (Gehrmann et al., 1995; Morganti-Kossman et al., 2007). Under normal conditions microglia are typically in a resting state and monitor the central nervous system environment for alterations indicative of damaged neurons and/or infectious agents (Gehrmann et al., 1995). In the case of traumatic brain injury, the primary insult to the brain results in damaged neurons and axons, and subsequent changes in the extracellular space such as ion fluctuations. These changes are detected by, and activate, microglia (Gehrmann et al., 1995; Morganti-Kossman et al., 2007). Activated microglia undergo a morphological change, taking on an amoeboid shape resembling peripheral macrophages, and scavenge the damaged central nervous system, performing phagocytic functions (Morganti-Kossman et al., 2007). However, activated microglia also produce and release pro-inflammatory cytokines, chemokines, adhesion molecules, and free radicals (Kadhim et al., 2009; Morganti-Kossman et al., 2007), all of which can contribute to secondary brain damage.

Common pro-inflammatory cytokines produced by microglia, astrocytes, and neurons during traumatic brain injury include tumor necrosis factor-α, interleukin-1β, and interleukin-6 (Kadhim et al., 2009; Morganti-Kossman et al., 2007). Taken together, these cytokines may contribute to secondary damage, either directly or indirectly, through

apoptotic and necrotic signaling, damage to myelin and its precursors, enhancement of blood-brain barrier permeability, edema, constriction of arteries, ischemia, and the further synthesis of pro-inflammatory cytokines, chemokines, and adhesion molecules (Kadhim et al., 2009; Morganti-Kossman et al., 2007; Schmidt et al., 2005).

Chemokines are chemoattractant cytokines that initiate the migration of peripheral inflammatory cells, such as neutrophils and macrophages, from the vascular system into the central nervous system and to the site of damaged tissue (Kadhim et al., 2009; Lu et al., 2009; Morganti-Kossman et al., 2007). The infiltration and accumulation of leukocytes in the brain also requires cell adhesion molecules such as integrins and selectins (Kadhim et al., 2009; Lu et al., 2009; Morganti-Kossman et al., 2007). Integrins are membrane-bound proteins expressed on peripheral leukocytes such as macrophages and neutrophils. Adhesion molecules expressed on endothelial cells bind with these integrins and assist in the extravasation of leukocytes into the brain (Lu et al., 2009). Neutrophils typically gain access to the central nervous system within 24 hrs post-injury and result in increased production of pro-inflammatory mediators and reactive oxygen species, lipid peroxidation, blood-brain barrier breakdown, and edema (Donnelly and Popovich, 2008; Lu et al., 2009; Schmidt et al., 2005). Macrophages infiltrate the central nervous system approximately three to five days post-injury and function similarly to activated microglia, clearing cellular debris while producing cytokines and other cytotoxins (Donnelly and Popovich, 2008; Lu et al., 2009). Overall, the infiltration of peripheral immune cells into the central nervous system can further weaken the blood-brain barrier, exacerbate the neuroinflammatory response, and worsen secondary damage (Lu et al., 2009; Schmidt et al., 2005).

In sum, traumatic brain injury can trigger both beneficial and damaging neuroinflammatory effects, and it is thought that the damaging neuroinflammatory effects can result in lasting impairments. However, further research is needed to better understand this complex cascade and its role in traumatic brain injury.

1.1.4. Traumatic brain injury treatment

Improvements in traumatic brain injury patient outcomes have largely resulted from advances in intensive care management, neurosurgical techniques, and emphasis on rehabilitation (Johnston and Gupta, 2002; Lollis et al., 2008; Morganti-Kossman et al., 2007; Young, 2009). Unfortunately, these strategies often result in limited functional benefits after traumatic brain injury (Lollis et al., 2008).

Although primary injury to the brain in traumatic brain injury is largely unavoidable, the prevention of some or all damage resulting from secondary injury represents a fruitful area for research in Neuroscience and Medicine. Pharmacological interventions might be a promising approach to patient care in traumatic brain injury given their potential to target such secondary injury mechanisms (Morganti-Kossman et al., 2007). However, despite the vast number of studies (> 1000) investigating possible neuroprotective treatments for traumatic brain injury, there is currently no safe and effective pharmacological therapy available for widespread clinical practice (Stein and Wright, 2010).

The difficulty in developing an effective traumatic brain injury pharmacotherapy likely results from a combination of factors. One problem lies in the complex heterogeneity of traumatic brain injury (Atif et al., 2009; Stein and Wright, 2010). As discussed earlier, several processes contribute to patient outcome in traumatic brain

injury. Furthermore, growing evidence indicates that traumatic brain injury is not isolated to the central nervous system and should be considered a systemic disorder (Lu et al., 2009; Masel and DeWitt, 2010; Stein and Wright, 2010). However, the majority of drugs developed to treat traumatic brain injury are monotherapies that target a single mechanism (e.g. blocking intracellular calcium; Atif et al., 2009). Given the failure of past treatments, therapeutic strategies might be improved by utilizing compounds capable of addressing several factors involved in traumatic brain injury or devising combinations of treatments to target the multiple issues in traumatic brain injury in a given patient.

Another problem in the development of an effective pharmacotherapy is that many of the secondary processes involved in traumatic brain injury remain poorly understood. For example, while it is now known that the neuroinflammatory response can be both beneficial and detrimental following traumatic brain injury, it is not yet clear how to best balance or alter these dual properties therapeutically (Kadhim et al., 2009; Morganti-Kossman et al., 2007). Thus, in order to improve traumatic brain injury treatments, further research is required to increase our understanding of the different processes involved.

1.2. Concussion

Concussion accounts for the large majority of traumatic brain injury cases – roughly 80% – and is now recognized as a serious public health concern (Anderson et al., 2006; Jennett, 1996). Concussion is most common in young adult males and is typically caused by sports, motor vehicle accidents, falls, and assaults (Anderson et al., 2006; Kraus and Nourjah, 1988). The term concussion is commonly used interchangeably with mild traumatic brain injury; however concussion is generally considered a narrower

category that emphasizes the impaired functional outcome resulting from the trauma (Anderson et al., 2006; Maroon et al., 2000). While no single agreed upon definition of concussion currently exists, it has recently been defined as a complex pathophysiological process affecting the brain that is induced by traumatic biomechanical forces (Anderson et al., 2006; McCrory et al., 2009). A consensus statement on concussion was recently composed by a panel of concussion experts, which identifies several common features that can be used to help define the nature of concussion injury (McCrory et al., 2009). According to this definition, a concussion (1) is caused by a direct blow or impact to the head, face, neck, or elsewhere on the body with a resulting force transmitted to the brain, (2) typically results in the rapid onset of transient neurologic impairments, (3) may result in neuropathological changes, although the acute symptoms largely reflect a functional disturbance rather than a structural brain injury, (4) results in symptoms that may or may not involve the loss of consciousness, and (5) does not result in abnormalities on standard structural neuroimaging studies (McCrory et al., 2009).

1.2.1. Symptoms and pathology

Signs and symptoms of concussion may include headache, dizziness, insomnia, fatigue, memory loss, cognitive impairment, poor concentration, impulsivity, disinhibition, depression, anxiety, irritability, inappropriate social behaviors, and personality change (Anderson et al., 2006; Boll and Barth, 1983; Giza and Hovda, 2001; Kim, 2002; Kushner, 1998). Concussion can be caused by a variety of mechanisms, ranging from blunt blows to rotational forces (Anderson et al., 2006; Young, 2009). As a result, concussion might involve both focal and more diffuse injury patterns (Young, 2009). Unlike moderate and severe traumatic brain injury, the neurological disturbances

associated with concussion often occur without any readily identifiable brain damage (McCrory et al., 2009; Young, 2009). Standard clinical imaging techniques used on traumatic brain injury patients, such as computed tomography and magnetic resonance imaging, typically reveal no signs of structural damage or loss of brain tissue in cases of concussion (Lollis et al., 2008; McCrory et al, 2009). Furthermore, as concussion is generally not associated with mortality, it is difficult to obtain post-mortem pathology that is not confounded by any number of factors (e.g. aging due to passage of time, other bodily injuries or disorders, intake of drugs or toxins, etc; Blumbergs et al., 1994). Consequently, there is a poor understanding of what occurs in the human brain following concussion injury.

Due to these limitations, more recent studies have used specialized imaging techniques to identify mechanisms that may be associated with concussion (Arfanakis et al., 2002; Benson et al., 2007; Chen et al., 2004; Kraus et al., 2007; Lovell et al., 2007; Umile et al., 2002). Diffusion imaging is a relatively new tool that uses magnetic resonance imaging technology. Diffusion imaging is based on the diffusivity of water molecules in the brain, which is directionally limited (anisotropic) in healthy axons (Arfanakis et al., 2002; Benson et al., 2007; Kraus et al., 2007). In unhealthy white matter, damaged axonal membranes become less constricting to molecules and are thus less anisotropic (Arfanakis et al., 2002; Benson et al., 2007; Kraus et al., 2007). Several studies have utilized diffusion imaging techniques in concussion patients and have found evidence of axonal injury in several white matter tracts, most consistently the corpus callosum (Arfanakis et al., 2002; Benson et al., 2007; Kraus et al., 2007). However, the

permanence of these injuries and their relation to behavioral changes remains poorly understood (Arfanakis et al., 2002; Benson et al., 2007; Kraus et al., 2007).

Imaging techniques that assess metabolic function, such as functional magnetic resonance imaging and positron emission tomography imaging, have also been applied in concussion patients (Chen et al., 2004; Lovell et al., 2007; Umile et al., 2002). These studies have successfully identified abnormalities occurring primarily in temporal, parietal, and frontal areas that may persist for months following injury (Chen et al., 2004; Lovell et al., 2007; Umile et al., 2002). However, the findings of these studies are inconsistent, reporting evidence for both increased and decreased metabolism after concussion (Chen et al., 2004; Lovell et al., 2007; Umile et al., 2002). Therefore, while metabolic abnormalities likely occur in the brains of concussed individuals, the nature of these changes and their relation to associated symptoms remains uncertain.

There is also some evidence indicating the presence of a neuroinflammatory response in concussed individuals. For example, evidence of a neuroinflammatory response, consisting of neutrophils, macrophages, and activated microglia, has been identified using immunohistochemical markers in brain tissue biopsies taken from human patients suffering contusion injuries (Holmin et al, 1998). Magnetic resonance spectroscopy is an imaging technique capable of identifying changes in brain metabolites, such as choline and lactate, that might indicate neuroinflammation (Carpentier et al., 2006; Marino et al., 2007). Magnetic resonance spectroscopy performed on concussed individuals has found small changes in these metabolites when compared to scans on non-injured controls (Marino et al., 2007). However, these abnormalities may also be

associated with other neural processes such as apoptosis and/or axonal injury (Marino et al., 2007).

Overall, these studies have provided some insight into mechanisms involved in concussion, and the techniques mentioned may prove useful as diagnostic tools in clinical practice. However, despite these advances, the pathophysiological response following concussion and its relation to any behavioral change remains poorly understood. Unfortunately, this lack of understanding is only magnified in the case of repeated concussion (Cantu, 2009).

1.2.2. Repeated concussion

As discussed, a single concussion typically does not result in long-term neurological changes. However, growing evidence suggests that repeated concussion can result in cumulative and chronic neurological damage and disease. While repeated concussion can occur in various circumstances such as motor vehicle accidents or falls, it is much more common in individuals, such as athletes, who engage in frequent competitive or combative bodily activities (Bailes and Cantu, 2001; McCrea et al., 2003; McKee et al., 2009). Estimates suggest that 20-30% of amateur athletes suffer repeated concussion and that this proportion is likely greater in professional athletes (Collins et al., 1999a; Matser et al., 1999). Studies using neuropsychological tests and questionnaires to assess athletes that had experienced repeated concussion have consistently found that those suffering three or more concussion injuries display worse acute symptoms, long-term cognitive impairments, and increased incidence of depression than athletes suffering a single concussion (Guskiewicz et al., 2003; Guskiewicz et al., 2005; Guskiewicz et al., 2007; Macciocchi et al., 2001).

For over 80 years it has been documented that repeated mild brain trauma is capable of inducing progressive neurological abnormalities (Corsellis et al., 1973; Courville, 1962; Martland, 1928; Millspaugh, 1937). Originally termed dementia pugilistica, this neurodegenerative disorder is now known as chronic traumatic encephalopathy (McKee et al., 2009). Chronic traumatic encephalopathy is thought to occur in approximately 20% of individuals suffering repeated concussion, though the exact incidence is unknown and may be much higher (McKee et al., 2009; Roberts et al., 1990). Symptoms associated with chronic traumatic encephalopathy typically begin with attention, concentration, and memory deficits, and progress to long-term cognitive impairments, depression, anxiety, impaired judgment and erratic behaviors, social instability, motor and gait disturbances, and dementia (Bailes and Cantu, 2001; Cantu, 2007; Collins et al., 2002; Corsellis et al., 1973; Guskiewicz et al., 2007; Jellinger, 2004; McKee et al., 2009; Omalu et al., 2005; Omalu et al., 2006). Pathological studies of post-mortem brain tissue taken from former athletes who had sustained repeated concussions and displayed symptoms of chronic traumatic encephalopathy have reported evidence of neuronal loss, brain atrophy, scarring, amyloid and tau deposition, neuroinflammation, and diffuse axonal injury (McKee et al., 2009; Omalu et al., 2005; Omalu et al., 2006).

Despite the growing evidence, it is still debated whether repeated concussion causes cumulative neurological damage and disease. This is likely due to the fact that little is known about the factors and mechanisms that might contribute to these long-term consequences (Cantu, 2009; McKee et al., 2009). As a result, there is currently much controversy concerning the proper management and treatment of concussion, particularly for athletes.

1.2.3. Concussion management

Given the transient nature of the symptoms, the absence of significant brain damage, the lack of a broadly accepted definition, and possible cumulative effects, the medical evaluation and treatment of concussion can often be challenging (Maroon et al., 2000). Traditionally, medical personnel have used one of three criteria for the evaluation of concussion and return-to-play decisions in athletes at risk for repeated concussion. These criteria are laid out in The Congress of Neurological Surgeons Guidelines for Cerebral Concussion, The Cantu Concussion Guidelines, and the American Academy of Neurology Guidelines (Maroon et al., 2000; Martineau et al., 2007; Quality of Standards Subcommittee, 1997).

Overall, there is much overlap amongst these guidelines, as each of them grades concussion severity from 1 (mild) to 3 (severe). These grades are determined based on whether or not the individual loses consciousness, as well as the duration of neurological disturbances such as amnesia and confusion. In general, Grade 1 concussion does not involve a loss of consciousness, and neurological symptoms take no longer than 5 to 30 min to resolve. The individual is instructed to rest. As soon as an athlete is asymptomatic they are eligible to return-to-play. If a second Grade 1 concussion occurs, an athlete is again instructed to rest and is withheld from play until asymptomatic for 1 to 2 weeks (Collins et al., 1999b; Maroon et al., 2000; Quality of Standards Subcommittee, 1997).

In Grade 2 concussion neurological symptoms are still present at 15 to 30 min post-injury. Loss of consciousness may or may not occur in Grade 2 concussion, but if it occurs the duration is limited to 5 min. Individuals are instructed to rest and athletes are able to return-to-play in 1 to 2 weeks if asymptomatic. If a second Grade 2 concussion

occurs, the individual is instructed to rest and is withheld from play for a minimum of 2 to 4 weeks, at which point they may return if asymptomatic. If an athlete suffers three or more Grade 1 or 2 concussions in a single season, they are withheld from play for the remainder of the season and may return-to-play the following year if asymptomatic (Collins et al., 1999b; Maroon et al., 2000; Quality of Standards Subcommittee, 1997).

Grade 3 concussion always involves a loss of consciousness (typically longer than 5 min) with neurological disturbances still present at 24 hrs post-injury. Grade 3 concussion is treated in a manner that is similar to a more severe traumatic brain injury, with mandatory computed tomography or magnetic resonance imaging. Athletes are withheld from play for a minimum of 2 to 4 weeks, at which point they may return-to-play if asymptomatic. If a second Grade 3 concussion is suffered, the athlete will be withheld from play for at least 4 weeks and will likely have their season terminated (Collins et al., 1999b; Maroon et al., 2000; Quality of Standards Subcommittee, 1997).

Unfortunately, these guidelines are not based on any kind of scientific knowledge regarding the determinants of injury severity or recovery progress (Maroon et al., 2000), and there is a growing body of literature identifying numerous limitations to these guidelines (Collins et al., 1999b; Lovell et al., 1999; Maroon et al., 2000). One limitation of these guidelines is the assumption that loss of consciousness is indicative of greater injury severity and worse outcome. To evaluate the guidelines in terms of this assumption, Lovell et al. (1999) investigated whether loss of consciousness predicted neuropsychological test performance in concussed patients. Findings from this study revealed that patients who had lost consciousness did not perform significantly worse

than those who had not. Additionally, this study found no evidence for the weighted use of other markers, such as amnesia and confusion in making return-to-play decisions.

Given these shortcomings, the medical community is beginning to use alternative approaches for concussion management in athletes. For example, many sports leagues have implemented the use of rigorous neuropsychological testing before and after concussion in return-to-play decisions (Lovell et al., 2007; Maroon et al., 2000). While this approach is an improvement over the former guidelines and may provide more informed return-to-play decisions then previous procedures, it remains true that little is known regarding the relationship between neuropsychological test performance and the underlying mechanisms that may contribute to the cumulative and neurodegenerative processes associated with repeated concussion (Lovell et al., 2007; Maroon et al., 2000). Clearly, more research is needed to address the lack of knowledge in this medically and socially important topic.

1.3. Animal models of traumatic brain injury

Animal models allow for the examination of factors involved in human disorders and their potential therapies using experiments that cannot be conducted in humans. In the case of traumatic brain injury, animal models have been utilized for over 50 years in a variety of species (Armstead and Kurth, 1994; Denny-Brown, 1945; Lindgren and Rinder, 1965; Sullivan et al., 1976; Whiting et al., 2006). Over time, these techniques have evolved to better model the mechanics and injury patterns of traumatic brain injury. Of the species available for use in experimental traumatic brain injury, the rat allows for the efficient investigation of both a wide range of behaviors and the pathological assessment of brain structures and pathways similar to those associated with traumatic

brain injury in humans (Jones et al., 2008; Paxinos and Watson, 1986; Pierce et al., 1998; Umile et al., 2002; Whiting et al., 2006). Consequently, a number of traumatic brain injury models, including weight-drop injuries, controlled cortical impact, as well as fluid percussion injury, have emerged for use with the rat (Prins and Hovda, 2003; Whiting et al., 2006).

1.3.1. Weight-drop models

Weight-drop injury models may or may not involve the exposure of the dura membrane that surrounds the brain (Prins and Hovda, 2003). Closed head weight-drop models typically make use of a weight, such as a small metal sphere, which is dropped onto the surface of the skull (see Fig. 1.1). The weight of the mass and the height of the release are varied depending on the desired severity of injury (Marmarou et al., 1994; Montasser et al., 1994; Prins and Hovda, 2003). In an attempt to avoid skull fractures and mortalities, closed head weight-drop techniques often utilize helmets or injury plates (Marmarou et al., 1994; Montasser et al., 1994; Prins and Hovda, 2003; Whiting et al., 2006). Although this technique is capable of modeling numerous aspects of traumatic brain injury, including edema and widespread injury to neurons, axons, and microvasculature (Prins and Hovda, 2003; Whiting et al., 2006), the technique is limited in its ability to model concussion due to high frequencies of skull fractures, convulsions, and mortalities at the injury levels required to produce behavioral changes similar to those observed in the human condition (DeFord et al., 2002; Marmarou et al., 1994; Montasser et al., 1994).

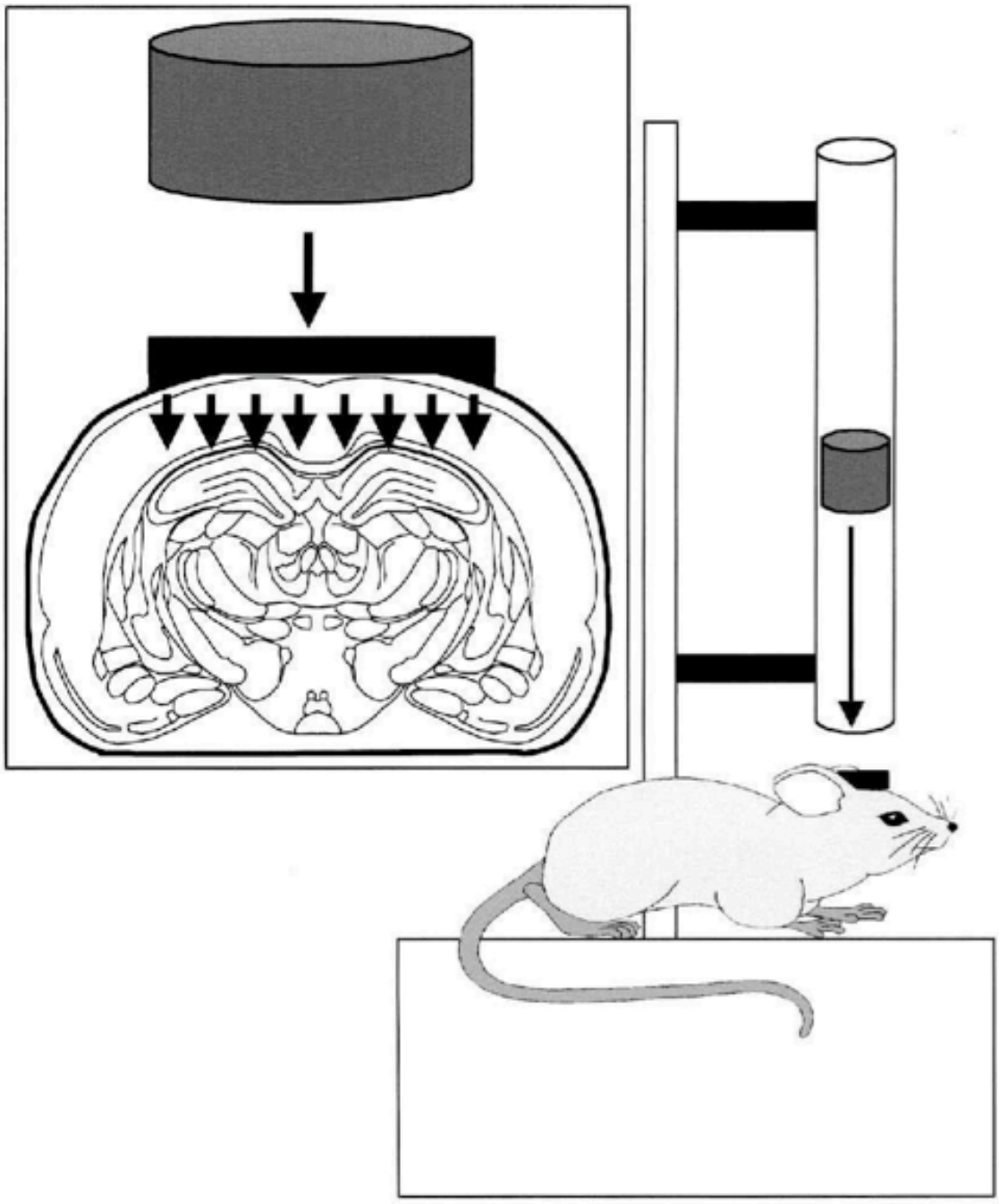

Figure 1.1. Illustration of the closed head weight-drop model. A weight is dropped onto the surface of the skull. An injury plate may or may not be used (Prins and Hovda, 2003).

Open head weight-drop models involve dropping an object through a tube onto a footplate resting directly on the exposed dura (Feeney et al., 1981; Prins and Hovda, 2003; see Fig. 1.2). As in the closed head technique, adjusting the weight of the dropped object or the distance from which it is released can alter injury severity. This technique successfully models some forms of traumatic brain injury as it generates a focal contusion that results in edema, inflammation, and a small necrotic cavity that evolves to maximum cavitation over a period of weeks (Prins and Hovda, 2003). However, considering the more diffuse nature and absence of structural brain damage in concussion, the localized loss of brain tissue associated with this technique appears to limit its usefulness in modeling concussion.

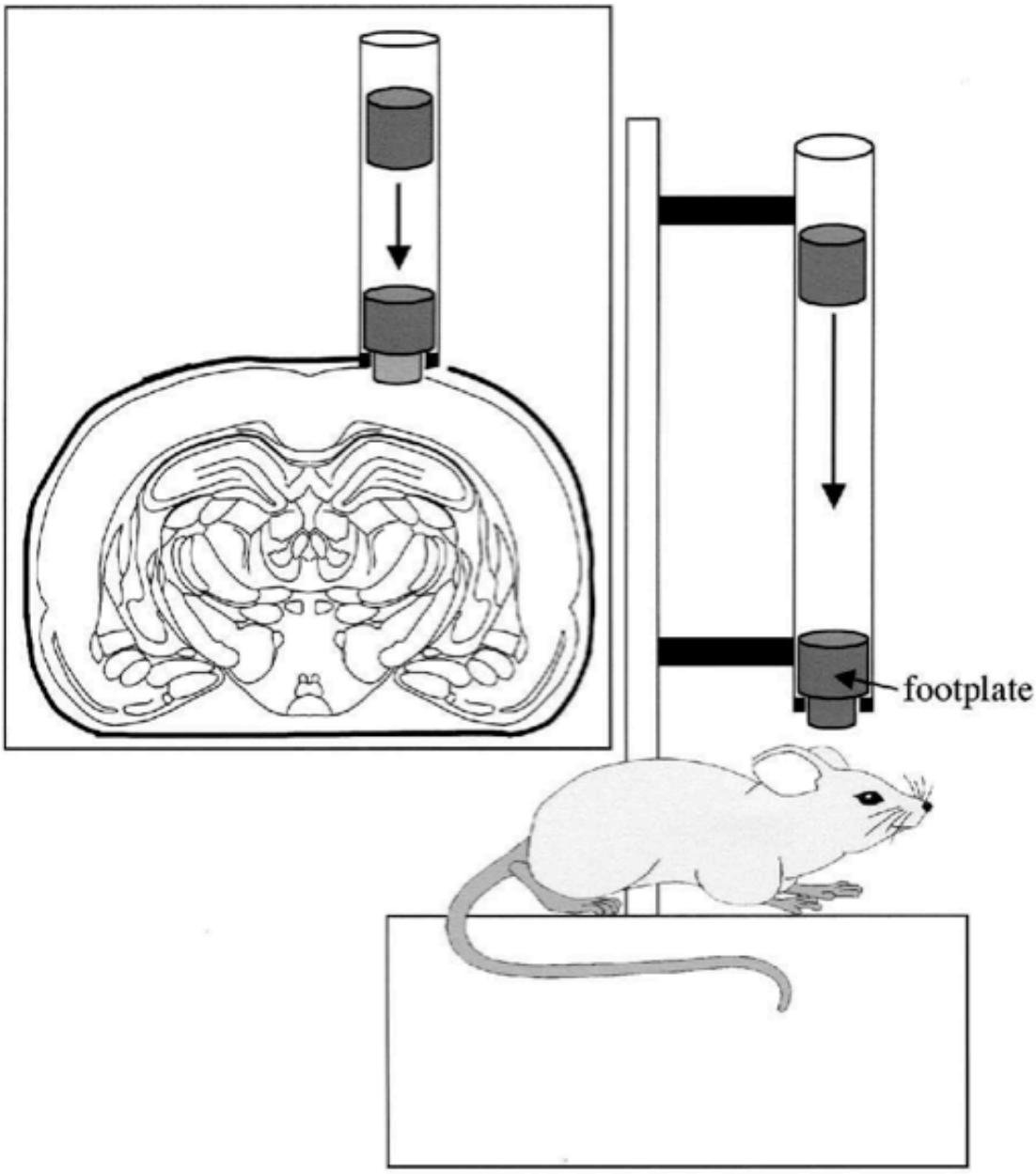

Figure 1.2. Illustration of the open head weight-drop model. A weight is dropped directly onto the exposed dura (Prins and Hovda, 2003).

1.3.2. Controlled cortical impact model

Controlled cortical impact is a modification of the open head weight-drop model that allows for more precise control over the velocity and depth of the injury impact. With controlled cortical impact a pneumatically driven piston penetrates the exposed dura (Dixon et al., 1991; Dixon et al., 1999; Prins and Hovda, 2003; Whiting et al., 2006; see Fig. 1.3). The impact tip of the piston may vary in size and shape to manipulate the degree of the injury (Prins and Hovda, 2003). Controlled cortical impact has been found to result in contusion, cavitation, edema, neuronal loss, axonal injury, and vascular changes (Dixon et al., 1991; Dixon et al., 1999; Prins and Hovda, 2003; Whiting et al., 2006). It is also capable of inducing long-term cognitive and motor deficits similar to those occurring in human traumatic brain injury (Dixon et al., 1991; Dixon et al., 1999; Whiting et al., 2006). However, as with the open head weight-drop model discussed above, the penetrating and focal nature of controlled cortical impact appears to limit its usefulness in modeling concussion.

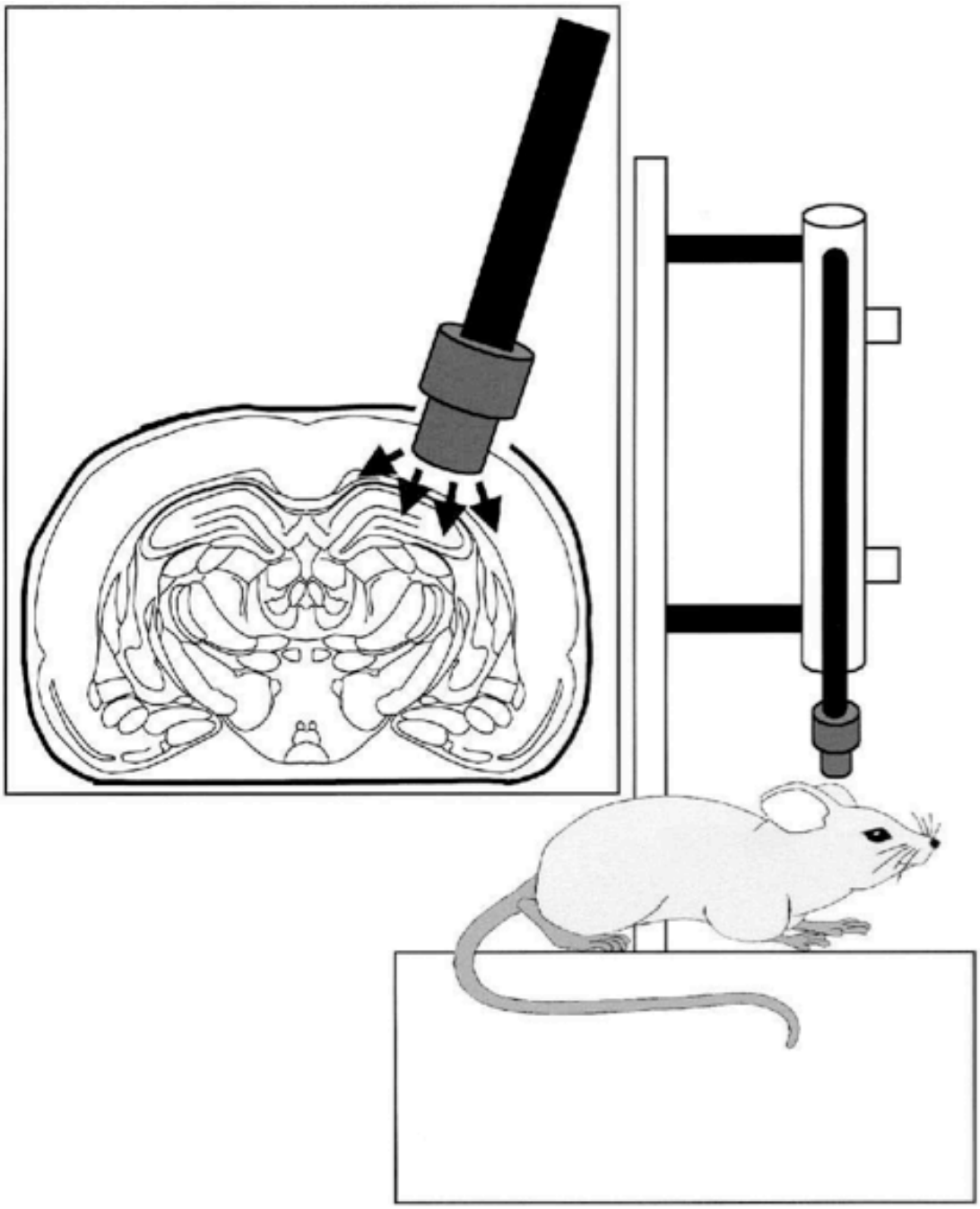

Figure 1.3. Illustration of the controlled cortical impact model. A pneumatically driven piston penetrates the dura at a known distance and velocity (Prins and Hovda, 2003).

1.3.3. Fluid percussion injury model

The most commonly used and well-characterized model of rodent traumatic brain injury is fluid percussion injury (Laurer et al., 2000; Thompson et al., 2005; see Fig. 1.4). To administer fluid percussion injury, rats must first undergo a craniotomy to expose the intact dural surface. The site of the craniotomy can be varied but is most often placed laterally over the parietal cortex (Thompson et al., 2005); such a preparation is referred to as lateral fluid percussion injury. Fluid percussion injury is then produced by the rapid impact of a fluid pulse to the dural surface, resulting in brief diffuse displacement of the brain (Prins and Hovda, 2003; Thompson et al., 2005; Whiting et al., 2006). The fluid pulse is typically generated by a fluid percussion device consisting of an adjustable hammer pendulum that when released strikes the piston end of a fluid-filled cylinder (Thompson et al., 2005; Whiting et al., 2006). The severity of fluid percussion injury can range from mild to severe, which can be altered by changing the release height of the hammer pendulum (Thompson et al., 2005; Whiting et al., 2006). The majority of lateral fluid percussion injury studies have used moderate to severe lateral fluid percussion injury forces (e.g. > 2.5 atm) to model features of moderate to severe closed head injury (Thompson et al., 2005). Taken together, these studies have demonstrated that lateral fluid percussion injury is capable of inducing physiological, pathological, and behavioral changes comparable to those in human traumatic brain injury patients (Laurer et al., 2000; Thompson et al., 2005). Depending on fluid percussion injury severity, these changes might include increased intracranial pressure, decreased cerebral blood flow, changes in ionic homeostasis, diffuse axonal injury, neuronal loss, hemorrhage, and cavitation with associated motor, cognitive, and emotional deficits that may persist for up

to a year (Dixon et al., 1987; Jones et al., 2008; Hallam et al., 2004; Hovda et al., 1990; Maxwell et al., 1997; McIntosh et al., 1989; Muir et al., 1992; Pierce et al., 1998; Prins et al., 1996; Rink et al., 1995).

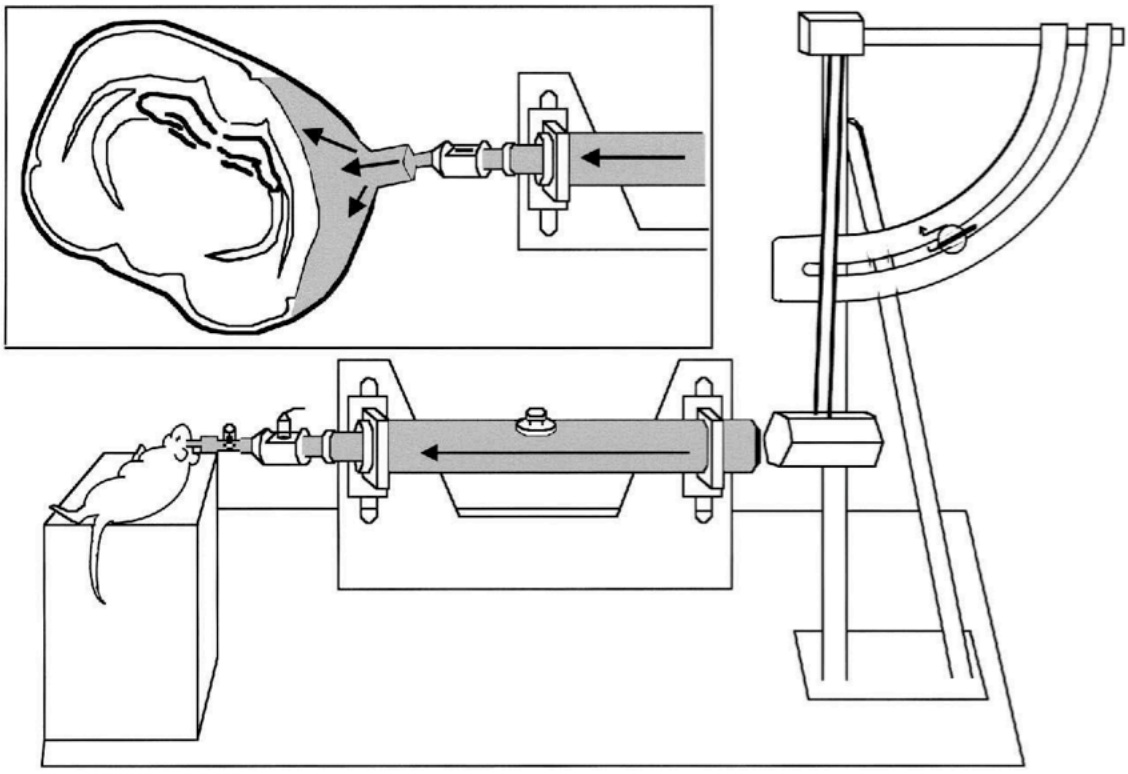

Figure 1.4. Illustration of the fluid percussion device. The rapid injection of a fluid pulse into the epidural space generates diffuse brain movement (Prins and Hovda, 2003).

1.4. Mild lateral fluid percussion injury as a model of single and repeated concussion

Given the aforementioned findings and its relatively non-invasive and diffuse nature, fluid percussion injury seems to be an appropriate technique for experimental concussion in the rat. Although the majority of fluid percussion injury research has utilized more severe percussion forces to model moderate to severe closed head injury, a number of previous studies have investigated the effects of a mild lateral fluid percussion injury and its ability to model concussion. For example, several studies have established that rats suffering mild lateral fluid percussion injury exhibit short-term cognitive impairments (DeRoss, 2002; Gurkoff et al., 2006, Wu et al., 2006; Wu et al., 2010). In addition, Gurkoff and colleagues (2006) found that cognitive deficits in the water maze occurred in the absence of significant neuronal loss. As transient cognitive deficits and lack of significant brain damage are key features of human concussion, these studies provide initial support for the use of the mild lateral fluid percussion injury to model concussion. However, these studies have included only limited behavioral and pathological analysis. As previously described, there are a number of other behavioral symptoms in addition to cognitive deficits associated with concussion, and there is growing evidence that concussion is linked to a number of pathological features. Given the limited studies and analysis done to date it is currently unknown whether mild lateral fluid percussion injury is capable of inducing additional behavioral impairments and pathologies similar to those occurring in human concussion.

As the dura remains intact following mild lateral fluid percussion injury the technique also appears applicable to repeated injury schedules that might model repeated

concussion. To date, only a single experiment has attempted to use repeated mild lateral fluid percussion injury to study the effects of repeated concussion. In this study, DeRoss and colleagues (2002) pre-trained rats to optimal performance levels in the water maze before administering mild lateral fluid percussion injury. Following the first mild lateral fluid percussion injury, rats were assessed in the water maze until they recovered to a new optimal level, defined as three consecutive days without improvement. At this point a second mild lateral fluid percussion injury was administered. This procedure was repeated until a maximum of three mild lateral fluid percussion injuries had been given. Rats were also tested on the beam task for sensorimotor ability before and after each injury. Findings from this study revealed that repeated mild lateral fluid percussion injury might result in cumulative cognitive deficits, as rats given repeated injuries displayed worsened performance levels in the water maze. Motor performance on the beam task appeared unaffected throughout the study.

Although De Ross and colleagues obtained preliminary evidence indicative of mild cumulative effects with repeated mild lateral fluid percussion injury, the study had a number of methodological limitations, many of which were acknowledged by the authors. Behavioral analysis was restricted to tasks of cognition and motor ability. However, as previously discussed, repeated concussion is associated with a number of other symptoms. Also, the study used only short recovery periods (10 to 14 days) following the final administered mild lateral fluid percussion injury. This recovery period is not adequate to determine whether the multiple injuries induced chronic changes similar to those observed in humans who have experienced repeated concussion. Further, any evidence for the acute cumulative effects of repeated mild lateral fluid percussion

injury may have been confounded by a number of factors, including the possible benefits of repeated behavioral testing during recovery. The study also failed to employ any statistical techniques for the analysis of data, and it failed to assess possible brain pathology in the test subjects. These omissions made it impossible to determine whether the reported behavioral impairments were statistically significant and what pathologies might be associated with these deficits. The maximum of three injuries suffered by rats in the study is also fewer than what human athletes and military personal may suffer over the course of their careers (Chen et al., 2004; McKee et al., 2009). Overall, the single study that has assessed the use of repeated mild lateral fluid percussion injury to model multiple concussion suffers from a number of significant shortcomings that limit its ability to assess and validate the model.

1.5. Present studies

In the present dissertation, mild lateral fluid percussion injury was used to model and study the effects of single and repeated concussion in the rat. The present studies were designed to expand on the findings of previous mild lateral fluid percussion injury studies by including both detailed behavioral analyses and pathological examination of brains, as well as repeated administration of mild lateral fluid percussion injury. In Study 1 (Chapter 2), the short- and long-term effects of a single mild lateral fluid percussion injury were investigated to determine the behavioral and pathological changes associated with the injury, and thereby further assess the validity of the single mild lateral fluid percussion injury model of concussion. In Study 2 (Chapter 3), a novel repeated mild lateral fluid percussion injury experimental design was used to assess repeated mild lateral fluid percussion injury as a model of the cumulative and chronic effects of

repeated concussion on behavior and brain pathology.

A novel pharmacological traumatic brain injury treatment, anti-CD11d integrin antibody, was also applied and assessed in experiments involving either single or repeated lateral fluid percussion injury. The anti-CD11d antibody targets integrins involved in the infiltration of peripheral inflammatory cells into the brain following traumatic brain injury (Utagawa et al., 2008). Previous studies that have administered anti-CD11d antibody following central nervous system injury in the rat have found that it was effective in both blocking the infiltration of peripheral leukocytes and decreasing associated secondary damage (Bao et al., 2004; Utagawa et al., 2008). Studies 3 and 4 assessed anti-CD11d antibody treatment following a single moderate lateral fluid percussion injury (Chapter 4), and following each of three mild lateral fluid percussion injuries in a repeated injury design (Chapter 5). These studies were intended to increase our understanding of the role of neuroinflammation in each of these models and to determine how the previously found pathological changes associated with anti-CD11d antibody treatment might translate into functional recovery.

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Chapter 2

2. A single mild fluid percussion injury induces short-term behavioral and neuropathological changes in the Long-Evans rat: support for an animal model of concussion[1]

2.1. Introduction

Brain concussion is a serious public health concern with an annual injury rate of approximately 1 in 150 individuals (Cassidy et al., 2004). Concussion can be defined as any transient neurological dysfunction resulting from a biomechanical force and is often associated with behavioral symptoms such as short-term cognitive impairment (e.g. anterograde amnesia), impulsivity, personality change, and emotional disturbances (Boll and Barth, 1983; Giza and Hovda, 2001; Kim, 2002; Kushner, 1998). These symptoms are typically observed in the absence of significant neuronal loss or structural damage (Benson et al., 2007; Giza and Hovda, 2001; Kushner, 1998). However, other findings suggest that axonal injury and neuroinflammatory processes may be common pathologies involved in concussion (Benson et al., 2007; Chen et al., 2004; Giza and Hovda, 2001; Holmin et al., 1998; Kushner, 1998; Lovell et al., 2007). Concussions have also been linked to the onset of a number of neurological disorders such as dementia, depression, and chronic traumatic encephalopathy (Cantu, 2007; Jellinger, 2004; Jones et al., 2008). A neuroinflammatory response has been associated with a number of these disorders and could be induced by brain concussion (Holmin et al., 1998; Lee et al., 2002; Nandoe et al., 2002; O'Sullivan et al., 2009; Stoll and Jander, 1999; Whitton, 2007; Zilka, 2006).

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Of the available techniques that may be suitable for modeling aspects of concussion, lateral fluid percussion injury is one of the most commonly used and well-characterized models of rodent traumatic brain injury (Laurer et al., 2000; Thompson et al., 2005). To administer lateral fluid percussion injury, rats first undergo a craniotomy to expose the intact dural surface of the parietal cortex. Lateral fluid percussion injury is then produced by the rapid impact of a fluid pulse to the dural surface generated by a fluid percussion device consisting of an adjustable hammer pendulum that when released strikes the piston end of a fluid-filled cylinder (Thompson et al., 2005). The majority of lateral fluid percussion injury studies have used moderate or severe levels of injury (e.g. > 2.5 atm; Thompson et al., 2005). Taken together, these studies have demonstrated that moderate or severe lateral fluid percussion injury is capable of inducing physiological, pathological, and behavioral changes comparable to those in humans who have suffered moderate to severe traumatic brain injury (Laurer et al., 2000; Thompson et al., 2005).

Relatively few lateral fluid percussion injury studies have investigated the effects of mild lateral fluid percussion injury. The available studies of mild lateral fluid percussion injury have found that rats given a mild lateral fluid percussion injury exhibited short-term cognitive impairments in the absence of significant neuronal loss (DeRoss, 2002; Griesbach et al., 2009; Gurkoff et al., 2006; Sanders et al., 2001; Wu et al., 2006, 2010). Although these findings are consistent with some of the symptoms and pathology observed after human concussion injury, most of these studies were limited to tasks of cognition (Griesbach et al., 2009; Gurkoff et al., 2006; Wu et al., 2006, 2010). In humans, concussion commonly induces other symptoms (e.g. impulsivity; emotional disturbances) in addition to those of a cognitive nature. Given the limited behavioral

analyses in previous studies, it is difficult to fully understand the extent of behavioral impairments induced by mild lateral fluid percussion injury and evaluate the validity of the model. Furthermore, few studies examined neuroinflammation and axonal injury after mild lateral fluid percussion injury in the rat, and those that did were limited in their ability to address the potential relationship between pathological changes and associated behavioral impairments (e.g. Aihara et al., 1995; Obenaus et al., 2007; Sanders et al., 2001).

To date no study has included a detailed examination of the behavioral and neuropathological effects of mild lateral fluid percussion injury at 1-1.5 atm. Therefore, to address the need to better understand the effects of mild lateral fluid percussion injury in an animal model of concussion the current study included a battery of behavioral tests of anxiety-like behavior, spatial cognition, depression-like behavior, social behavior, locomotion, and sensorimotor ability, as well as immunohistochemical evaluation of neuroinflammation and axonal injury. As concussion is typically associated with transient changes, the current study included short- and long-term recovery periods to assess recovery after mild lateral fluid percussion injury.

2.2. Materials and methods

2.2.1. Subjects

Subjects were 64 young adult male Long-Evans hooded rats obtained from Charles River Laboratories (Quebec, Canada). Prior to surgery rats weighed between 250-300 g, were housed in pairs in standard acrylic cages (26 cm x 48 cm x 21 cm) at a controlled temperature (21 ±1.0 °C), and were naïve to all experimental procedures. In order to maintain the integrity of the injury cap, rats were housed individually post-

surgery for the remainder of the study. The light/dark cycle was a 12:12 cycle with lights on from 7:00 to 19:00 hrs and animals were allowed access to food and water *ad libitum*. Behavioral test procedures for the current studies were in accordance with guidelines of the Canadian Council on Animal Care and approved by the University of Western Ontario Animal Use Committee.

2.2.2. Surgery: craniotomy and injury cap

Rats were placed in a sealed Plexiglas box into which 4% isoflurane and 2 L/min oxygen flow was introduced for anesthesia. Rats were then placed in a standard stereotaxic device equipped with a gas anesthesia nose cover to maintain anesthesia throughout surgery with 2% isoflurane and 500 ml/min oxygen flow. Under aseptic conditions rats underwent a craniotomy surgery. All craniotomies were circular windows (3 mm diameter) centered over the following coordinates with reference to Bregma: anterior/posterior -3.0 mm; medial/lateral 6.0 mm (Paxinos and Watson, 1986). A hollow plastic injury cap was sealed over the craniotomy with silicone adhesive, cyanoacrylate, and dental acrylic. Three small stainless steel screws were inserted into the skull surrounding the injury cap to provide anchors for dental acrylic, which attached the injury cap to the skull. Once the dental acrylic hardened the scalp was sutured and a removable plug was inserted into the injury cap to seal the craniotomy until, and after, the injury was administered. Immediately post-surgery all rats received a subcutaneous injection of analgesic (Ketoprofin, 5 mg/kg).

2.2.3. Injury groups

Rats were randomly assigned to one of two injury groups: Concussion (CONC, 1.00 – 1.50 atm) or Sham-Control (SHAM). In addition, rats were assigned to one of two

recovery groups: short recovery (SR; 24 hrs), or long recovery (LR; 4 weeks). Thus, there were a total of 4 experimental groups in the current study: CONC-SR (n = 16); SHAM-SR (n = 16); CONC-LR (n = 16); and SHAM-LR (n = 16). The mild fluid percussion force generated by the fluid percussion device, as listed in atm above, to produce the injury level was chosen based on previous rodent studies (DeRoss et al., 2002; Li et al., 2006; Wu et al., 2006, 2010) and pilot work.

2.2.4. Lateral fluid percussion injury

Approximately 24 hrs post-surgery rats were placed in a sealed Plexiglas box into which 4% isoflurane and 2 L/min oxygen flow was introduced for anesthesia. Once under anesthetic, rats were placed under aseptic conditions, the integrity of the dura was confirmed, and the injury cap was filled with sterile saline and attached to the fluid percussion device (FP302, *AmScien Instruments*, Richmond, VA). At the first response of hind-limb withdrawal to a toe pinch, SHAM rats were removed from the device while CONC rats were administered one mild lateral fluid percussion injury pulse. Apnea, return of hind-limb withdrawal, and self-righting reflex were all monitored immediately following injury (Griesbach et al., 2009; Griesbach et al., 2004; Gurkoff et al., 2006). Apnea times were determined as the time from injury to the return of spontaneous breathing. Return of hind-limb withdrawal time was used as a measure of unconsciousness time and determined by response to a toe pinch (Griesbach et al., 2009; Griesbach et al., 2004). Self-righting was determined as the time from injury to return to an upright position. Following injury, rats received their assigned recovery times before behavioral testing.

2.2.5. Behavioral test apparatus

Anxiety-like behavior was assessed using an elevated-plus maze consisting of two arms intersecting at a 90° angle, thereby creating 4 individual arms each 55 cm long and 12 cm wide. The two opposing closed arms were shielded by 46 cm high walls; the two opposing open arms contained no walls. The maze was placed 50 cm above the ground. An overhead video camera recorded all trials. Following testing, the videotape was scored and the number of entries into and amount of time spent on each arm were recorded.

Spatial cognition was assessed using a water maze consisting of a circular pool (1.5 m in diameter, 45 cm deep) filled with tap water at 29 ±1.0 °C. A clear Plexiglas escape platform (9 cm X 9 cm) was hidden approximately 2 cm below the water surface in the center of the south-east quadrant of the pool during acquisition, and in the center of the north-west quadrant during reversal. Polypropylene beads floating on top of the water prevented the rats from seeing the hidden platform (Cain et al., 1993). Doors, cabinets, and posters on the walls provided a variety of distal cues. Behavior was recorded by a video camera mounted to the ceiling above the centre of the pool. The camera was connected to a computer and behavior was objectively analyzed by a tracking system that digitized each swim trial (*Poly-Track*, *San Diego Instruments*, San Diego, CA).

Locomotor and social behaviors were evaluated in a circular open field arena (90 cm diameter, 40 cm high) with Beta Chip bedding covering the floor of the arena. A CD camera was mounted above the centre of the arena. The camera was connected to a computer, allowing behavior to be recorded using the *EthoVision 3.0.15 Behavioral Monitoring and Analysis System* at a rate of 5.994 frames/sec. This program is capable of tracking the x-y coordinates of each animal and it allows for the computation of several

quantitative variables. The camera was also connected to a VCR, allowing behavior to be recorded onto VHS cassettes.

Sensorimotor ability was evaluated using a narrow wooden beam, which was 1 m long and was rigidly suspended at each end 1 m above the floor, with soft padding on the floor underneath in case a rat fell off the beam (Kolb and Whishaw, 1985). One edge of the beam was 4 cm wide and was placed facing up for initial acclimation to the task. The other edge was 2 cm wide and was placed facing up during the actual beam task. The lights in the testing room were turned off and a halogen lamp was placed at the start end to illuminate the beam and provide incentive for the rats to walk along the beam, which led to a dark platform at the far end of the beam as a goal. These conditions provide ample incentive for rats to traverse the beam (Beiko et al., 1997; Kolb and Whishaw, 1985; Shultz et al., 2009). Experience with the water maze does not affect performance on the beam task (Beiko et al., 1997).

Depression-like behaviors were assessed using the forced swim test, which made use of a test apparatus that was similar to those previously described for rats (Christianson et al., 2008; Jones et al., 2008) and consisted of a clear glass cylinder (diameter 20 cm) filled with 25 ±1.0 °C water to a depth of 30 cm. Behavior during the 5-min test session was recorded by a side-view video camera and later scored. To ensure that rats were physically able to complete testing, and to induce learned helplessness, a 15 min training session was given 24 hrs before the test session started (Jones et al., 2008; Porsolt et al., 1977).

2.2.6. Experimental procedure: day 1

Behavioral testing commenced either after a SR of 24 hrs or a LR of 4 weeks following mild lateral fluid percussion injury. To avoid fatigue, testing occurred over a period of 4 days and rats were tested on no more than two behavioral tasks per day.

Rats were individually placed in the center of the elevated-plus maze facing an open arm and allowed to explore the maze freely for 5 min.

Water maze acquisition training began immediately following elevated-plus maze testing and consisted of 10 training trials, with each trial beginning with the rat being placed gently in the pool adjacent to, and facing, the pool wall, and ending when the rat stood on the hidden platform. Each trial began at one of the four pool wall start locations (North, South, East, or West), with start locations pseudo-randomly ordered to prevent sequential starts from the same location. As this resulted in start locations that varied in distance from the hidden platform, for graphic presentation of search time data in Results, the time to reach the platform was averaged for every block of 2 trials (e.g. Block 1= (Trial 1 + Trial 2)/2). Rats that failed to reach the hidden platform within 60 sec of the commencement of the trial were placed on the platform by the experimenter. Rats remained on the platform for 15 sec before they were placed in a drying chamber that was heated from above by an infrared lamp. Rats were run in squads of five so that the inter-trial interval for the 10 acquisition trials was not more than 6 min.

2.2.7. Experimental procedure: day 2

Individual rats were placed gently in the centre of the open field apparatus and allowed to explore the arena freely for 10 min. Immediately following open field testing rats underwent a second water maze session for reversal training. The procedures for the

reversal session were identical to acquisition except that the hidden platform was now located in the opposite quadrant of the pool.

2.2.8. Experimental procedure: day 3

Two rats that had been given the same treatment were placed in the open field and social behavior data were collected for 30 min. On the day before testing, the dorsal surface of one rat from each pair was colored black using black hair dye so that the *EthoVision Tracking System* could distinguish and track each rat separately (Lazar et al., 2008; Shultz et al, 2008).

Twenty-four hrs prior to beam testing (see Day 4), rats were given a training session with both the 4 cm edge and the 2 cm edge of the beam for acclimation to the task. After beam training, rats also completed forced swim training. Training for the forced swim task required each rat to complete a 15 min swimming session in the forced swim apparatus.

2.2.9. Experimental procedure: day 4

The beam task testing session began approximately 24 hrs post-training and consisted of 10 trials. A trial began with the rat being placed on the illuminated end of the beam and ended when the animal successfully reached the dark goal platform. A maximum of 60 sec was allowed for each trial. Rats were run in squads of five so that the inter-trial interval for the 10 trials was not more than 5 min. Immediately following beam testing each rat was placed in the forced swim apparatus for a 5 min test session.

2.2.10. Behavioral analyses

For the elevated-plus maze, time spent in the open and closed arms of the maze were used to evaluate anxiety levels in rats. All four of the rat's paws had to enter an arm

for it to be considered an entry (Walf and Frye, 2007). As time spent in the open arm is decreased in rats that exhibit greater stress-associated behaviors, a percentage score was calculated for the time spent in the open arm as follows: time in the open arm/[time in the open arm + time closed arm] (Saucier et al., 2008; Steimer and Driscoll, 2003; Zhu et al., 2006). The number of entries into the closed arm of the maze was also calculated as a measure of locomotion.

For water maze analysis, search time and direct and circle swims were used as measures of spatial place memory (Morris, 1989; Whishaw and Jarrard, 1995). Search time was defined as the time in sec from release until the rat climbed onto the hidden platform. A maximum of 60 sec was allowed for each trial. Direct and circle swims were measured because they represent efficient swim paths that are normally generated by control rats swimming to a fixed visible platform (Beiko et al., 2004; Cain and Boon, 2003; Cain et al., 2006; Cain et al., 1996). In hidden platform paradigms, well-trained control rats generate direct and circle swims in approximately 40% of the trials (e.g. Cain and Boon, 2003; Shultz et al., 2009). This measure has the advantage of providing data from each trial, and is not confounded by changes in swim speed. A direct swim was defined as a swim that remained entirely within an 18 cm wide virtual alley from the start point to the hidden platform without crossing over itself. A circle swim was defined as a swim that approximated an arc of a circle without exceeding 360° or crossing over itself (Beiko et al., 2004; Cain and Boon, 2003; Cain et al., 2006; Whishaw and Jarrard, 1995). Direct and circle swims were summed and calculated as a percentage of the total swims for each test session. Percent of time spent in the periphery of the pool was used as a measure of thigmotaxis and was objectively calculated by the *Poly-track* system. For

reversal training the percent of time spent in the acquisition platform quadrant was used as a measure to indicate perseveration of responding to the platforms original location. Swim speed was used as a measure of motor ability and was objectively calculated in cm/sec by the *Poly-track* system.

For open field and social behavior analysis, *EthoVision* automatically collected and calculated the total distance traveled (cm) by each rat (open field and social), and the mean distance apart (cm) between the rats in each pair (social).

For beam task analysis, traverse time and the number of slips and falls were used as measures of sensorimotor function. Traverse time was defined as the time required to traverse the beam, with a maximum allowed time of 60 sec. Slips and falls were scored when a rat slipped from the beam or when a rat fell completely off the beam. Rats that fell from the beam were given a maximum time of 60 sec (Shultz et al., 2009).

The following measures were scored to assess depression-like behaviors in the forced swim task: the time spent immobile (primary measure), defined as the rat making only the necessary movements to keep its head above water; time spent climbing, defined as the rat actively struggling to escape the cylinder with its forepaws breaking the surface of the water; and time spent swimming, defined as the rat remaining active in the cylinder but is not struggling (Jones et al., 2008).

2.2.11. Brain tissue preparation and neuropathological procedures

Once the final behavioral testing day was completed (post-injury day 4 for SR rats; post-injury day 32 for LR rats), animals were deeply anaesthetized with sodium pentobarbital (270 mg/ml, ip) and transcardially perfused with ice cold PBS followed by 4% paraformaldehyde in PBS. Brains were removed and placed in 4% paraformaldehyde

solution and stored at 4±1.0 °C for 24 hrs. Following the fixation period, brains were placed in an 18% sucrose solution for cryoprotection prior to paraffin embedding for immunohistochemical analysis.

Serial 4 μm coronal sections were obtained through the cortex, including adjacent white matter, at the level of injury (approximately -3.0 mm posterior to Bregma). This anatomical site was chosen because it allowed reliable quantification of possible changes induced by mild lateral fluid percussion injury in cortex and white matter areas that have been shown to be altered in human concussion (Arfanakis et al., 2002; Umile et al., 2002). Anti-GFAP (1:500, rabbit polyclonal, DakoCytomation, Glostrup, Denmark) and anti rat CD68 (1:200 monoclonal, Serotec, Oxford, U.K.) antibodies were used as markers for reactive astrogliosis and activated microglia, respectively (MacFabe et al., 2007). APP (1:1200 monoclonal, Millipore, Temecula, United States) was used as a marker for axonal injury (Li et al., 2006). Tissue sections were mounted on positively charged glass slides (SurgiPath, Canada) and dried overnight at 37±1.0 °C. Sections were deparaffinized and rehydrated using standard procedures (Shi et al., 2001). Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide in distilled water solution for 5 min. For antigen recovery, sections were immersed in boiling 0.21% citric acid buffer (pH 6.0) for 30 min in a 1250 W microwave oven. Slides were counterstained with Gill haematoxylin (EMD Biosciences) and rinsed with PBS for 5 min. A 10% normal horse serum in PBS solution was applied for 5 min followed by the primary antibodies for 1 hr at room temperature. Following the incubation period, sections were washed with PBS and incubated with biotinylated antirabbit IgG (Vector Laboratories, Burlingame CA - BA1000) as a secondary antibody for anti-GFAP, and biotinylated anti- mouse

(Vector Laboratories, Burlingame, CA-2000) as a secondary antibody for CD68 for 30 min. Tissues were again washed with PBS and incubated in the avidin-biotin complex (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA - PK6100) for 30 min at room temperature. Following incubation, slides were washed with PBS and 3,3 – diaminobenzidine DAB chromagen (Sigma – D8001) was applied for 5 min. To increase the resolution for APP staining, an additional run was completed using VIP as chromagen (Vector Laboratories, Burlingame, CA). After final rinsing, slides were dehydrated, cleared, and coverslipped (MacFabe et al., 2008; Shultz et al., 2008; Shultz et al., 2009).

2.2.12. Immunohistochemistry quantification

Using a standard light microscope, a technician blinded to group membership of the subjects captured photomicrographs under fixed microscope illumination settings and exposure times to ensure objective and consistent image quality across all pictures. For GFAP and CD68 analysis, non-overlapping digital photomicrographs (area = 160,000 μm^2), from both hemispheres were captured at 25X magnification. Extending from the pial surface and using the rhinal fissure as the originating landmark (Paxinos and Watson, 1986), a total of 10 sequential digital photomicrographs were obtained from each hemisphere from the ventral-most area to the dorsal-most area of cortex. Two photomicrographs from each hemisphere were obtained from the perirhinal cortex (PR; see Fig. 2.1; Paxinos and Watson, 1986). Six photomicrographs from each hemisphere were obtained from the parietal/temporal cortex (PT; see Fig. 2.1; Paxinos and Watson, 1986). Two photomicrographs from each hemisphere were obtained from the frontal/hindlimb cortex (FH; see Fig. 2.1; Paxinos and Watson, 1986).

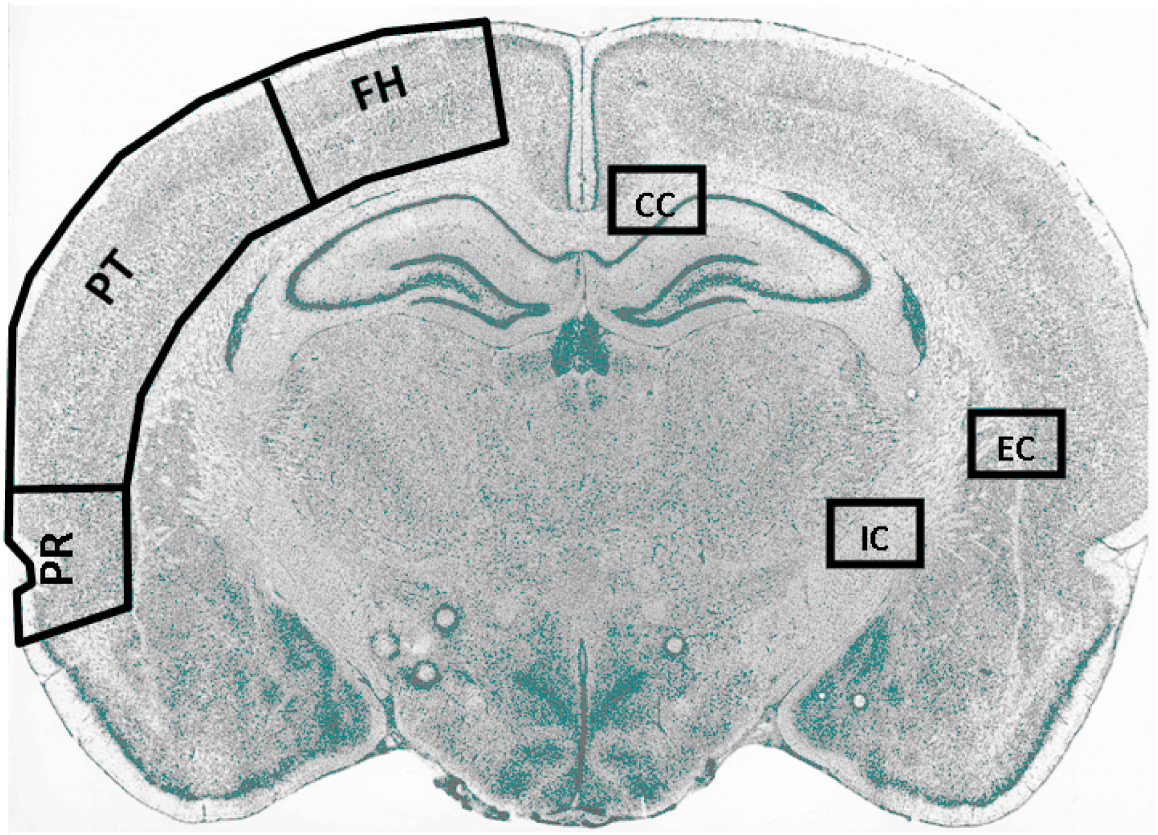

Figure 2.1. Brain areas used for neuropathological analyses. To assess neuroinflammation both hemispheres were separated into areas consisting of FH, PT, and PR cortex. Axonal injury was assessed in the white matter from the EC, IC, and CC of both hemispheres (Paxinos and Watson, 1986).

Due to the diffuse nature of GFAP staining, the 'area stained' function within ImagePro Plus 5.1 software was used as a semi quantitative index of immunoreactivity. This function sums the immunopositive area within a digital image to provide a total immunopositive area per image in μm^2. To quantify CD68 staining, immunopositive nucleated cells were counted by the 'cell count' function within ImagePro Plus 5.1 software after a color recognition criteria was set. A technician blinded to the study procedure randomly selected a sample of 3 photomicrographs from each of the 3 treatment groups to manually create and save a set of best-fit color recognition criteria (RGB) for each antibody to counter the effects of variance in the intensity of DAB labeling. For quantification, the 'count/size' measure was selected within Image-Pro Plus 5.1. The 'area stained' or 'cell count' measures were then selected under the measurement menu. Previously saved color recognition criteria were loaded onto photomicrographs and 'area stained' or 'cells count' were calculated. Area is defined as the immunopositive area per image in μm^2.

For APP analysis of axonal injury, a digital image (area = 160,000 μm^2) was captured from each of three areas containing white matter from both ipsilateral (I) and contralateral (C) hemispheres. Photomicrographs were obtained within each of the external capsule (EC), internal capsule (IC), and corpus callosum (CC; see Fig. 2.1). These white matter areas were identified using a standard rat brain atlas (Paxinos and Watson, 1986) and have been included in a previously established model of APP immunoreactivity following mild lateral fluid percussion injury (Li et al., 2006). In total, 26 digital photomicrographs were taken from each brain of the 12 randomly selected animals (CONC, n=6; SHAM, n=6). For APP staining analysis, a 'total area stained'

measure was adapted based on previously used APP quantification methods (Gerber et al., 2009; Mochizuki et al., 1996). Specifically, the 'limit to threshold area' function within Image J software was used to calculate a semi-quantitative index of APP accumulation. This function sets a threshold to isolate APP staining before summing the stained area within the digital image to provide a total stained area per image in μm^2.

2.2.13. Statistical analyses

Search time, traverse time, and distance traveled (open field) were analyzed by SPSS 17.0 using mixed design ANOVAs with injury as the between-subjects factor and trial as the within-subjects factor. Immunohistochemical analyses were carried out using mixed design ANOVAs with injury as the between-subjects factor and brain area as the within-subjects factor. Simple effects *F*-tests and Fisher's LSD post hoc pair-wise comparisons were carried out when appropriate. One-way ANOVAs, with injury as the between-subjects factor, were used to analyze apnea, hind-limb withdrawal, return of self-right reflex, direct and circle swims, percent of time spent in periphery, swim speed, percent of time spent in platforms acquisition quadrant, distance traveled (social behavior), mean distance apart, slips and falls, and forced swim measures. Statistical significance was set at $p < .05$.

2.3. Results

2.3.1. Injury force and post-injury measures

As shown in Table 2.1, the mean injury force for the CONC group measured by the fluid percussion device was 1.20 ± .03 atm. There was no group difference in the duration of apnea ($p > .05$). One-way ANOVAs revealed that CONC rats took significantly longer to regain hind-limb withdrawal ($F(1, 63) = 28.643, p < .001$) and

return of the self-righting reflex ($F(1, 39) = 6.952, p < .05$) compared to SHAM rats.

There were no cases of mortality resulting from mild lateral fluid percussion injury. However two rats were removed from the study prior to the onset of behavioral testing because of a rupture of the dura prior to mild lateral fluid percussion injury or loss of their injury cap.

INJURY GROUP	**FORCE (atm)** *M ± SEM*	**APNEA (sec)** *M ± SEM*	*Range*	**HIND-LIMB (sec)** *M ± SEM*	*Range*	**SELF-RIGHTING (sec)** *M ± SEM*	*Range*
CONC	1.20 ± .03	.15 ± .13	0 - 4	3.96 ± .63 *	0 - 15	92.95 ± 18.42 *	9 – 346
SHAM	N/A	0	N/A	0	N/A	39.04 ± 10.04	0 – 210

Table 2.1. Injury force and post-injury measures. The CONC group displayed significantly longer mean (± SEM) hind-limb withdrawal and self-righting reflex times than the SHAM group. * = different from SHAM ($p < .05$). For additional statistical detail see Results.

2.3.2. Elevated-plus maze

As shown in Fig. 2.2A, CONC-SR rats exhibited increased time spent in the open arms compared to the SHAM-SR group. This was confirmed by a one-way ANOVA with a significant injury effect ($F(1, 31) = 4.213, p < .05$). There was no difference between groups in the number of closed arm entries (Fig. 2.2B; $p > .05$). The LR groups did not differ on any elevated-plus maze measure (all $ps > .05$; see Fig. 2.2C-D).

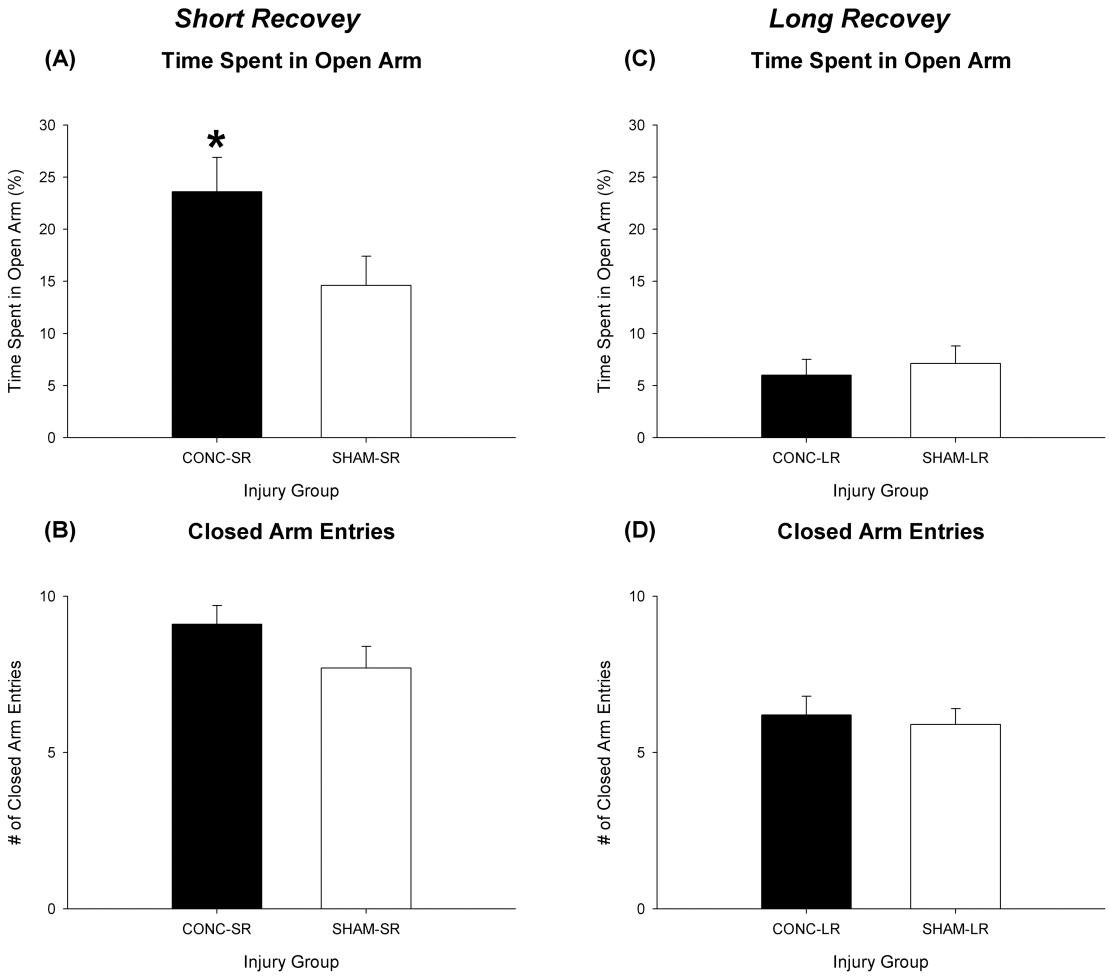

Figure 2.2. Short recovery and long recovery elevated-plus maze results. (A) The CONC-SR group spent a significantly greater percent of time in the open arm than the SHAM-SR group. (B) The SR groups did not differ in the number of entries into the closed arm. (C) The LR groups did not differ in the percent of time spent in the open arm. (D) The LR groups did not differ in the number of entries into the closed arm. Histogram bars represent group means (± SEM). * = different from the SHAM-SR, $p < .05$. For additional statistical detail see Results.

2.3.3. Water maze

As shown if Fig. 2.3A, during acquisition training, search times decreased in all groups as testing progressed with no apparent between-group differences. These impressions were confirmed by ANOVA with a significant main effect found for trial ($F(9, 270) = 7.938, p < .001$) but not injury ($p > .05$). The percent direct and circle swim data were consistent with the search time data in indicating no group differences during acquisition (see Fig. 2.3B).

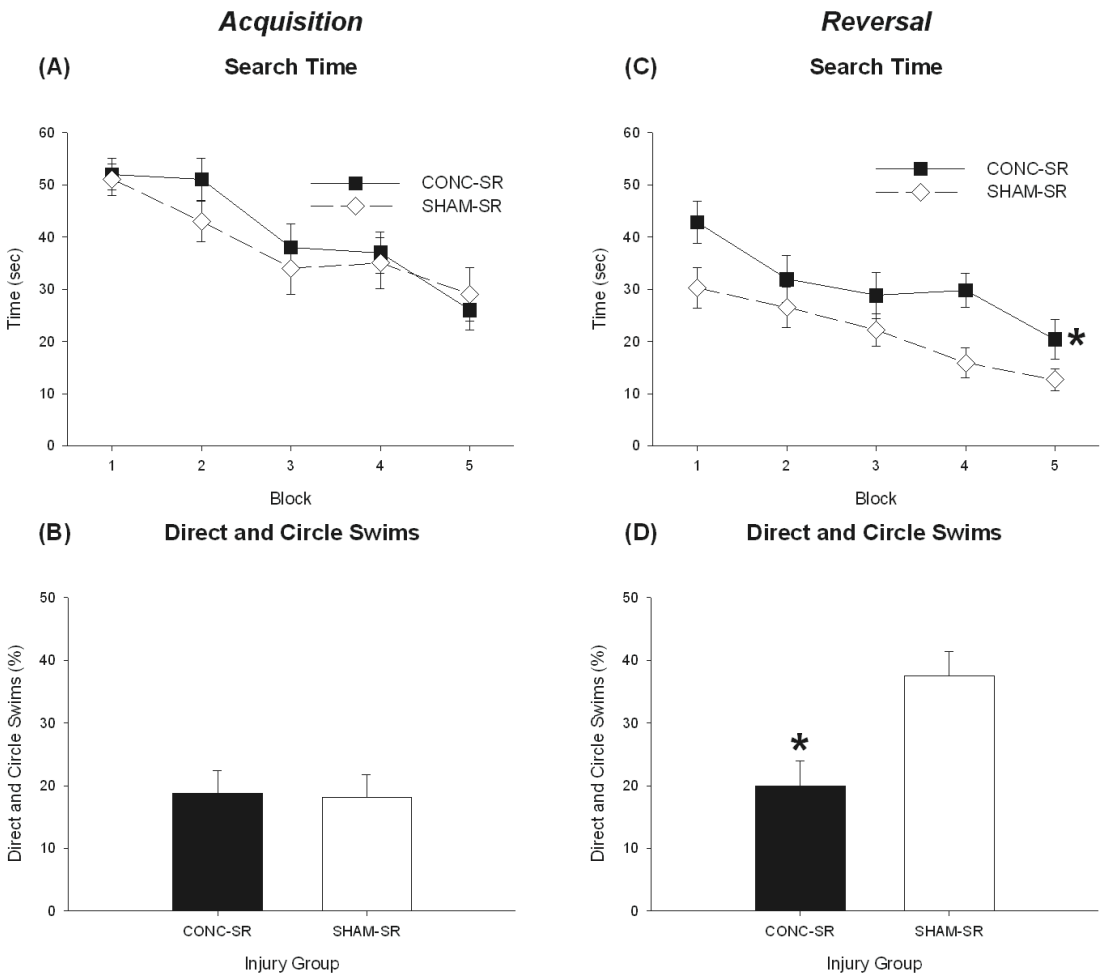

Figure 2.3. Short recovery water maze results. (A) The groups did not differ in search times during acquisition training. (B) The groups did not differ in direct and circle swims during acquisition training. (C) The CONC-SR group displayed significantly longer search times during reversal training than the SHAM-SR group. (D) The CONC-SR group displayed significantly fewer direct and circle swims during reversal training than the SHAM-SR group. In panels (A) and (C) data points represent means of data collected for each block of two trials (± SEM). In panels (B) and (D) histogram bars represent means of data collected during the 10 water maze trials (± SEM). * = different from SHAM-SR, *ps* < .01. For additional statistical detail see Results.

As shown in Fig. 2.3C, during reversal training search times decreased in both groups as testing progressed. However, search times decreased less in the CONC-SR group than the SHAM-SR group. ANOVA confirmed these impressions with significant main effects of both trial ($F(9, 270) = 6.317, p < .001$) and injury ($F(1, 31) = 7.671, p < .01$). The percent direct and circle swim data were consistent with the search time data in indicating fewer direct and circle swims in the CONC-SR group during reversal (see Fig. 2.3D). ANOVA confirmed a significant injury effect during reversal training ($F(1, 31) = 9.800, p < .01$). There were no SR group differences in percent of time spent in the periphery of the pool or swim speed during both acquisition and reversal training sessions (all *ps* > .05; data not shown).

The LR groups did not differ on any water maze measure (all *ps* > .05; see Fig. 2.4).

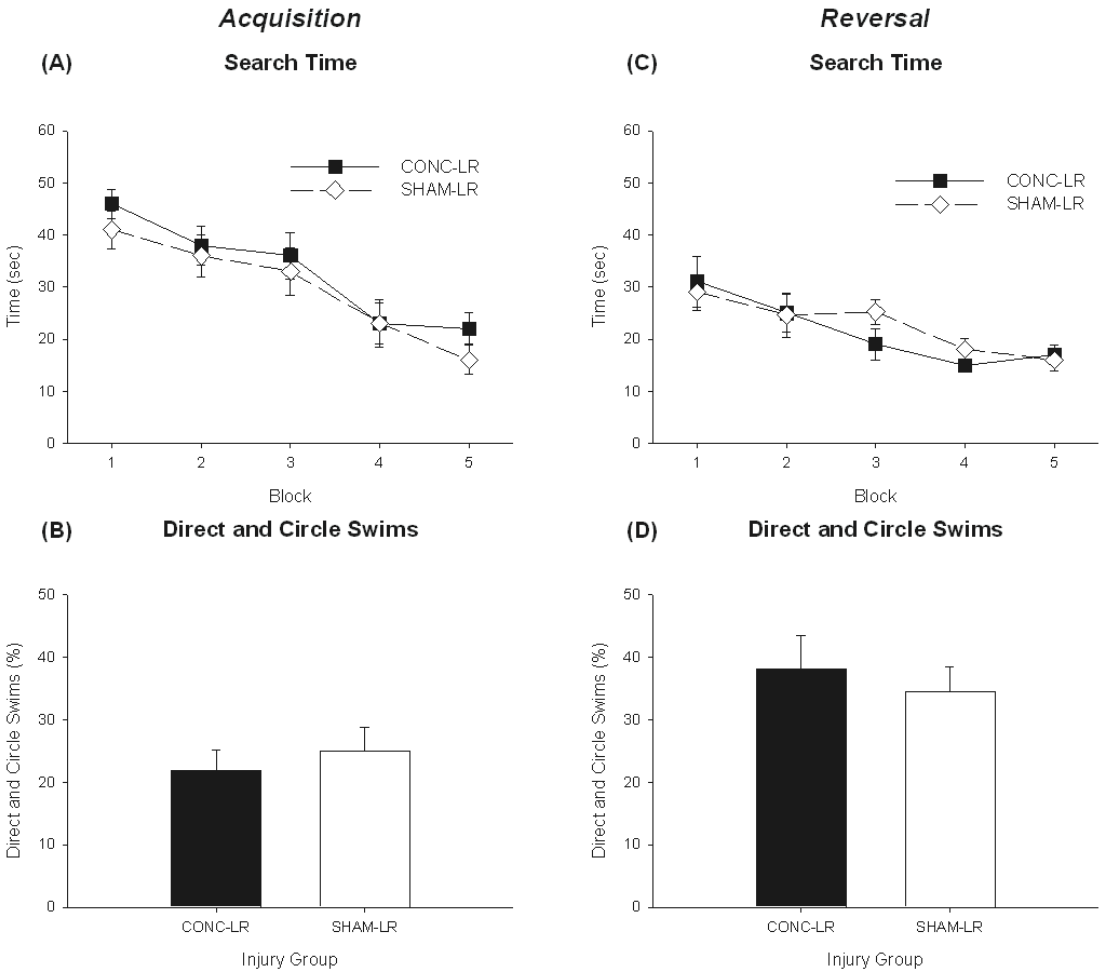

Figure 2.4. Long recovery water maze results. The groups did not differ in search times during acquisition (A) or reversal (C) training. The groups did not differ in direct and circle swims during acquisition (B) or reversal (D) raining. In panels (A) and (C) data points represent means of data collected for each block of two trials (± SEM). In panels (B) and (D) histogram bars represent means of data collected during the 10 water maze trials (± SEM). For additional statistical detail see Results.

2.3.4. Open field, social behavior, beam task, and forced swim

There were no SR or LR between-group differences on any measures in the open field, social behavior, beam, and forced swim tasks (all *ps* > .05; data not shown).

2.3.5. Neuropathology

As shown in Fig. 2.5, at 4 days post-injury CONC-SR rats showed a significant increase in CD68 labeled activated microglia/macrophages, with the greatest immunoreactivity occurring in the I hemisphere. ANOVA confirmed these impressions with significant main effects of both brain area ($F(5, 45) = 8.361, p < .001$) and injury group ($F(1, 9) = 20.881, p < .001$), and a significant brain area x injury group interaction ($F(5, 45) = 4.533, p < .005$). Post hoc tests revealed that the CONC-SR group displayed significantly increased activation compared to the SHAM-SR group in I-PT and I-FH ($p < .05$).

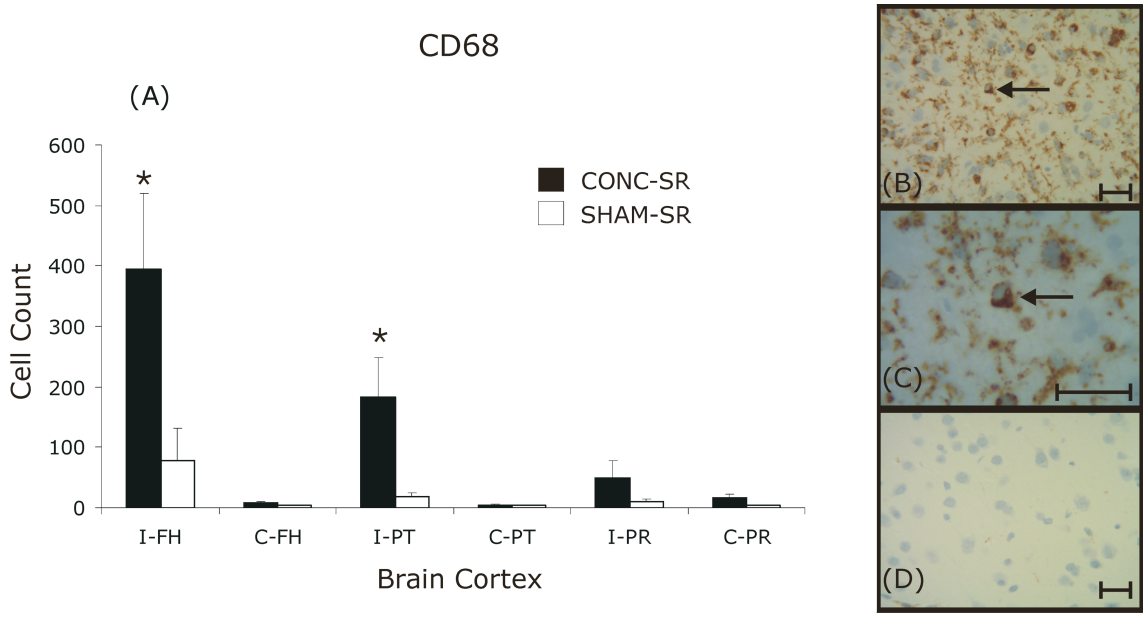

Figure 2.5. Short recovery CD68 results. (A) The CONC-SR group displayed significantly more CD68 labeled activated microglia/macrophages than the SHAM-SR group, with the greatest activation occurring in the I hemisphere. Histogram bars represent mean CD68 labeled activated microglia in brain cortex areas (± SEM). * = different from SHAM-SR group, ps < .05. (B-C) Representative photomicrographs showing CD68 immunoreactivity in I-PT from the CONC-SR group at 25X magnification (B) and 63X magnification (C). Arrow indicates CD68-labeled activated microglia. (D) Representative photomicrograph showing CD68 immunoreactivity in I-PT from the SHAM-SR group at 25X magnification. Scale bars = 100 μm. For additional statistical detail see Results.

As shown in Fig. 2.6, at 4 days post-injury CONC-SR rats showed a significant increase in GFAP immunoreactivity, with the greatest immunoreactivity occurring in the I hemisphere. ANOVA confirmed these impressions with significant main effects of both brain area ($F(5, 45) = 17.846, p < .001$) and injury group ($F(1, 9) = 5.209, p < .05$), and a significant brain area x injury group interaction ($F(5, 45) = 5.541, p < .001$). Post hoc tests revealed that the CONC-SR group displayed significantly increased activation compared to the SHAM-SR group in I-PT ($p < .001$; see Fig. 2.6A).

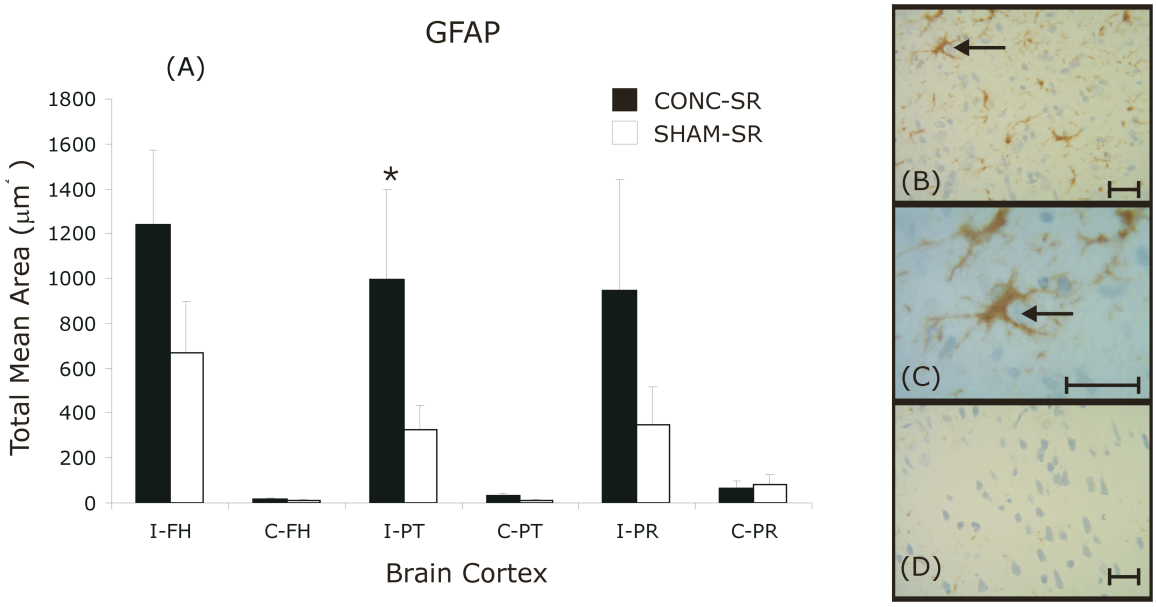

Figure 2.6. Short recovery GFAP results. (A) The CONC-SR group displayed significantly more GFAP immunoreactivity than the SHAM-SR group, specifically in the I-PT cortex. Histogram bars represent mean GFAP immunoreactivity in brain cortex areas (± SEM). * = different from SHAM-SR group, $p < .001$. (B-C) Representative photomicrographs showing GFAP immunoreactivity in I-PT from the CONC-SR group at 25X magnification (B) and 63X magnification (C). Arrow indicates GFAP-labeled reactive astrocyte. (D) Representative photomicrograph showing GFAP immunoreactivity in I-PT from the SHAM-SR group at 25X magnification. Scale bars = 100 μm. For additional statistical detail see Results.

As shown in Fig. 2.7, at 4 days post-injury CONC-SR rats showed a significant increase in APP immunoreactivity with the greatest immunoreactivity occurring in the I-CC. ANOVA confirmed these impressions with significant main effects of both brain area ($F(5, 45) = 8.878, p < .001$) and injury group ($F(1, 9) = 8.098, p < .05$), and a significant brain area x injury group interaction ($F(5, 45) = 7.287, p < .001$). Post hoc tests revealed that the CONC-SR group displayed significantly increased staining compared to the SHAM-SR groups in I-CC ($p < .05$; see Fig. 2.7A).

The LR groups did not differ on any neuropathological measures (all $ps > .05$; data not shown).

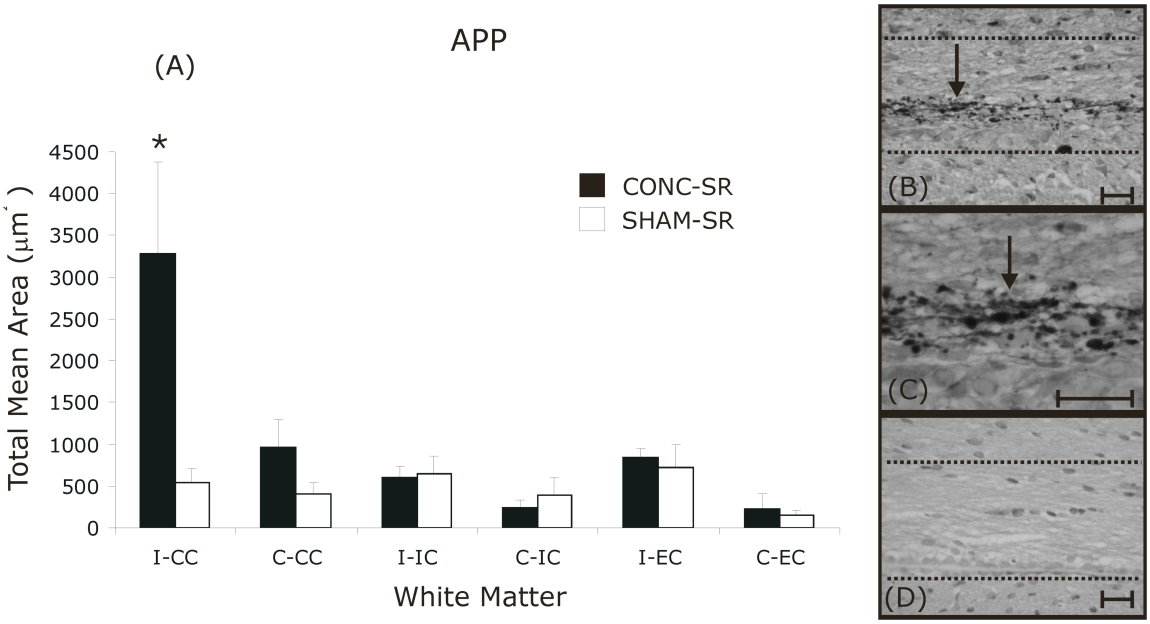

Figure 2.7. Short recovery APP results. (A) The CONC-SR group displayed significantly more APP immunoreactivity than the SHAM-SR group with the greatest accumulation occurring in the I-CC. Histogram bars represent mean APP immunoreactivity in white matter areas (± SEM). * = different from SHAM-SR group, $p < .05$. (B-C) Representative photomicrographs showing APP immunoreactivity in I-CC from the CONC-SR group at 25X (B) and 63X magnification (C). Arrow indicates APP accumulation. (D) Representative photomicrograph showing APP immunoreactivity in I-CC from SHAM-SR group at 25X magnification. Scale bars = 100 μm. For additional statistical detail see Results.

2.4. Discussion

The results show that a single mild lateral fluid percussion injury produced short-term behavioral and neuropathological changes in the Long-Evans rat that were consistent with features of human concussion. CONC-SR rats spent a greater amount of time in the open arm of the elevated-plus maze compared to the SHAM-SR rats, suggesting a decrease in anxiety-like behavior. Furthermore, the CONC-SR group was cognitively impaired during reversal training of the water maze, displaying longer search latencies and fewer direct and circle swims compared to the SHAM-SR group. Given that there were no group differences in the number of closed arm entries in the elevated-plus maze, swim speed in the water maze, open field exploration, and beam task measures, it appears that hyperactivity and/or impaired locomotion were not confounding factors in these findings. Neuropathological analyses revealed increased APP staining in the CONC-SR group compared to the SHAM-SR group, suggesting that axonal injury may be associated with the behavioral deficits observed in CONC-SR rats. Additionally, CONC-SR rats had a greater neuroinflammatory response compared to SHAM-SR rats as indicated by increased CD68 immunoreactivity. CONC-SR rats also displayed reactive astrocytosis, as indicated by increased GFAP immunoreactivity, compared to the SHAM-SR rats. Rats subjected to mild lateral fluid percussion injury appeared to recover from any short-term disturbances, as there were no LR group differences on any behavioral or neuropathological measures.

2.4.1. Nature of behavioral impairments

Past studies reporting increased time spent in the open arm of the elevated-plus maze in the absence of hyperactivity have commonly concluded that this behavior is

indicative of decreased anxiety, and possibly increased disinhibition and/or impulsivity (Dalvi and Rodgers, 1999; Fan et al., 2008; Hasenöhrl et al., 1998; Hogg, 1996; Lindemann et al., 2008; Meyer et al., 2008; Ognibene et al., 2007). The current finding that mild lateral fluid percussion injury increased time spent on the open arm of the elevated-plus maze is novel in mild lateral fluid percussion injury research, as few previous studies have incorporated behavioral tasks specific for measures of anxiety. Therefore, it appears that mild lateral fluid percussion injury induced a decrease in anxiety-like behaviors, and possibly increased disinhibition and/or impulsivity, in the CONC-SR group.

Our finding of cognitive impairments in adult rats following mild lateral fluid percussion injury is consistent with previous findings (DeRoss, 2002; Wu et al., 2006, 2010). In the current study, CONC-SR rats displayed no impairments during initial acquisition of the water maze task. However, following a 24 hr delay, impairments occurred during reversal training in this task. This pattern of water maze results observed in the CONC-SR group might represent several forms of cognitive impairment. For example, the deficits observed during the reversal session may be indicative of a form of anterograde amnesia for the behavioral strategy and spatial place learning that occurred during acquisition (Shultz et al., 2009). Similar anterograde cognitive deficits, including reversal impairments, have been observed in studies involving lesions to the medial temporal lobe (Kaut and Bunsey, 2001; Mumby and Glenn, 2000; Wiig et al., 1996), an area found to be affected by mild lateral fluid percussion injury in the current study.

Alternatively, the cognitive deficits observed during reversal training may represent perseveration of behavior. Specifically, as reversal training requires the

suppression of responses to the platform location used during acquisition, a deficit during reversal training might imply perseverative behavior with respect to the original platform location (Shultz et al., 2009). However, a perseveration effect appears improbable, as there were no significant group differences during reversal training in the measure of *percent of time spent in the acquisition platform quadrant*.

The fact that the CONC-SR group experienced a decrease in anxiety-related behavior suggests that anxiety behaviors may need to be considered when interpreting water maze results. However, CONC-SR rats were not impaired during acquisition of the water maze, the training session that occurred immediately following the decrease in anxiety-like behaviors found during elevated-plus maze testing. In addition, there were no significant group differences found on the measure of time spent in the periphery of the pool, a measure thought to be affected by anxiety level, during either the acquisition or reversal sessions. Previous studies have also failed to find a consistent relationship between cognition and a decrease in anxiety (Fan et al., 2008; Hasenöhrl et al., 1998). Therefore, taken together it appears unlikely that the decrease in anxiety-like behavior during elevated-plus maze testing directly accounted for the cognitive impairments observed in the water maze.

2.4.2. Role of neuroinflammation, reactive astrocytosis, and axonal injury

There are a number of ways that mild lateral fluid percussion injury might have disrupted brain function to cause the behavioral impairments reported in the current study. Here, mild lateral fluid percussion injury was found to induce reactive astrocytosis and an acute neuroinflammatory response consisting of activated microglia/macrophages in various regions of cortex. A similar neuropathological response has been observed in

human traumatic brain injury, with evidence suggesting that these processes might alter normal brain function (Hein and O'Banion, 2009; Lenzlinger et al., 2001; Morganti-Kossmann et al., 2002; Schmidt et al., 2005). Previous research from our laboratory has also revealed a cognitive deficit in reversal training of the water maze in the presence of a neuropathological response similar to the one occurring in the current study (Shultz et al., 2009). In addition, previous studies of mild lateral fluid percussion injury have also implicated an inflammatory response in subsequent cognitive deficits. Specifically, increased oxidative stress following mild lateral fluid percussion injury has been associated with a worse cognitive outcome (Wu et al., 2006, 2010). As activated microglia are capable of producing reactive oxygen species (Dringen, 2005), the neuroinflammatory response occurring in the current study may have negatively affected cognitive performance via increased oxidative stress. Taken together, it seems possible that reactive astrocytosis and acute neuroinflammation could contribute to the transient behavioral changes following mild lateral fluid percussion injury.

Axonal injury, as indicated by increased APP accumulation in CONC-SR rats, may have also contributed to the subsequent behavioral deficits. Li and colleagues (2006) also found that cognitive deficits following mild lateral fluid percussion injury in gerbils were associated with axonal injury to white matter areas including the CC, and axonal injury in rats has been found to generate behaviors on the elevated-plus maze similar to the one observed in the current study (Fan et al., 2008). As the CC has been shown to be involved in brain networks associated with cognition and emotionality in the rat (Amagdei et al., 2010; Miu et al., 2006), it is possible that the finding of axonal injury in the CC in the current study is a contributing factor to the behavioral deficits observed.

2.4.3. Relation to human concussion

The findings from the current study indicate that mild lateral fluid percussion injury induces short-term behavioral and neuropathological changes in the rat, which bear similarity to features of human concussion. Humans suffering from a concussion often display short-term cognitive deficits that recover within weeks (Barth et al., 1989; Lovell et al., 2007). The short-term cognitive deficits displayed by CONC-SR rats in the water maze are consistent with this. In particular, the finding that CONC-SR rats performed well in the initial water maze test session but were impaired in the second session 24 hrs later resembles the commonly observed anterograde cognitive disturbances suffered by humans following a concussion (Young, 2009). Additionally, the decrease in anxiety-like behaviors in CONC-SR rats might resemble the human symptoms of increased impulsivity and disinhibition following brain concussion (Boll and Barth, 1983; Kim, 2002). The finding that mild lateral fluid percussion injury resulted in a loss of consciousness, as determined by hind-limb reflex, in some but not all CONC rats is also similar to human concussion (Cantu, 2007; Lovell et al., 1999).

The proposed mechanisms underlying the behavioral abnormalities in the current study are also linked to the human condition. Recent evidence suggests that axonal injury may be a common pathological feature of concussion (Arfanakis et al., 2002; Benson et al., 2007; Kraus et al., 2007). Specifically, human concussion studies utilizing diffusion imaging have found a relationship between the degree of axonal injury in the CC and cognitive performance (Arfanakis et al., 2002; Kraus et al., 2007). Neuroinflammation is another pathology found following traumatic brain injury in humans (Hein and O'Banion, 2009; Holmin et al., 1998; Morganti-Kossmann et al., 2002). The current findings

indicate pathological changes in the rat following mild lateral fluid percussion injury in the CC, as well as the frontal, parietal, and temporal lobes, all of which are areas typically affected in humans following a concussion (Arfanakis, et al., 2002; Chen et al., 2004; Lovell et al., 2007; Umile et al., 2002).

Concussion has been linked to long-term neurological disorders such as dementia, depression, and chronic traumatic encephalopathy (Cantu, 2007; Jellinger, 2004; Jones et al., 2008). While there were no long-term behavioral or pathological changes following a single injury in the current study, recent evidence suggests that multiple concussive blows might result in cumulative effects and long-term neurological disorders such as chronic traumatic encephalopathy (Cantu, 2007; McKee et al., 2009). A neuroinflammatory response similar to the one found in the current study, including deposition of beta amyloid, reactive astrogliosis, migration of peripheral macrophages and their transformation into activated microglia, and the production of nitric oxide and cytokines, has been linked to a number of neurological disorders and could contribute to a progressive encephalopathic condition following repeated concussions (Holmin et al., 1998; Lee et al., 2002; Nandoe et al., 2002; O'Sullivan et al., 2009; Stoll and Jander, 1999; Whitton, 2007; Zilka, 2006). Future research could utilize a repeated mild lateral fluid percussion injury schedule to better understand these potential chronic effects associated with multiple concussion injuries.

2.4.4. Conclusions

A single mild lateral fluid percussion injury at the force of 1-1.5 atm induced short-term cognitive deficits and a decrease in anxiety-related behaviors in adult male Long-Evans rats. As this injury also induced neuroinflammation and axonal injury, it

seems possible that these mechanisms may be involved in the behavioral changes observed. Overall, the current findings of short-term cognitive deficits, decreased anxiety-like behavior, neuroinflammation, and axonal injury in rats suffering a single mild lateral fluid percussion injury are consistent with common symptoms and pathological changes seen in humans suffering a concussion and support the use of mild lateral fluid percussion injury to model the human condition.

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Chapter 3

3. Repeated mild fluid percussion brain injury in the rat causes cumulative long-term behavioral impairments and pathological changes in a rodent model of repeated concussion[2]

3.1. Introduction

Concussion can be defined as a complex pathophysiological process affecting the brain that is induced by traumatic biomechanical forces (McCrory et al., 2009), and is often associated with transient behavioral symptoms such as cognitive impairments, disinhibition, and emotional disturbances (Boll and Barth, 1983; Giza and Hovda, 2001; Kushner, 1998; McCrory et al., 2009). These symptoms are typically observed in the absence of significant structural damage (Giza and Hovda, 2001; Kushner, 1998; McCrory et al., 2009), although other findings suggest that axonal injury, neuroinflammation, and neurometabolic dysfunction may be involved (Blumbergs et al., 1994; Chen et al., 2004; Giza and Hovda, 2001; Holmin et al., 1998; Li et al., 2006; Lovell et al., 2007; Marino et al., 2007; Povlishock et al., 1983; Povlishock et al.,1979).

Effects of concussion are of particular concern to individuals engaged in combative or bodily contact activities, such as military personnel and athletes, who are at increased risk of repeated concussion (Cantu, 2003; Cassidy et al., 2004; Collins et al., 1999; Fear et al., 2009; Kelly, 1999; McCrea et al., 2003; McKee et al., 2009; Okie, 2005; Thurman and Guerrero, 1999). Studies of athletes found that those who experienced three or more concussions displayed more severe acute symptoms, long-term cognitive deficits, and increased incidence of depression compared to athletes who

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[2] A version of this chapter is currently being prepared for publication. Shultz, S.R., Bao, F., Omana, V., Chiu, C., Brown, A., and Cain, D.P.

experienced a single concussion (Guskiewicz et al., 2003; Guskiewicz et al., 2005; Guskiewicz et al., 2007; Macciocchi et al., 2001). Repeated concussion has also been associated with chronic traumatic encephalopathy, a neurodegenerative disorder with symptoms that include long-term cognitive impairment, dementia, depression, anxiety, and motor abnormalities (Bailes and Cantu, 2001; Cantu, 2007; Collins et al., 2002; Corsellis et al., 1973; Guskiewicz et al., 2007; Jellinger, 2004; McKee et al., 2009). Recent pathological studies of post-mortem brain tissue taken from former athletes who had sustained repeated concussion and displayed symptoms of chronic traumatic encephalopathy have reported neuronal loss, atrophy, scarring, amyloid and tau deposition, neuroinflammation, and diffuse axonal injury (McKee et al., 2009; Omalu et al., 2005; Omalu et al., 2006). However, despite growing evidence that repeated concussion can result in cumulative damage and neurological disease, little is known about the factors and mechanisms that underlie these long-term consequences (Cantu, 2009; McKee et al., 2009).

Animal models provide a means to systematically examine factors and mechanisms that may be involved in traumatic brain injury through experimentation that is not possible with humans. Fluid percussion injury is the most commonly used injury technique to model closed head traumatic brain injury (Laurer et al., 2000; Thompson et al., 2005). Work from our laboratory and others has found that a single mild lateral fluid percussion injury in the rat can model a single concussion, resulting in transient cognitive impairments, anxiety-related abnormalities, axonal injury, neuroinflammation, but no neuronal loss (DeRoss, 2002; Griesbach et al., 2009; Gurkoff et al., 2006; Sanders et al., 2001; Wu et al., 2006, 2010; see Chapter 2). These findings suggest that repeated mild

lateral fluid percussion injury might hold promise as a means to study the effects of multiple concussion and chronic traumatic encephalopathy in an animal model. It appears that only a single experiment has used repeated mild lateral fluid percussion injury to study the effects of multiple concussion. In this experiment DeRoss and colleagues (2002) trained rats to optimal performance levels in a water maze prior to administering mild lateral fluid percussion injury. Following injury, rats were assessed daily in the water maze and beam task until they recovered to asymptomatic levels, at which point another mild lateral fluid percussion injury was administered, for a maximum of three mild lateral fluid percussion injuries. Although this study reported preliminary evidence that repeated mild lateral fluid percussion injuries may have cumulative effects, it had a number of methodological shortcomings, including limited evaluation of cognitive and behavioral abilities, no evaluation of long-term effects of repeated mild lateral fluid percussion injuries, the potential confound of benefits from repeated behavioral training given during the recovery period, no statistical analysis of the data, and no neuropathological assessment of the brains.

In order to provide useful information about the behavioral impairments and associated brain damage that might result from the cumulative effects of repeated concussion, the present study was undertaken to determine whether repeated mild lateral fluid percussion injury in the rat is a suitable model to study repeated concussion. Here rats received either 1, 3, or 5 mild lateral fluid percussion injuries, or sham treatment, at intervals of 5 days. Previous work has shown that the cerebral pathophysiology at 5 days after lateral fluid percussion injury in the rat is equivalent to pathophysiology at approximately 2-4 weeks post-concussion in humans (Giza and Hovda, 2001). This

sequence of injuries is similar to what might occur in athletes and military personnel under common concussion management guidelines (Maroon et al., 2000). We previously found that effects of a single mild lateral fluid percussion injury were present at 24 hrs but not 4 weeks post-injury (see Chapter 2). Here we expected that repeated mild lateral fluid percussion injuries might result in cumulative effects that would manifest as greater enduring behavioral impairments and brain pathology than a single mild lateral fluid percussion injury.

3.2. Materials and methods

3.2.1. Subjects

Subjects were 122 young adult male Long-Evans hooded rats obtained from Charles River Laboratories (Quebec, Canada). Prior to surgery rats weighed between 250-300 g, were housed in pairs in standard acrylic cages (26 cm x 48 cm x 21 cm) at 21 ±1.0 °C, and were naïve to all experimental procedures. After surgery rats were housed individually for the remainder of the study under a 12:12 light/dark cycle, with lights on from 7:00 to 19:00 hrs. Animals were allowed access to food and water *ad libitum*. Behavioral test procedures were in accordance with guidelines of the Canadian Council on Animal Care and approved by the University of Western Ontario Animal Use Subcommittee.

3.2.2. Surgery: craniotomy and injury cap

Rats were placed in a sealed Plexiglas box into which 4% isoflurane and 2 L/min oxygen flow was introduced for anesthesia. Rats were then placed in a standard stereotaxic device equipped with a gas anesthesia nose cover to maintain anesthesia throughout surgery with 2% isoflurane and 500 ml/min oxygen flow. Under aseptic

conditions rats underwent a craniotomy surgery to create a 3 mm diameter circular opening centered over cortex at -3.0 mm A/P and 6.0 mm lateral with reference to Bregma (Paxinos and Watson, 1986). A hollow plastic injury cap was sealed over the craniotomy with silicone adhesive, cyanoacrylate, and dental acrylic. Three small stainless steel screws were inserted into the skull surrounding the injury cap to provide anchors for dental acrylic, which attached the injury cap to the skull. After the dental acrylic hardened the scalp was sutured, topical antibiotic ointment was applied, and a removable plug was inserted into the injury cap to seal the craniotomy. A subcutaneous injection of analgesic (Ketoprofin, 5 mg/kg) was given immediately after surgery.

3.2.3. Injury groups

Rats were randomly assigned to one of four injury groups: one concussion (1C), three concussion (3C), five concussion (5C), or Sham-Control (SHAM) (see Table 3.1). After the last assigned injury, rats were randomly assigned to receive either a short recovery period (SR) of 24 hrs or a long recovery period (LR) of 8 weeks before the start of behavioral testing. After mild lateral fluid percussion injury, seven rats died and five rats were removed from the study prior to the onset of behavioral testing because of a rupture of the dura, loss of the injury cap, or failure to maintain normal body weight. There were a total of 8 experimental groups, with final group sizes as follows: 1C-SR (n = 14); 3C-SR (n = 14); 5C-SR (n=14); SHAM-SR (n = 17); 1C-LR (n = 12); 3C –LR, (n = 14); 5C-LR (n=13); and SHAM-LR (n = 12).

INJURY GROUP	***DAY***				
	1	**6**	**11**	**16**	**21**
SHAM	S	S	S	S	S
1C	S	S	S	S	mLFP
3C	S	S	mLFP	mLFP	mLFP
5C	mLFP	mLFP	mLFP	mLFP	mLFP

Table 3.1. Repeated mild lateral fluid percussion injury schedule. S = Sham injury; mLFP = mild lateral fluid percussion injury.

3.2.4. Injury schedule

Repeated mild lateral fluid percussion injury methods were adapted from initial work done by DeRoss and colleagues (2002). On Day 1, approximately 24 hrs post-surgery, rats were placed in a sealed Plexiglas box into which 4% isoflurane and 2 L/min oxygen flow was introduced for anesthesia (Utagawa et al., 2008; see Chapter 2). After rats were anesthetized the plug was removed from the injury cap, and under aseptic conditions the injury cap was filled with sterile saline and connected to the fluid percussion device. At the first response of hind-limb withdrawal to a toe pinch, SHAM, 1C, and 3C rats were removed from the device, which constituted sham treatment, and 5C rats received mild lateral fluid percussion injury. Rats were again anesthetized and received either a sham or mild lateral fluid percussion injury on days 6, 11, 16, and 21 according to the schedule in Table 3.1. All treatments were separated by a 5 day interval. Thus, as shown in Table 3.1, the 1C group received sham injuries on days 1, 6, 11 and 16, and received mild lateral fluid percussion injury on day 21. The 3C group received sham injuries on Days 1 and 6, and received mild lateral fluid percussion injuries on days 11, 16, and 21.

A fluid percussion force of 1.0-1.5 atm was delivered by the fluid percussion device to produce each mild lateral fluid percussion injury. This percussion force was chosen based on previous rodent studies that have validated the use of mild lateral fluid percussion injury in a rat model of concussion (DeRoss et al., 2002; Griesbach et al., 2009; Li et al., 2006; Wu et al., 2010; see Chapter 2). No percussion force was delivered during sham treatments. Acute injury measures consisting of duration of apnea, duration of unconsciousness, and latency to occurrence of the self-righting reflex were all

monitored beginning immediately following each sham or mild lateral fluid percussion injury (Griesbach et al., 2009; Gurkoff et al., 2006; see Chapter 2). Duration of apnea was determined as the time from injury to the return of spontaneous breathing. Duration of unconsciousness was determined as the time from injury to the return of the hind-limb withdrawal in response to toe pinch. Time to the occurrence of the self-righting reflex was determined as the time from injury to return to an upright position (Griesbach et al., 2009; Gurkoff et al., 2006). Following their last assigned treatment, rats received their designated SR or LR recovery period before the start of behavioral testing.

3.2.5. Behavioral test apparatus

Anxiety-like behavior was assessed using an elevated-plus maze consisting of two arms intersecting at a 90° angle, thereby creating 4 individual arms each 55 cm long and 12 cm wide. Two opposing arms were enclosed by 46 cm high walls. The remaining two opposing open arms had no walls. The maze was placed 50 cm above the ground. An overhead video camera recorded all trials. Following testing, the videotape was scored and the number of entries into and amount of time spent on each arm were recorded.

Spatial cognition was assessed using a water maze consisting of a circular pool (1.5 m in diameter, 45 cm deep) filled with tap water at 29 ±1.0 °C. A clear Plexiglas escape platform (9 cm X 9 cm) was hidden approximately 2 cm below the water surface in the center of the south-east quadrant of the pool during acquisition, and in the center of the north-west quadrant during reversal. Polypropylene beads floating on top of the water prevented the rats from seeing the hidden platform (Cain et al., 1993). Doors, cabinets, and posters on the walls provided a variety of distal cues. Behavior was recorded by a video camera mounted to the ceiling above the centre of the pool. The camera was

connected to a computer and behavior was objectively analyzed by a tracking system that created a digital record of each swim trial (*Poly-Track*, *San Diego Instruments*, San Diego, CA).

Locomotor and social behaviors were evaluated in a circular open field arena (90 cm diameter, 40 cm high) with wood chip bedding (Beta Chip) covering the floor of the arena. A CD camera was mounted above the centre of the arena and sent feed to a computer, allowing behavior to be recorded using the *EthoVision 3.0.15 Behavioral Monitoring and Analysis System* at a rate of 5.994 frames/sec. This system is capable of continuously tracking the x-y coordinates of each animal and it computing several quantitative variables. The camera also sent feed to a VCR, allowing behavior to be recorded onto VHS cassettes.

Sensorimotor ability was evaluated using a 1 m long narrow wooden beam that was rigidly suspended at each end 1 m above the floor, with soft padding on the floor underneath in case a rat fell off the beam (Kolb and Whishaw, 1985). One edge of the beam was 4 cm wide and was placed facing up for initial acclimation to the task. The other edge was 2 cm wide and was placed facing up during beam task data collection. The lights in the testing room were off and a halogen lamp was placed above the start end of the beam to illuminate it and provide incentive for the rats to walk along the beam, which led to a dark goal platform at the far end of the beam. These conditions provide ample incentive for rats to traverse the beam (Beiko et al., 1997; Kolb and Whishaw, 1985; Shultz et al., 2009). Experience with the water maze does not affect performance on the beam task (Beiko et al., 1997).

Depression-like behaviors were assessed using a forced swim test apparatus similar to that previously used with the rat (Christianson et al., 2008; Jones et al., 2008; Porsolt et al., 1977), consisting of a clear glass cylinder (20 cm diameter) filled to a depth of 30 cm with water at 25 ±1.0 °C. A training session was performed 24 hrs before the test session began. Behavior during the test session was recorded by a side-view video camera and later scored by an individual blind to group membership.

3.2.6. Behavioral testing: day 1

Behavioral testing began at the end of the assigned recovery period, either 24 hrs or 8 weeks after the last mild lateral fluid percussion injury. Rats were placed in the center of the elevated-plus maze facing an open arm and allowed to explore the maze freely for 5 min.

Acquisition training began shortly after completion of elevated-plus maze testing and consisted of 10 training trials, with each trial beginning with the rat being placed gently in the pool adjacent to, and facing, the pool wall, and ending when the rat stood on the hidden platform. Each trial began at one of the four pool wall start locations (North, South, East, or West), with start locations pseudo-randomly ordered to prevent sequential starts from the same location. As this resulted in start locations that varied in distance from the hidden platform, for graphic presentation of search time data in Results, the search time to reach the platform was averaged for each block of 2 trials (e.g. Block 1= (Trial 1 + Trial 2)/2). Rats that failed to reach the hidden platform within 60 sec of the commencement of the trial were placed on the platform by the experimenter. Rats remained on the platform for 15 sec before they were placed in a drying chamber that was

heated from above by an infrared lamp. Rats were run in squads of five so that the inter-trial intervals were not more than 6 min.

3.2.7. Behavioral testing: day 2

Individual rats were placed gently in the centre of the open field apparatus and allowed to explore the arena freely for 10 min.

Shortly after completion of open field testing rats underwent a second water maze session for reversal training. The procedures for the reversal session were identical to acquisition except that the hidden platform was now located in the opposite quadrant of the pool.

3.2.8. Behavioral testing: day 3

Two rats that had received the same number of injuries were placed in the open field and behavioral data were collected for 30 min. On the day before testing, the dorsal surface of one rat from each pair was colored black using black hair dye so that the *EthoVision Tracking System* could distinguish and track each rat separately (Lazar et al., 2008; Shultz et al, 2008).

24 hrs prior to beam task testing rats were given a training session with the 4 cm edge, followed by the 2 cm edge of the beam for acclimation to the task. Approximately 5 trials were given with each.

After beam training, rats underwent forced swim training consisting of a 15 min swimming session in the forced swim apparatus.

3.2.9. Behavioral testing: day 4

Beam testing began approximately 24 hrs after beam task training and consisted of 10 trials. A trial began with the rat being placed on the illuminated end of the beam

and ended when the animal successfully reached the dark goal platform. A maximum of 60 sec was allowed for each trial. Rats were run in squads of five so that the inter-trial intervals were not more than 5 min.

Shortly after beam testing each rat was placed in the forced swim apparatus for a 5 min test session.

3.2.10. Behavioral analyses

For the elevated-plus maze, time spent in the open and closed arms of the maze were used to evaluate anxiety levels in rats. All four of the rat's paws had to enter an arm for it to be considered an entry (Walf and Frye, 2007). As time spent in the open arm is decreased in rats that exhibit greater anxiety-like behaviors, the following percentage score was calculated for the time spent in the open arm: time in the open arm/[time in the open arm + time closed arm] (Saucier et al., 2008; Steimer and Driscoll 2003; Zhu et al. 2006). The number of entries into the closed arm of the maze was also calculated as a measure of locomotion.

For the water maze analysis, search time and direct and circle swims were used as measures of spatial place memory (Morris, 1989; Whishaw and Jarrard, 1995). Search time was defined as the time in sec from release until the rat climbed onto the hidden platform. A maximum of 60 sec was allowed for each trial. Direct and circle swims were measured because they represent efficient swim paths that are normally generated by control rats swimming to a fixed visible or hidden platform (Beiko et al., 2004; Cain and Boon, 2003; Cain et al., 2006; Cain et al., 1996). This measure has the advantage of providing data from each trial, and is not confounded by changes in swim speed. A direct swim was defined as a swim that remained entirely within an 18 cm wide virtual alley

from the start point to the hidden platform without crossing over itself. A circle swim was defined as a swim that approximated an arc of a circle without exceeding 360° or crossing over itself (Beiko et al., 2004; Cain and Boon, 2003; Cain et al., 2006; Whishaw and Jarrard, 1995). Direct and circle swims were summed and calculated as a percentage of the total swims for each test session. Swim speed was used as a measure of motor ability and was objectively calculated in cm/sec by the *Poly-track* system.

For open field and social behavior analysis, *EthoVision* automatically collected and calculated the total distance traveled (cm) by each rat (open field and social), and the mean distance apart (cm) between the rats in each pair (social).

For beam task analysis, traverse time and the number of slips and falls were used as measures of sensorimotor function. Traverse time was defined as the time required to traverse the beam, with a maximum allowed time of 60 sec. Slips and falls were scored when one or more paws slipped off the beam or when a rat fell completely off the beam. Rats that fell off the beam were given a maximum time of 60 sec.

The following measures were scored to assess depression-like behaviors: the time spent immobile (the primary outcome), defined as the rat making only the necessary movements to keep its head above water; time spent escaping, defined as the rat actively struggling to escape the cylinder with its forepaws breaking the surface of the water; and time spent swimming, defined as the rat remaining active in the cylinder but not struggling (Jones et al., 2008; Porsolt et al., 1977).

3.2.11. Brain tissue preparation and immunohistochemical procedures

After the completion of behavioral testing, animals were deeply anaesthetized with sodium pentobarbital (270 mg/ml, ip) and transcardially perfused with ice cold PBS

followed by 4% paraformaldehyde in PBS. Brains were removed, placed in 4% paraformaldehyde solution, and stored at 4±1.0 °C for 24 hrs. Following the fixation period, brains were placed in an 18% sucrose solution for cryoprotection.

Randomly selected brains from 5 or 6 rats per injury group were used for immunohistochemical analysis. Serial 35 μm coronal cross-sections were obtained through the cortex at the level of injury (approximately -3.0 mm posterior to Bregma). This anatomical site was chosen because it allowed reliable quantification of possible mild lateral fluid percussion injury induced changes in cortical areas that have been shown to be altered in human concussion (Umile et al., 2002). Monoclonal mouse anti-ED1 (1:500, Serotec, Raleigh, NC) and NeuN (1:500, Chemican, Temecular, CA) antibodies were used for immunohistochemical staining. Randomly selected, representative sections of the injured area from each animal were processed free-floating for staining as described previously (Weaver et al., 2001). Immunoreactivity was revealed with a glucose-diaminobenzidine-nickel solution. The stained sections were rinsed in PBS, mounted on slides, dehydrated through a gradient of ethanol baths, cleared, and coverslipped with DPX mountant. PBS was substituted for the primary antibody on control sections in each reaction.

3.2.12. Immunohistochemical analyses

An experimenter blinded to the injury groups completed the ED1 immunoreactivity analysis to assess the number of activated microglia/macrophages in the injured cortex. Using a standard light microscope, a photomicrograph image of the injured cortex was taken at 1.25X magnification for each rat (see Fig. 3.1). These photomicrographs were captured from the coronal cross-section closest to the level of

injury (approximately -3.0 mm posterior to Bregma) and placed in the same orientation, with the longitudinal fissure oriented vertically (see Fig. 3.1). Using Image Pro Plus software, one line was drawn from the most dorsal point of the longitudinal fissure to the third ventricle (see Fig. 3.1.). A second line was drawn from the third ventricle at a 60˚ angle to the surface of the injured cortex. It was known that this 60˚ angle would indicate the approximate epicenter of the injury (Paxinos and Watson, 1986). In the event that the epicenter of the injury had shifted from the standard 60˚ angle, an additional 20˚ was allotted to either side of the 60˚ line and the region of greatest immunoreactivity was located within these boundaries (see Fig. 3.1). A photomicrograph image at 20X magnification was then obtained for quantification. All photomicrographs were captured under fixed microscope illumination settings and exposure times to ensure objective and consistent image quality across all pictures. To count the total number of activated microglia/macrophages, the Image Pro Plus color detection function was used to identify the ED1 positively-stained cells. The color threshold was adjusted to detect inflammatory cells while excluding the background.

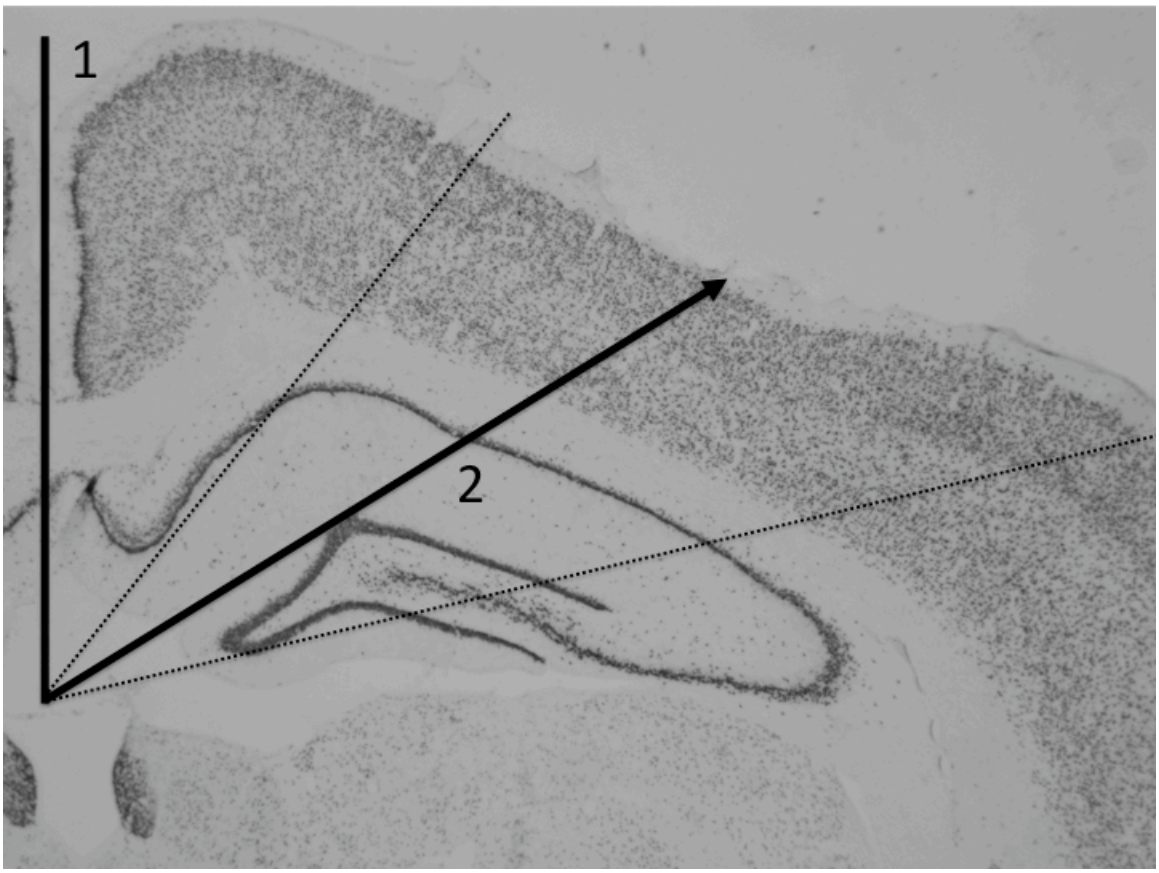

Figure 3.1. Locating the epicenter of the injury and the boundaries for immunohistochemical quantification. The first solid black line was drawn from the most dorsal point of the longitudinal fissure to the third ventricle. The second line was drawn from the third ventricle at a 60˚ angle to the surface of the cortex, indicating the approximate epicenter of the injury (Paxinos and Watson, 1986). The dashed lines represent the additional 20˚ allotted to either side of the 60˚ line in the event that the epicenter of the injury had shifted. Images for immunohistochemical quantification were obtained from the cortex within these boundaries.

An experimenter blinded to the injury groups also completed a semi-qualitative analysis that assessed damage in the injured cortex. A 1.25X magnification photomicrograph image of NeuN-stained injured cortex was obtained from each coronal cross-section using a standard light microscope. Each image was placed in the same orientation, with the longitudinal fissure oriented vertically, and spanned to the parietal/temporal cortex (Paxinos and Watson, 1986; see Fig. 3.1). A score of 1 was given for no or mild (e.g. slight depression) cortical damage. A score of 2 was given for moderate cortical damage (e.g. slight cavitation/cortical loss). A score of 3 was given for severe cortical damage (e.g. obvious cavitation/cortical loss). This rating scale was adapted from previous studies (Braak and Braak, 1991; Lemstra et al., 2007; Li et al., 2006).

3.2.13. Statistical analyses

Water maze search time, beam traverse times, and acute injury measures were analyzed by SPSS 17.0 using mixed design ANOVA with injury group as the between-subjects factor and trial (search and traverse times) or treatment number (acute injury measures) as the within-subjects factor. Simple effects post hoc *F*-tests were carried out when appropriate. One-way ANOVAs, with injury group as the between-subjects factor, were used to analyze elevated-plus maze measures, direct and circle swims, swim speed, distance traveled, mean distance apart, slips and falls, forced swim measures, and ED1 quantification. Fisher's LSD post hoc pair-wise comparisons were carried out when appropriate. Statistical significance was set at $p < .05$.

3.3. Results

3.3.1. Acute injury measures

Apnea time, unconsciousness time, and self-righting reflex time were used as acute measures of injury severity. Apnea time increased with additional treatments, as indicated by a significant effect of *treatment number* ($F(4, 228) = 3.792, p < .005$). However, apnea time increased more in the 5C group, as indicated by a significant *injury group x treatment number* interaction ($F(12, 228) = 1.868, p < .05$; see Fig. 3.2A). Specifically, the fifth treatment in the 5C group induced longer apnea time than the fifth treatment in all other groups and previous treatments the 5C group received (all *ps* < .05; see Fig. 3.2A).

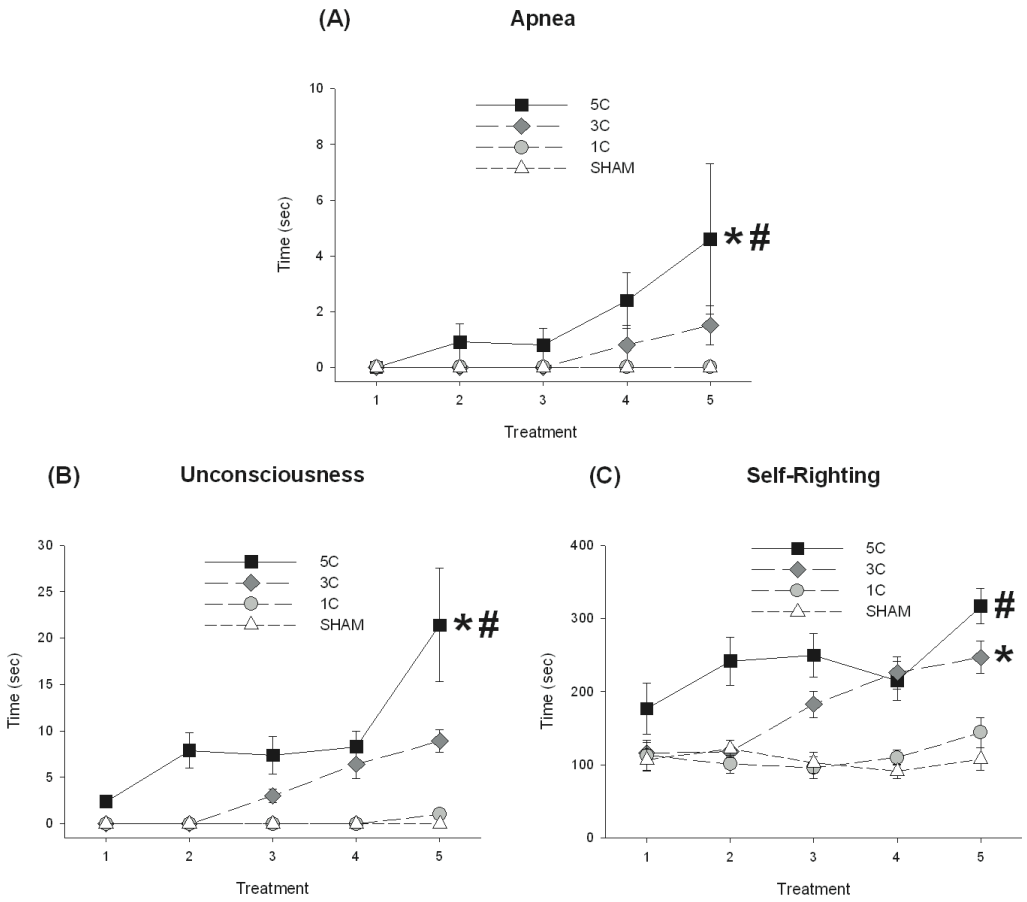

Figure 3.2. Acute injury measures. (A) Apnea times increased with additional treatments. The 5C group displayed significantly longer apnea time than all other groups. (B) Unconsciousness times increased with additional treatments. The 5C group displayed significantly longer unconsciousness time than all other groups. (C) Self-righting reflex times increased with additional treatments. The 3C group displayed significantly longer self-righting reflex times than the 1C and SHAM groups. The 5C group displayed significantly longer self-righting reflex times than all other groups. For panels (A) and (B) # = different from all other groups; * = different from previous treatments; all *ps* < .05. For panel (C) # = different from all other groups; * = different from 1C and SHAM groups; all *ps* < .05. Histogram bars represent means (± SEM). For additional statistical detail see Results.

Unconsciousness time increased with additional treatments, as indicated by a significant effect of *treatment number* ($F(4, 228) = 7.998, p < .001$). However, as treatments progressed unconsciousness time increased more in the 5C group, as indicated by a significant effect of *injury group* ($F(3, 57) = 10.057, p < .001$; 5C > all other groups, all *ps* < .01) and a significant *injury group x treatment number* interaction ($F(12, 228) = 3.269, p < .001$; see Fig. 3.2B). Specifically, the fifth treatment in the 5C group induced longer unconsciousness time than the fifth treatment in all other groups and previous treatments the 5C group received (all *ps* < .05; see Fig. 3.2B).

Self-righting reflex time increased with additional treatments, as indicated by a significant effect of *treatment number* ($F(4, 228) = 7.915, p < .001$). However, as treatments progressed self-righting reflex time increased more in the 5C and 3C groups, as indicated by a significant effect of *injury group* ($F(3, 57) = 24.863, p < .001$; 5C > all other groups, all *ps* < .005; 3C > 1C and SHAM, all *ps* < .001) and a significant *injury group x treatment number* interaction ($F(12, 228) = 3.714, p < .001$; see Fig. 3.2C). The fourth and fifth treatments in the 5C and 3C groups induced longer self-righting reflex time than the fourth and fifth treatment in the 1C and SHAM groups (all *ps* < .05). The second and third treatments in the 5C group induced longer self-righting reflex time then the second and third treatments in all other groups (all *ps* < .05; see Fig. 3.2C).

3.3.2. Elevated-plus maze

Time spent in open arms of the elevated-plus maze was used to measure anxiety-like behavior. The number of closed arm entries was used as a measure of locomotion. During SR testing the 5C-SR group spent significantly less time in the open arms compared to the SHAM-SR group, as indicated by a significant effect of *injury group*

($F(3, 54) = 2.788, p < .05$; 5C-SR < SHAM-SR rats, $p < .01$; see Fig. 3.3A). There was no difference between groups in the number of closed arm entries (Fig. 3.3B; $p > .05$).

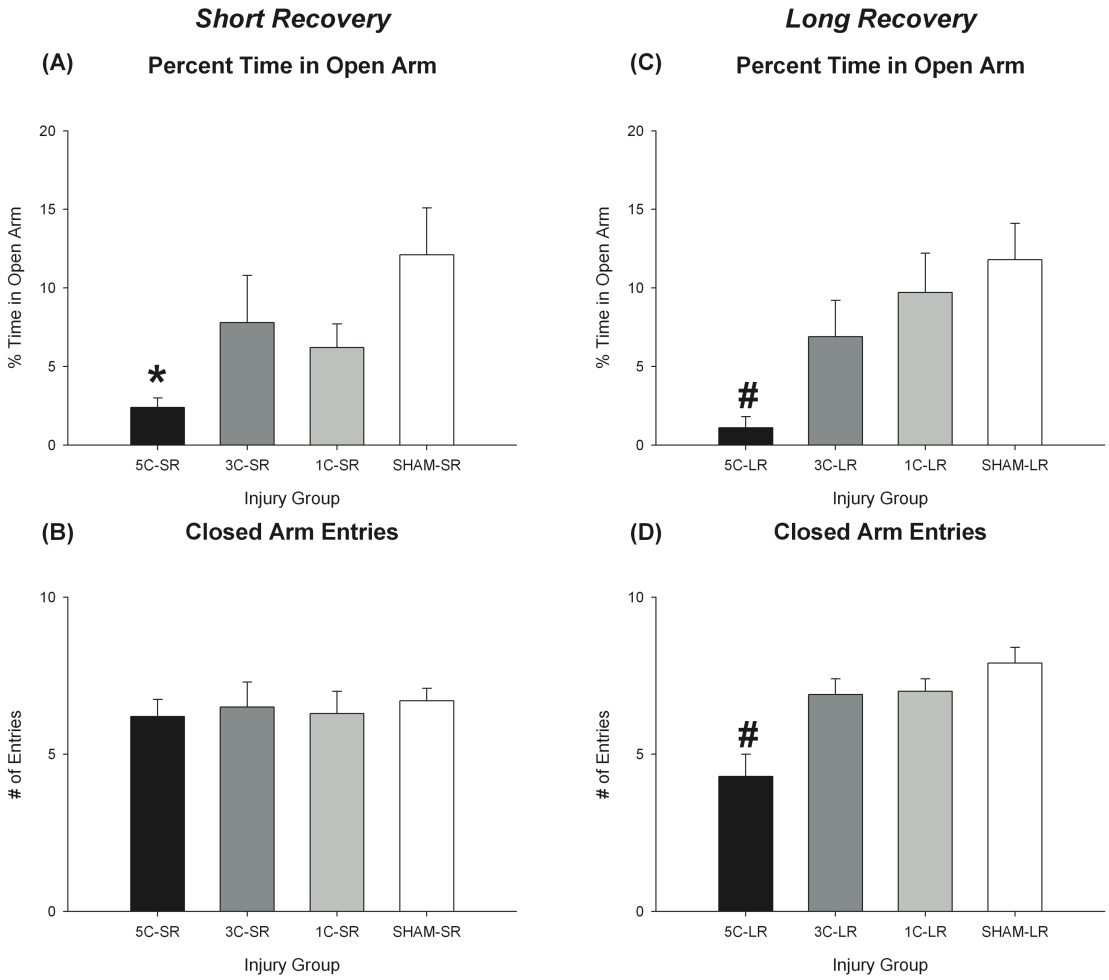

Figure 3.3. Short recovery and long recovery groups elevated-plus maze results. (A) The 5C-SR group spent a significantly smaller percent of time in the open arm than the SHAM-SR group. (B) The groups did not differ in the number of entries into the closed arm. (C) The 5C-LR group spent a significantly smaller percent of time in the open arm than all other groups. (D) The 5C-LR group displayed significantly fewer closed arm entries than all other groups. Histogram bars represent means (± SEM). # = different from all other groups, all *ps* < .05 or better; * = different from the SHAM-SR, $p < .01$. For additional statistical detail see Results.

During LR testing the 5C-LR group spent significantly less time in the open arms compared to all other groups, as indicated by a significant effect of *injury group* ($F(3, 47) = 5.140, p < .01$; 5C-LR < all other groups, all $ps < .05$; see Fig. 3.3C). 5C-LR rats also displayed fewer entries into the closed arm compared to all other groups, as indicated by a significant effect of *injury group* ($F(3, 47) = 10.771, p < .001$; 5C-LR < all other groups, all $ps < .001$; see Fig. 3.3D).

3.3.3. Water maze: short recovery

Search time and direct and circle swims were used as measures of cognitive ability in the water maze. Swim speed was included as a measure of motor ability. During SR acquisition training search time decreased in all groups as testing progressed, as indicated by a significant effect of *trial* ($F(9, 486) = 11.867, p < .001$). Search time decreased less in all injured groups than in the SHAM-SR group, as indicated by a significant effect of *injury group* ($F(3, 54) = 8.126, p < .001$; 5C-SR, 3C-SR, and 1C-SR > SHAM-SR, all $ps < .05$; see Fig. 3.4A). 5C-SR rats also displayed longer search time than 1C-SR rats ($p < .05$; see Fig. 3.4A)

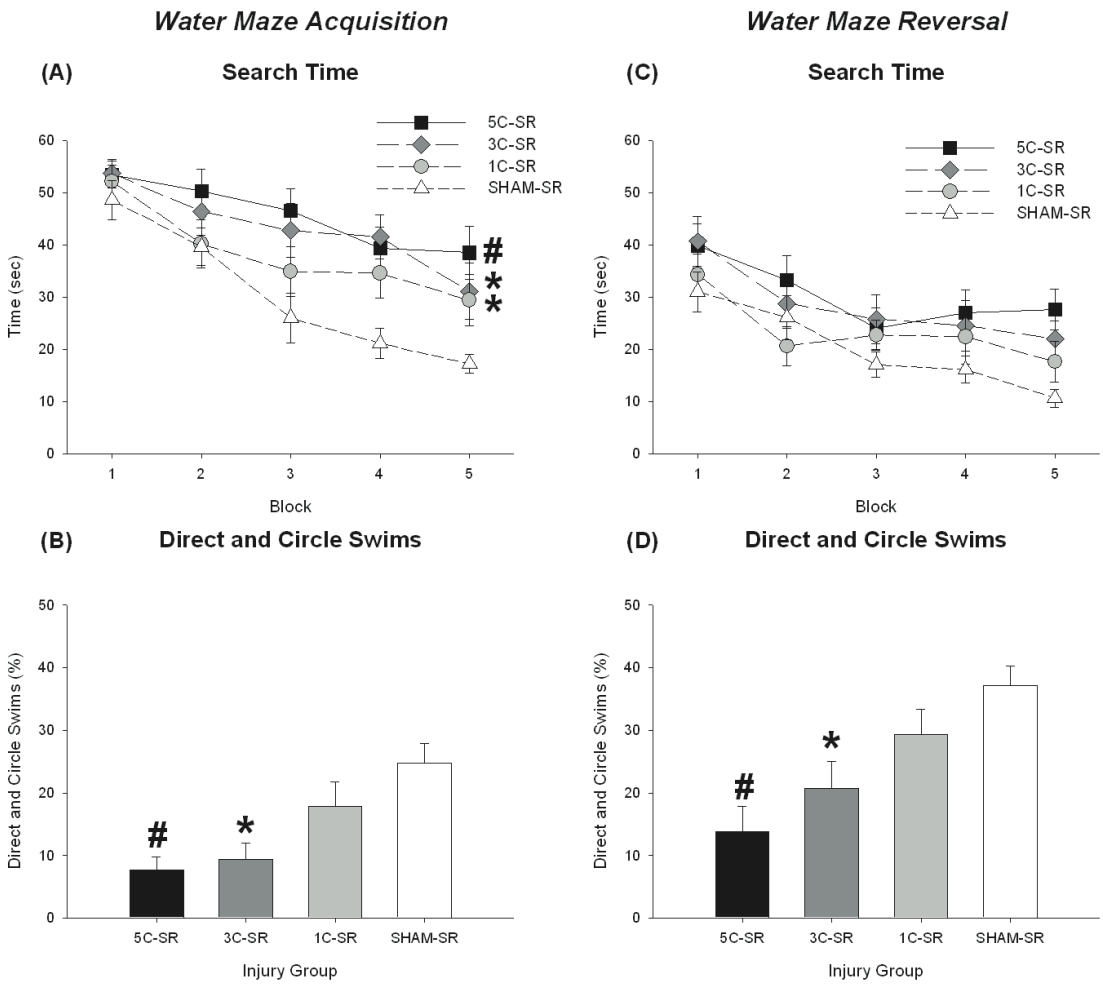

Figure 3.4. Short recovery groups water maze results. (A) The 1C-SR and 3C-SR groups displayed significantly longer search time during acquisition than the SHAM-SR group. The 5C-SR group displayed significantly longer search time than the 1C-SR and SHAM-SR groups. (B) The 3C-SR group displayed significantly fewer direct and circle swims during acquisition than the SHAM-SR group. The 5C-SR group displayed significantly fewer direct and circle swims than the 1C-SR and SHAM-SR groups. (C) During reversal training all groups displayed shorter search time as training progressed. (D) The 3C-SR group displayed significantly fewer direct and circle swims during reversal training than the SHAM-SR group. The 5C-SR group displayed significantly fewer direct and circle swims than the 1C-SR and SHAM-SR groups. In panels (A) and

(C) data points represent means of data collected for each block of two trials (± SEM). In panels (B) and (D) histogram bars represent means of data collected during the 10 water maze trials (± SEM). # = different from 1C-SR and SHAM-SR, all *ps* < .05; * = different from SHAM-SR, all *ps* < .05 or better. For additional statistical detail see Results.

The direct and circle swim data were consistent with the search time data in revealing fewer direct and circle swims in the 5C-SR and 3C-SR groups during acquisition compared to the SHAM-SR group, as indicated by a significant effect of *injury group* ($F(3, 54) = 6.769, p < .001$; 5C-SR and 3C-SR < SHAM-SR, all $ps < .01$; see Fig. 3.4B). The 5C-SR group also displayed significantly fewer direct and circle swims than the 1C-SR group ($p < .05$).

During SR reversal training search time decreased in all groups as testing progressed, as indicated by a significant effect of *trial* ($F(9, 486) = 10.547, p < .001$; see Fig. 3.4C). There was also a non-significant trend for an effect of *injury group* ($F(3, 54) = 2.488, p = .07$) on search time.

The 5C-SR and 3C-SR groups displayed fewer direct and circle swims during reversal training compared to the SHAM-SR group, as indicated by a significant effect of *injury group* ($F(3, 54) = 6.786, p < .001$; 5C-SR and 3C-SR < SHAM-SR group, all $ps < .01$; see Fig. 3.4D). The 5C-SR group also displayed significantly fewer direct and circle swims than the 1C-SR group ($p < .05$). There were no SR group differences in swim speed during either acquisition or reversal training sessions (all $ps > .05$; data not shown).

3.3.4. Water maze: long recovery

During LR water maze acquisition training search time decreased in all groups as testing progressed, as indicated by a significant effect of *trial* ($F(9, 423) = 12.135, p < .001$). Search time decreased less in 5C-LR and 3C-LR groups than in the 1C-LR and SHAM-LR groups, as indicated by a significant effect of *injury group* ($F(3, 47) = 9.793$, $p < .001$; 5C-LR and 3C-LR > 1C-LR and SHAM-LR, all $ps < .01$; see Fig. 3.5A).

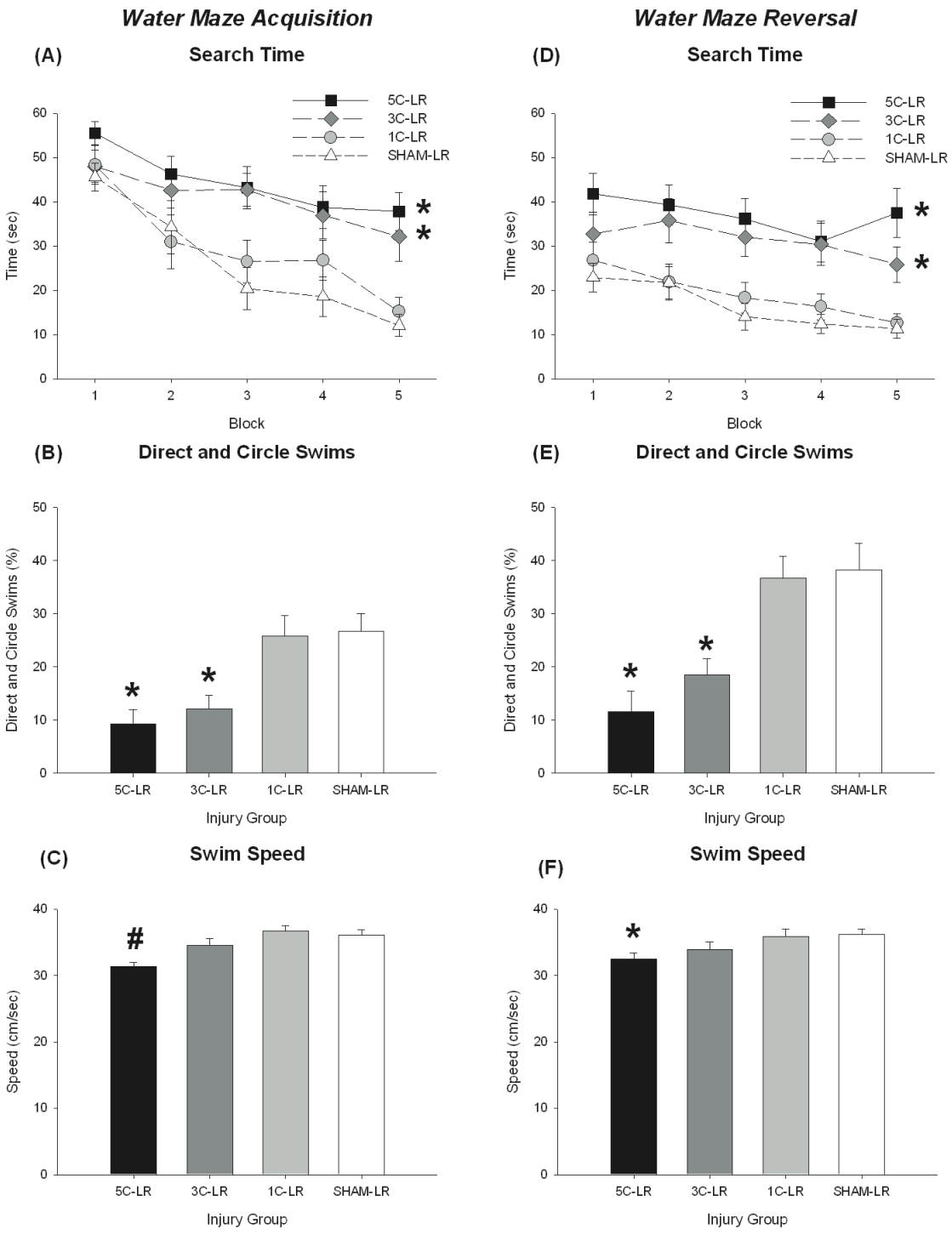

Figure 3.5. Long recovery groups water maze results. (A) The 3C-LR and 5C-LR groups displayed significantly longer search time during acquisition than both the 1C-LR and the SHAM-LR groups. (B) The 3C-LR and 5C-LR groups displayed significantly

fewer direct and circle swims during acquisition than both the 1C-LR and SHAM-LR groups. (C) The 5C-LR group displayed significantly slower swim speed during acquisition than all other groups. (D) The 3C-LR and 5C-LR groups displayed significantly longer search time during reversal than both the 1C-LR and the SHAM-LR groups. (E) The 3C-LR and 5C-LR groups displayed significantly fewer direct and circle swims during reversal than both the 1C-LR and SHAM-LR groups. (F) The 5C-LR group displayed significantly slower swim speed during reversal than the 1C-LR and SHAM-LR groups. In panels (A) and (D) data points represent means of data collected for each block of two trials (± SEM). In panels (B), (C), (E), and (F) histogram bars represent means of data collected during the 10 water maze trials (± SEM). # = different from all other groups, all *ps* < .05; * = different from 1C-LR and SHAM-LR, all *ps* < .05. For additional statistical detail see Results.

The direct and circle swim data were consistent with the search time data in revealing fewer direct and circle swims in the 5C-LR and 3C-LR groups during acquisition compared to the 1C-LR and SHAM-LR groups, as indicated by a significant effect of *injury group* ($F(3, 47) = 8.297, p < .001$; 5C-LR and 3C-LR < 1C-LR and SHAM-LR, all *ps* < .01; see Fig. 3.5B).

During LR reversal training search time decreased in all groups as testing progressed, as indicated by a significant effect of *trial* ($F(9, 423) = 4.595, p < .001$). Search time decreased less in 5C-LR and 3C-LR groups than in the 1C-LR and SHAM-LR groups, as indicated by a significant effect of *injury group* ($F(3, 47) = 13.488, p < .001$; 5C-LR and 3C-LR > 1C-LR and SHAM-LR, all *ps* < .01; see Fig. 3.5D).

The direct and circle swim data were consistent with the search time data in revealing fewer direct and circle swims in the 5C-LR and 3C-LR groups during acquisition compared to the 1C-LR and SHAM-LR groups, as indicated by a significant effect of *injury group* ($F(3, 47) = 10.702, p < .001$; 5C-LR and 3C-LR < 1C-LR and SHAM-LR, all *ps* < .01; see Fig. 3.5E). The 5C-LR group also displayed slower swim speed during both LR training sessions, as indicated by a significant effect of *injury group* during acquisition ($F(3, 47) = 6.998, p < .01$; 5C-LR < all other groups, all *ps* < .05; see Fig. 3.5C) and reversal ($F(3, 47) = 2.876, p < .05$; 5C-LR < 1C-LR and SHAM-LR, all *ps* < .05; see Fig. 3.5F).

3.3.5. Beam task

Traverse time and the number of slips and falls on the beam task were used as measures of sensorimotor ability. There was a non-significant trend for *injury group* on the measure of slips and falls during both SR ($F(3, 54) = 2.560, p = .065$; see Fig. 3.6A)

and LR testing ($F(3, 47) = 2.490, p = .072$; see Fig. 3.6B). The groups did not differ on traverse time ($p > .05$; data not shown).

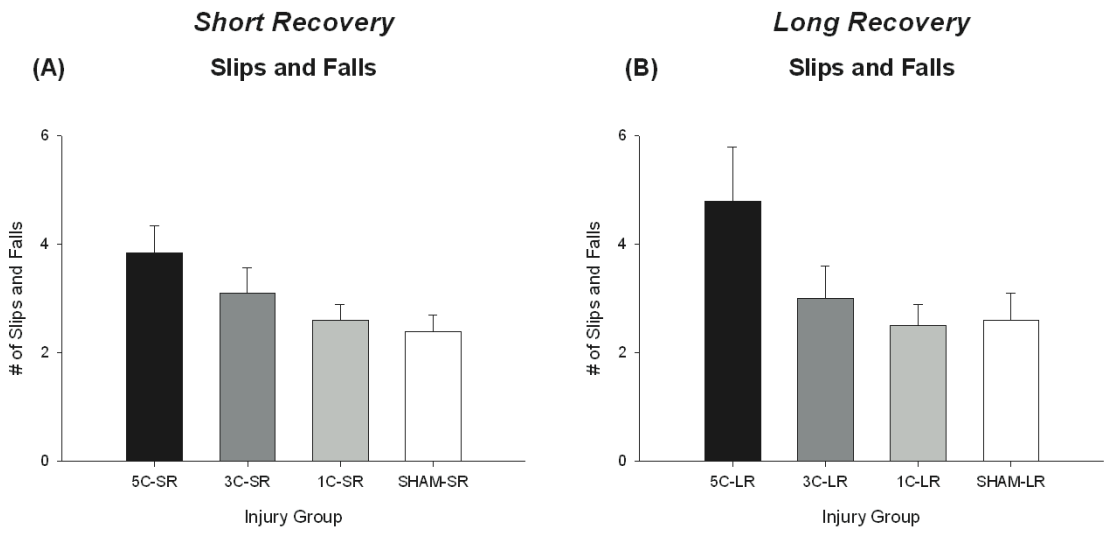

Figure 3.6. Short and long recovery groups beam task slips and falls results. There were non-significant trends for both the SR (A), $p = .065$, and LR (B), $p = .072$, groups to differ in slips and falls. Histogram bars represent means of data collected during the 10 beam task trials (± SEM). For additional statistical detail see Results.

3.3.6. Forced swim

The time spent immobile in the forced swim task was used to measure depression-like behavior. The groups did not differ on any behavioral measure during SR forced swim testing (all *ps* > .05; data not shown).

During LR forced swim testing 5C-LR rats displayed increased time immobile, as indicated by a significant effect of *injury group* ($F(3, 47) = 2.898$, $p < .05$; 5C-LR < SHAM-LR, $p < .01$; see Fig. 3.7). There was also a non-significant trend suggesting 3C-LR rats spent more time immobile than SHAM-LR rats ($p = .062$). There were no group differences on the measures of time spent swimming or escaping (all *ps* > .05; data not shown).

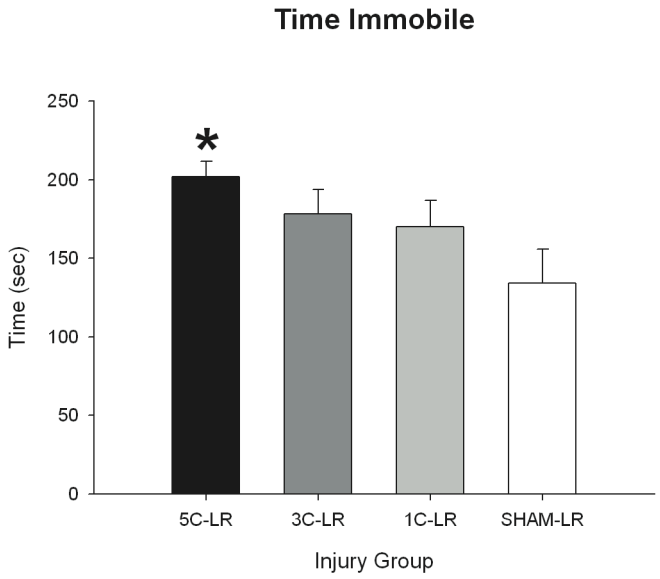

Figure 3.7. Long recovery groups forced swim time immobile results. The 5C-LR rats spent significantly more time immobile than the SHAM-LR group. Histogram bars represent means of data collected during the forced swim test trial (± SEM). * = different from SHAM-LR, $p < .01$. For additional statistical detail see Results.

3.3.7. Open field and social behavior

Distance traveled in the open field task and the social behavior task was used as a measure of locomotion. Mean distance apart and proximity were used as measures of social behavior. The groups did not differ on any behavioral measure in the open field or social behavior tasks (all *ps* > .05; data not shown).

3.3.8. Neuroinflammation

The number of ED1-labeled microglia/macrophages in the injured cortex was used to assess neuroinflammation. The 5C-SR and 3C-SR groups displayed significantly more ED1-labeled activated microglia/macrophages than both the SHAM-SR and 1C-SR groups, as indicated by a significant effect of *injury group* ($F(3, 23) = 13.023$, $p < .001$; 5C-SR and 3C-SR > SHAM-SR and 1C-SR, all *ps* < .01; see Fig. 3.8A). There was also a non-significant trend suggesting that 1C-SR rats displayed more ED1-labeled activated microglia/macrophages than the SHAM-SR group ($p = .081$).

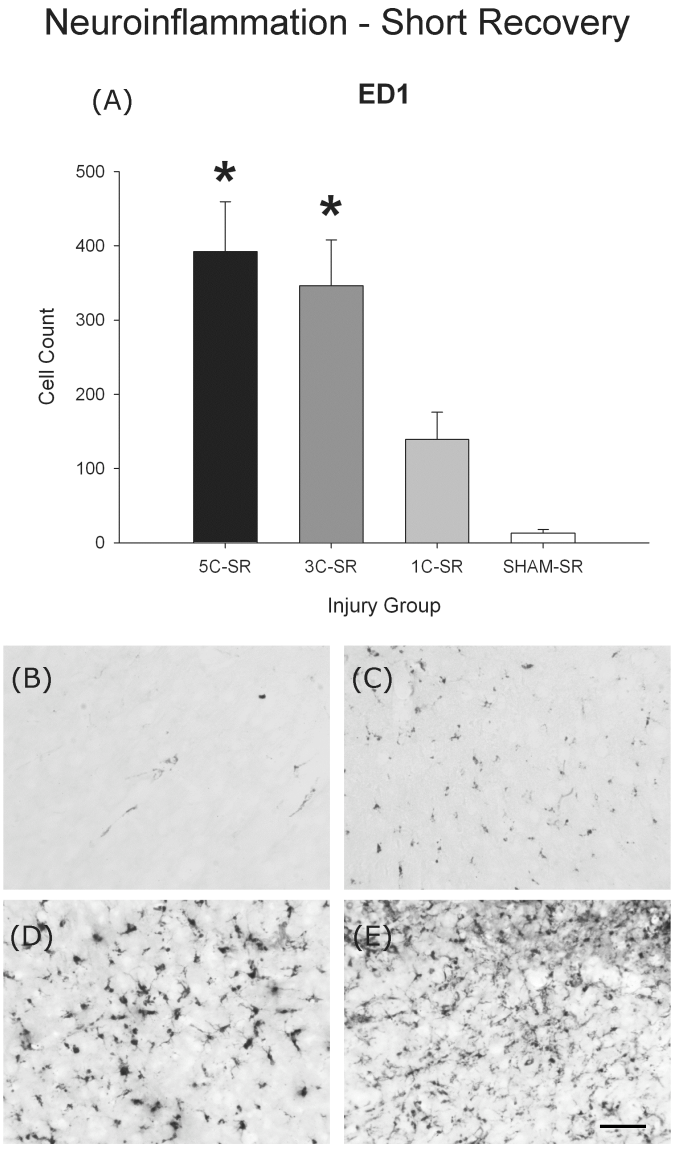

Figure 3.8. Short recovery ED1 results. (A) The 5C-SR and 3C-SR groups displayed significantly more ED1-labeled activated microglia/macrophages in the injured cortex than both the SHAM-SR and 1C-SR groups. Histogram bars represent mean ED1 labeled cells (± SEM). * = different from the SHAM-SR and 1C-SR groups, *ps* < .01. (B-E) Representative photomicrographs at 20X magnification showing ED1 immunoreactivity from the SHAM-SR (B), 1C-SR (C), 3C-SR (D), and 5C-SR (E) groups. Scale bar = 100 μm. For additional statistical detail see Results.

The 5C-LR group displayed significantly more ED1-labeled activated microglia/macrophages than all the other groups, as indicated by a significant effect of *injury group* ($F(3, 22) = 7.074$, $p < .05$; 5C-LR > all other groups, all *ps* < .05; see Fig. 3.9).

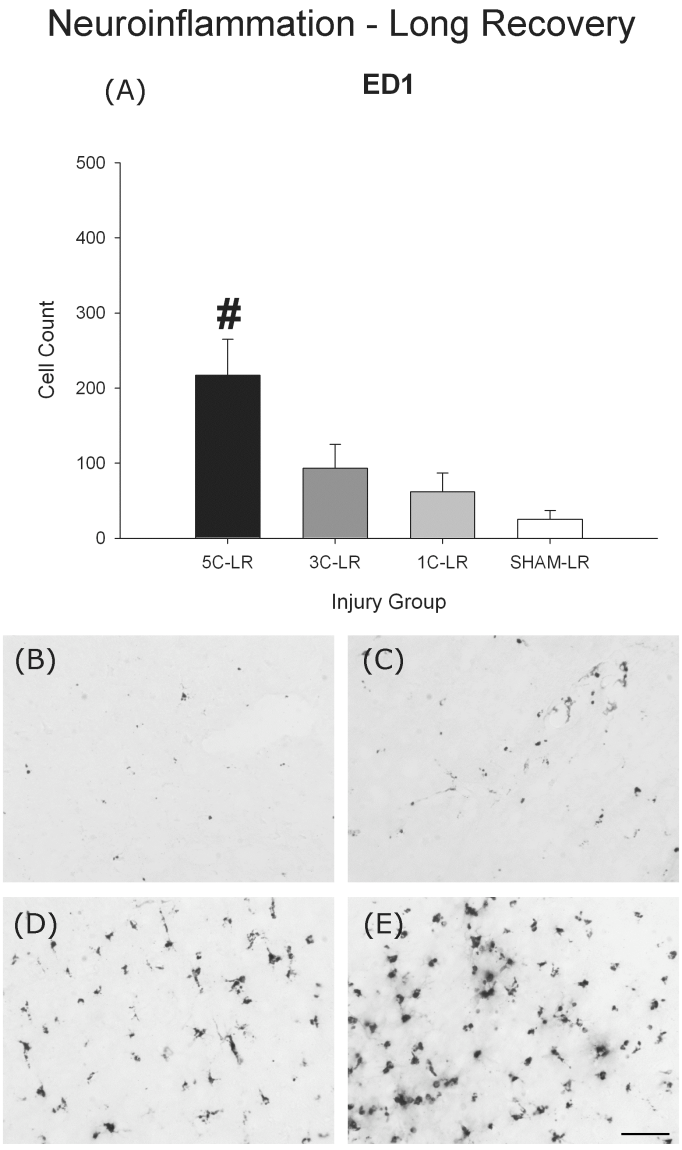

Figure 3.9. Long recovery ED1 results. (A) The 5C-LR group displayed significantly more ED1-labeled activated microglia/macrophages in the injured cortex than all other groups. Histogram bars represent mean ED1 labeled cells (± SEM). # = different from all other groups, *ps* < .01. (B-E) Representative photomicrographs at 20X magnification showing ED1 immunoreactivity from the SHAM-LR (B), 1C-LR (C), 3C-LR (D), and 5C-LR (E) groups. Scale bar = 100 μm. For additional statistical detail see Results.

3.3.9. Cortical Damage

Cortical damage was assessed using a semi-qualitative analysis, with three levels: no/mild damage; moderate damage; severe damage. The majority of 5C-SR and 3C-SR rats displayed moderate or severe cortical damage (see Fig 3.10). There was no indication of moderate or severe damage in the 1C-SR and SHAM-SR groups.

Among LR groups, 84% of 5C-LR rats and 80% of 3C-LR rats displayed moderate or severe cortical damage (see Fig. 3.10). A single 1C-LR rat displayed severe damage. The remaining 1C-LR and SHAM-LR rats displayed little or no indication of cortical damage.

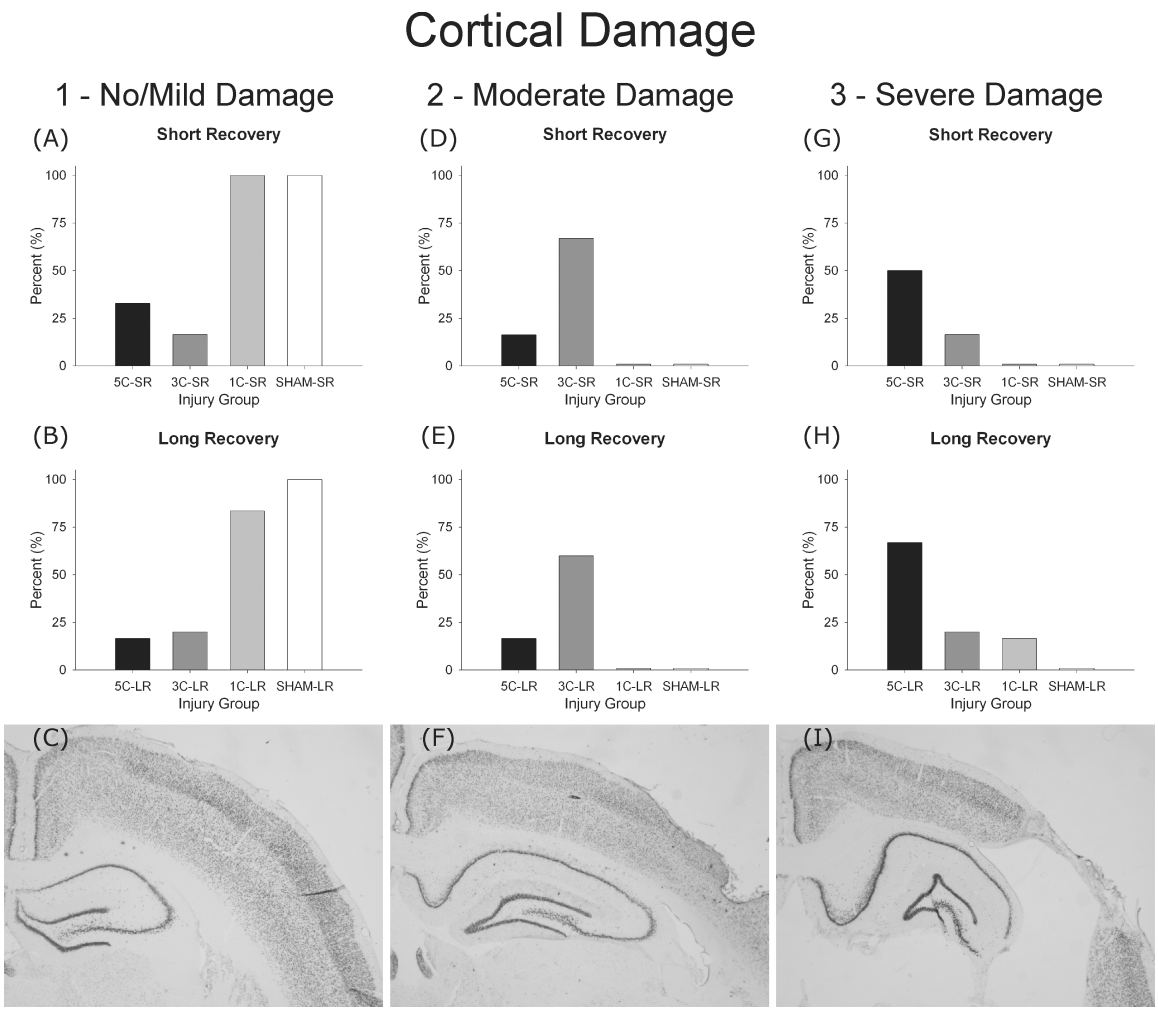

Figure 3.10. Semi-qualitative analysis of cortical damage. The histogram bars in panels (A), (D), and (G) show the percent of each short recovery injury group scoring 1 (A), 2 (D), or 3 (G) on the semi-qualitative scale of cortical damage. The histogram bars in panels (B), (E), and (H) show the percent of each long recovery injury group scoring 1 (B), 2 (E), or 3 (H) on the semi-qualitative scale of cortical damage. Panels (C), (F), and (I) show representative photomicrographs at 1.25X magnification of cortical damage scored 1-no/mild damage (C), 2-moderate damage (F), or 3-severe damage (I). For additional detail see Results.

3.4. Discussion

The current findings suggest that repeated mild lateral fluid percussion injury can produce cumulative and long-term changes in the rat that appear consistent with features of repeated concussion. Acute injury assessment found that rats in the 5C groups displayed increased apnea, a longer time unconscious, and a longer latency to the self-righting reflex. Rats given three mild lateral fluid percussion injuries also displayed longer latency to the self-righting reflex. Both 5C groups spent less time in the open arm of the elevated-plus maze, indicating increased anxiety-like behavior. In the water maze task all 5C and 3C groups were impaired on most or all measures relative to SHAM controls, and the 1C-SR group had scores on certain measures that were intermediate between the 5C-SR and SHAM-SR groups. Taken together, these data suggest that repeated mild lateral fluid percussion injuries resulted in both cumulative and long-term cognitive impairments. The 5C-LR group also displayed significantly more immobility in the forced swim task, suggesting increased depression-like behavior.

Immunohistochemical examination found that the 5C-SR and 3C-SR groups displayed increased neuroinflammation after short recovery. Increased neuroinflammation was still present after long recovery in the 5C-LR group. Semi-qualitative analysis of cortical damage found that the majority of rats experiencing repeated mild lateral fluid percussion injury displayed moderate or severe damage. Although a single rat from the 1C-LR group displayed severe damage, all other 1C and SHAM rats displayed little or no cortical damage.

3.4.1. Nature of the behavioral impairments

Past studies that found decreased time spent in the open arm of the elevated-plus maze have interpreted this outcome as an increase in anxiety-like behavior (Jones et al, 2008; Walf and Frye, 2007). The current finding that the 5C groups spent less time on the open arm of the elevated-plus maze is novel in mild lateral fluid percussion injury research, and suggests increased anxiety in these groups. Our use of a specific test for anxiety-like behavior allows us to evaluate the suggestion that cognitive impairment in the water maze might be secondary to anxiety in rats given repeated mild lateral fluid percussion injuries (DeRoss et al., 2002). Although the 5C groups displayed both anxiety-like behavior in the elevated-plus maze and impairment in the water maze, both 3C groups displayed water maze impairments in the absence of anxiety-like behavior. This suggests that impairment in the water maze task may not be secondary to anxiety, and strengthens the conclusion that repeated concussion can cause cognitive impairment in a rat model.

There were no SR group differences in the number of closed arm entries in the elevated-plus maze, swim speed in the water maze, open field exploration, or beam task measures, suggesting that locomotor abnormalities were not confounding factors in the SR findings. However, 5C-LR rats displayed fewer closed arm entries in the elevated-plus maze and slower swim speeds during water maze testing. Thus, the possibility that motor deficits contributed to the behavioral deficits observed in the 5C-LR group must be considered. However, Walf and Frye (2007) suggest that arm entries are not an optimal measure of locomotion, and direct measures of locomotor ability in the present study found no significant differences between groups in locomotion in either the open field task or the beam task. Further, the 5C-SR group spent significantly less time on the open

arms of the plus maze, yet made as many closed arm entries as the other SR groups, and the 3C-LR group was impaired to the same extent as the 5C-LR group in the water maze task yet displayed no indication of locomotor abnormalities. Taken together, the available evidence suggests that it is unlikely that motor or other abnormalities directly account for either the decreased time spent in the open arm during elevated-plus maze testing or the water maze impairments displayed by the 5C-LR group.

Cognitive impairments occurred after both single and repeated mild lateral fluid percussion injury treatments. Our finding that 1C-SR, but not 1C-LR, rats were impaired during water maze testing is consistent with similar findings in previous single mild lateral fluid percussion injury water maze studies, and is also consistent with the transient impairments often observed in humans suffering a single concussion (DeRoss et al., 2002; Gurkoff et al., 2006; McCrory et al., 2009; see Chapter 2). Importantly, the results from both the SR and LR groups show that repeated mild lateral fluid percussion injury can induce cumulative cognitive impairments in the water maze, and the results from the LR groups show that the cumulative cognitive impairments persist for at least 8 weeks in the 3C-LR and 5C-LR groups.

Increased time spent immobile in the forced swim task is considered to be indicative of depression-like behavior in the rat (Jones et al., 2008; Porsolt et al., 1977). The current finding that 5C-LR rats spent more time immobile in this task is novel in mild lateral fluid percussion injury research. As 5C-LR rats displayed swim speed impairments in the water maze and fewer closed arm entries in the elevated-plus maze, both indirect measures of locomotor ability, it is possible that motor deficits may have contributed to increased immobility in the forced swim task. Although 5C-LR rats did not

display significant impairments on direct measures of locomotor ability in the open field or beam tasks, and were able to complete the rigorous 15 min forced swim training session, future studies might avoid this potential confound by utilizing measures of depression that are minimally sensitive to motor impairments such as the sucrose preference test (Jones et al., 2008).

3.4.2. Pathology and its relation to behavioral changes

In the current study it was found that repeated mild lateral fluid percussion injury is capable of producing moderate or severe cortical damage. Previous studies have found that damage to cortex similar to that observed in the current study is capable of impairing cognitive and emotional function in both humans and rats (Bissiere et al., 2006; Drevets et al., 2008; Jones et al., 2008; Levin et al., 1987; Shamay-Tsoory et al., 2004; Wahl et al., 2000). For example, Wahl and colleagues (2000) found that lateral fluid percussion injury induced lesions to rat parietal and temporal cortex resulted in cognitive impairment, and that the impairment was reduced by decreasing the lesion size. In addition, another study found that a single severe lateral fluid percussion injury resulted in both cortical damage and increased anxiety-like behaviors (Jones et al., 2008). Overall, it seems possible that cortical damage induced by repeated mild lateral fluid percussion injury is capable of producing the behavioral changes found in the current study.

Repeated mild lateral fluid percussion injury was also found to induce a neuroinflammatory response consisting of activated microglia/macrophages in the injured cortex. Neuroinflammation has been observed in rat and human traumatic brain injury, with evidence suggesting that these processes might alter normal brain function (Hein and O'Banion, 2009; Lenzlinger et al., 2001; Morganti-Kossmann et al., 2007; Schmidt

et al., 2005; Spalletta et al., 2006). For example, previous research from our laboratory and others has revealed a cognitive deficit in the water maze in the presence of a neuroinflammatory response similar to the one found in the current study (Shultz et al., 2009; Wu et al., 2006, 2010; see Chapter 2). Neuroinflammation also may have contributed to the cortical damage observed in repeated mild lateral fluid percussion injury rats, as neuroinflammation is capable of inducing secondary brain damage in traumatic brain injury through processes such as apoptosis and the production of reactive oxygen species (Dringen, 2005; Morganti-Kossmann et al., 2007; Schmidt et al., 2005). The finding that five mild lateral fluid percussion injuries often resulted in long-term cortical damage in the presence of a neuroinflammatory response may be important for understanding the cumulative and chronic effects of repeated concussion, as neuroinflammation has been linked to a number of neurodegenerative disorders (Lee et al., 2002; Nandoe et al., 2002; O'Sullivan et al., 2009; Stoll and Jander, 1999; Whitton, 2007; Zilka, 2006). Taken together, it seems possible that neuroinflammation could contribute to the behavioral changes found in the current study.

3.4.3. Relation to repeated concussion in humans

The current study found that repeated mild lateral fluid percussion injury induced behavioral and neuropathological changes in the rat that bear similarity to features of repeated concussion in humans. Athletes who experienced three or more concussions displayed more severe acute symptoms, long-term cognitive impairments, and increased incidence of depression compared to athletes who experienced a single concussion, and repeated head trauma can lead to chronic traumatic encephalopathy with long-term cognitive impairments, dementia, depression, and anxiety (Bailes and Cantu, 2001;

Cantu, 2007; Collins et al., 2002; Guskiewicz et al., 2003; Guskiewicz et al., 2005; Guskiewicz et al., 2007; Macciocchi et al., 2001; McKee et al., 2009). Analysis of post-mortem brain tissue taken from individuals who had sustained repeated concussion and displayed chronic behavioral symptoms have reported that neuronal loss, atrophy, and neuroinflammation are pathologies associated with repeated concussion and chronic traumatic encephalopathy (McKee et al., 2009; Omalu et al., 2006). The current findings of progressively worse acute injury measures, cumulative and long-term cognitive deficits, persistent anxiety-like behavior, long-term depression-like behavior, neuroinflammation, and cortical damage in rats after repeated mild lateral fluid percussion injury are similar to many of the above symptoms and pathologies in humans.

Taken together, the current findings provide support for the use of repeated mild lateral fluid percussion injury as a model of repeated concussion. Future studies might utilize this model to better understand the factors involved in the cumulative and chronic nature of these injuries and provide insight into their management and treatment through the development and application of new guidelines and therapies.

3.4.4. Conclusions

Repeated mild lateral fluid percussion injury induced cumulative and long-term cognitive deficits, anxiety, and depression in adult male Long-Evans rats. As these injuries also resulted in neuroinflammation and cortical damage, it seems possible that these pathologies may be involved in the behavioral changes observed. These behavioral and pathological changes in rats suffering repeated mild lateral fluid percussion injury are consistent with symptoms and pathologies seen in humans that have experienced repeated

concussion and provide support for the further use of repeated mild lateral fluid percussion injury in the rat to investigate repeated concussion.

3.5. References

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Chapter 4

4. Treatment with anti-CD11d integrin antibody reduces cognitive, emotional, and motor impairments following traumatic brain injury in the Long-Evans rat[3]

4.1. Introduction

Traumatic brain injury is an international health concern often resulting in chronic neurological abnormalities including cognitive deficits, emotional disturbances, and motor impairments (Maas et al., 2000; Marshall, 2000; Rao and Lyketsos, 2000). To date there has been limited success in the development of effective pharmacological interventions for traumatic brain injury (Doppenberg et al., 2004; Stein and Wright, 2010; Thompson et al., 2005). This lack of effective treatment is likely due to the complex neural processes that follow traumatic brain injury (Atif et al., 2009), which can involve both primary and secondary injury mechanisms (Morganti-Kossman et al., 2002; Schmidt et al., 2005). Primary injuries consist of damage induced by the mechanical forces applied to the brain at the time of impact (Graham et al., 2000; Marshall, 2000). Secondary injuries result from processes that are initiated by the primary insult, such as activation of neuroinflammatory processes that can induce secondary damage by apoptosis, free radical formation, and lipid peroxidation (Bao et al., 2004a, 2004b; Farkas et al., 1998; Hall, 1995; Juurlink and Paterson, 1998; Morganti-Kossman et al., 2001; Schmidt et al., 2005; Taoka and Okajima, 1998). The neuroinflammatory response is characterized by the activation of microglia and astrocytes, the release of pro-inflammatory cytokines and chemokines, and the migration of leukocytes (e.g. neutrophils and macrophages) across the blood-brain barrier (Ghirnikar et al., 1998;

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[3] A version of this chapter is currently being prepared for publication. Shultz, S.R., Bao, F., Hepburn, J.D., Omana, V., Ernst, E., Brown, A., and Cain, D.P.

Holmin et al., 1998; Kadhim et al., 2009; Laird et al., 2008; Morganti-Kossman et al., 2007). These infiltrating leukocytes can further exacerbate the neuroinflammatory response and worsen secondary damage through the production of pro-inflammatory mediators and free radicals (Juurlink and Paterson, 1998; Schoettle et al., 1990; Taoka and Okajima, 1998; Zhuang et al., 1993).

The infiltration of leukocytes into the central nervous system is mediated by CD11/CD18 integrins, a family of membrane-bound glycoproteins. The CD11/CD18 heterodimer is composed of a common CD18 (β) subunit and one of four CD11 subunits (a-d). The CD11d/CD18 integrin is expressed on monocytes/macrophages and neutrophils and binds to adhesion molecules in both rats (vascular adhesion molecule-1) and humans (vascular adhesion molecule-1 and intercellular adhesion molecule-1; Bevilacqua, 1993; Hogg and Leitinger, 2001). Previous work has used anti-CD11d integrin antibody to block the CD11d/CD18 integrin and vascular adhesion molecule-1 interaction following experimental spinal cord injury in rats (Bao et al., 2004a, 2004b; Bao et al., 2005; Gris et al., 2004). These studies have found that anti-CD11d antibody treatment beginning two hours after spinal cord injury improves functional recovery while reducing the number of neutrophils and macrophages, the formation of reactive free radicals, and lipid peroxidation (Bao et al., 2004a, 2004b; Gris et al., 2004; Saville et al., 2004). As many of these mechanisms also contribute to secondary injury following traumatic brain injury, it is possible that anti-CD11d antibody treatment may also be utilized as a traumatic brain injury therapy. Indeed, an initial study examining anti-CD11d antibody treatment following experimental traumatic brain injury in the rat found that it reduced contusion volume and macrophage infiltration (Utagawa et al., 2008).

Lateral fluid percussion injury in the rat is a well-characterized rat model of traumatic brain injury for the preclinical evaluation of pharmacological therapies (Bullock et al., 1999; Laurer et al., 2000; Thompson et al, 2005). To administer lateral fluid percussion injury, rats first undergo a craniotomy to expose the intact dural surface. Lateral fluid percussion injury is produced by the rapid impact of a fluid pulse to the dural surface generated by a fluid percussion device consisting of an adjustable hammer pendulum that when released strikes the piston end of a fluid-filled cylinder (Thompson et al., 2005). Lateral fluid percussion injury is capable of inducing physiological, pathological, and behavioral changes comparable to those seen in humans who have experienced moderate to severe traumatic brain injury (Laurer et al., 2000; Thompson et al., 2005). These changes include increased intracranial pressure and decreased cerebral blood flow (Muir et al., 1992; Prins et al., 1996), diffuse axonal injury, neuronal loss, neuroinflammation (Hallam et al., 2004; Pierce et al., 1998, see Chapter 2), and neuromotor, emotional, and cognitive deficits (Jones et al., 2008; Pierce et al., 1998; see Chapter 2).

To evaluate the therapeutic potential of anti-CD11d antibody treatment following traumatic brain injury, the current study investigated the effects of anti-CD11d antibody treatment following a 2.5 - 3.0 atm lateral fluid percussion injury in the rat. To optimize the potential benefits of anti-CD11d antibody treatment, a moderate lateral fluid percussion injury force was chosen for the current study in order to induce more severe behavioral and pathological changes than those produced by mild lateral fluid percussion injury (see Chapter 2). As traumatic brain injury is associated with acute and chronic changes, both short and long recovery periods were used. Following recovery, rats were

tested on behavioral tasks of spatial cognition, anxiety-like behavior, and sensorimotor ability. Post-mortem pathological analysis of rat brains examined neuroinflammation.

4.2. Materials and Methods

4.2.1. Subjects

Subjects were 114 young adult male Long-Evans hooded rats obtained from Charles River Laboratories (Quebec, Canada). Prior to surgery rats weighed between 250-300 g, were housed in pairs in standard acrylic cages (26 cm x 48 cm x 21 cm) at 21 ±1.0 °C, and were naïve to all experimental procedures. After surgery rats were housed individually for the remainder of the study under a 12:12 light/dark cycle, with lights on from 7:00 to 19:00 hrs. Animals were allowed access to food and water *ad libitum*. Behavioral test procedures were in accordance with guidelines of the Canadian Council on Animal Care and approved by the University of Western Ontario Animal Use Subcommittee.

4.2.2. Treatment groups

Rats were randomly assigned to one of three treatment conditions: treatment with anti-CD11d integrin monoclonal antibody (aCD11d), treatment with isotype-matched 1B7 control monoclonal antibody (1B7), or sham injury with PBS injection (SHAM). Rats received their assigned anti-CD11d antibody or 1B7 control antibody treatment two hrs post-injury via tail vein injection (1.0 mg/kg in PBS). SHAM rats received an equivalent volume of PBS. After treatment, groups were randomly subdivided to begin behavioral testing after either a short recovery time of 24 hrs (SR groups) or a long recovery time of four weeks (LR groups). To provide brain tissue for the temporally relevant histological analysis of neuroinflammation after injury (Carlos et al., 1997; Clark

et al., 1996; Donnelly and Popovich, 2008; Utagawa et al., 2008), approximately half of the SR rats were perfused 24 hrs post-injury after the completion of elevated-plus maze testing. All other rats were perfused immediately after the completion of behavioral testing approximately 72 hrs or 30 days post-injury. Four rats died as a result of lateral fluid percussion injury. One rat was removed from the study prior to the start of behavioral testing due to the loss of its injury cap. There were a total of six experimental groups, with final group sizes as follows: aCD11d-SR, n=25; aCD11d-LR, n=14; 1B7-SR, n=24; 1B7-LR, n=14; SHAM-SR, n=24; SHAM-LR, n=14.

4.2.3. Surgery and injury

Rats were placed in a sealed Plexiglas box into which 4% isoflurane and 2 L/min oxygen flow was introduced for anesthesia. Rats were then placed in a standard stereotaxic device equipped with a gas anesthesia nose cover to maintain anesthesia throughout surgery with 2% isoflurane and 500 ml/min oxygen flow. Under aseptic conditions rats underwent a craniotomy surgery. All craniotomies were circular windows (3 mm diameter) centered over the following coordinates with reference to Bregma: anterior/posterior -3.0 mm; medial/lateral 6.0 mm (Paxinos and Watson, 1986). A hollow plastic injury cap was sealed over the craniotomy with silicone adhesive and cyanoacrylate. Three small stainless steel screws were inserted into the skull surrounding the injury cap to provide anchors for dental acrylic, which attached the injury cap to the skull. After the dental acrylic hardened the scalp was sutured, topical antibiotic ointment was applied, the injury cap was filled with sterile saline and the rat was attached to the fluid percussion device. At the first response of hind-limb withdrawal to a toe pinch, rats in the aCD11d or 1B7 groups received a single fluid percussion pulse of 2.5-3.0 atm. This

fluid percussion force was chosen based on force values used in previous rodent studies that induced short- and long-term behavioral impairments with associated pathologies modeling moderate closed head injury in humans (Passineau et al., 2001; Pillay et al., 2007; Shojo and Kibayashi, 2006; Thompson et al., 2005). SHAM rats were treated similarly but were removed from the fluid percussion device without receiving lateral fluid percussion injury. After injury a plug was inserted into the injury cap to seal the craniotomy.

Apnea, time of unconsciousness, and self-righting reflex were monitored immediately following injury (Griesbach et al. 2004; Griesbach et al., 2009). Apnea times were determined as the time from injury to the return of spontaneous breathing. Time of unconsciousness was determined by the return of hind-limb withdrawal in response to toe pinch. Self-righting was determined as the time from injury to return to an upright position from lying on the side. After these acute injury measures were completed all rats received a subcutaneous injection of analgesic (Ketoprofin, 5 mg/kg). Two hrs post-injury, rats received a tail injection of their assigned treatment or saline. Behavioral testing began after each group's respective recovery time.

4.2.4. Behavioral test apparatus

Anxiety-like behavior was assessed using an elevated-plus maze consisting of two arms intersecting at a 90° angle, thereby creating four individual arms each 55 cm long and 12 cm wide. The two opposing closed arms were shielded by 46 cm high walls; the two opposing open arms contained no walls. The maze was placed 50 cm above the floor. An overhead video camera recorded all trials. A person blinded to group membership later scored the video and recorded the number of entries into and amount of time spent

on each arm.

Spatial cognition was assessed using a circular water maze (1.5 m in diameter, 45 cm deep) filled with tap water at 29 ±1.0 °C. A clear Plexiglas escape platform (9cm X 9cm) was hidden approximately 2 cm below the water surface in the center of the south-east quadrant of the pool during acquisition, and in the center of the north-west quadrant during reversal. Polypropylene beads floating on the water prevented the rats from seeing the hidden platform (Cain et al., 1993). Doors, cabinets, and posters on the walls provided a variety of distal visual cues. Behavior was recorded by a video camera mounted to the ceiling above the centre of the pool. The camera was connected to a computer and behavior was objectively analyzed by a tracking system that digitized each swim trial (*Poly-Track*, *San Diego Instruments*, San Diego, CA).

Sensorimotor ability was evaluated using a narrow wooden beam, which was 1 m long and was rigidly suspended at each end 1 m above the floor, with soft padding on the floor underneath in case a rat fell off the beam (Kolb and Whishaw, 1985). One edge of the beam was 4 cm wide and was placed facing up for initial acclimation to the task. The other edge was 2 cm wide and was placed facing up during the actual beam task. The lights in the testing room were turned off and a halogen lamp was placed at the start end to illuminate the beam and provide incentive for the rats to walk along the beam, which led to a dark platform at the far end of the beam as a goal. These conditions provide ample incentive for rats to traverse the beam (Beiko et al., 1997; Kolb and Whishaw, 1985; Shultz et al., 2009). Behavior was recorded by a video camera mounted beside the beam. Experience with the water maze does not affect performance on the beam task (Beiko et al., 1997).

4.2.5. Experimental procedure: day 1

Rats were placed in the center of the elevated-plus maze facing an open arm and allowed to explore the maze freely for 5 min. As previously noted, approximately half of the SR rats were perfused 24 hrs post-injury, after the completion of elevated-plus maze testing.

Water maze acquisition training began shortly after elevated-plus maze testing and consisted of 10 training trials. A trial began by gently placing the rat in the pool adjacent to, and facing, the pool wall, and ended when the rat stood on the hidden platform. Each trial began at one of four pool wall start locations (North, South, East, or West) according to a pseudo-random schedule of start locations that prevented repeated sequential starts from the same location. For graphic presentation of search time data in Results, the time to reach the platform was averaged for every block of two trials (e.g. Block 1= (Trial 1 + Trial 2)/2). Rats that failed to reach the hidden platform within 60 sec of the start of the trial were placed on the platform by the experimenter. Rats remained on the platform for 15 sec before they were placed in a drying chamber that was heated from above by an infrared lamp. Rats were run in squads of 4-6 so that the inter-trial interval for the 10 acquisition trials was not more than 6 min.

Beam training began shortly after water maze acquisition. For acclimation to the beam task, rats were given a training session consisting of 5 trials to traverse the beam with the 4 cm edge facing up, and a further 5 trials with the 2 cm edge of the beam facing up.

4.2.6. Experimental procedure: day 2

The beam test session began approximately 24 hrs after the beam training session and consisted of 10 trials. A trial was begun by placing a rat on the illuminated end of the beam and ended when the animal successfully reached the dark goal platform. A maximum of 60 sec was allowed for each trial. Rats were tested in squads of 5 so that the inter-trial interval for the 10 trials was not more than 5 min.

Soon after beam testing, rats underwent a second water maze session for reversal training. The procedures for the reversal session were identical to acquisition except that the hidden platform was located in the opposite quadrant of the pool relative to the location during acquisition.

4.2.7. Behavioral analyses

For the elevated-plus maze, time spent in the open and closed arms of the maze was used to evaluate anxiety-like behavior in rats. All four of the rat's paws had to enter an arm for it to be considered an entry (Walf and Frye, 2007). As time spent in the open arm is decreased in rats that exhibit greater anxiety-related behaviors, a percentage score was calculated for the time spent in the open arm, as follows: time in the open arm/[time in the open arm + time in the closed arm] (Saucier et al., 2008; Steimer and Driscoll 2003; Zhu et al. 2006). The number of entries into the closed arm of the maze was also calculated as a measure of locomotion.

For the water maze, search time and direct and circle swims were used as measures of spatial place memory (Morris, 1989; Whishaw and Jarrard, 1995). Search time was defined as the time in sec from release until the rat climbed onto the hidden platform. A maximum of 60 sec was allowed for each trial. Direct and circle swims were measured because they represent efficient swim paths that are normally generated by

control rats swimming to a fixed visible or hidden platform (Beiko et al., 2004; Cain et al., 1996; Cain and Boon, 2003; Cain et al., 2006). This measure has the advantage of providing data from each trial, and is not confounded by changes in swim speed. A direct swim was defined as a swim that remained entirely within an 18 cm wide virtual alley from the start point to the hidden platform without crossing over itself. A circle swim was defined as a swim that approximated an arc of a circle without exceeding 360° or crossing over itself (Beiko et al., 2004; Cain and Boon, 2003; Cain et al., 2006; Whishaw and Jarrard, 1995). Direct and circle swims were summed and calculated as a percentage of the total swims for each test session. Swim speed was used as a measure of motor ability and was objectively calculated in cm/sec by the *Poly-track* system.

For beam task analysis, traverse time and the number of slips and falls were used as measures of sensorimotor function. Traverse time was defined as the time required to traverse the beam, with a maximum allowed time of 60 sec. Slips and falls were scored when one or more paws slipped off the beam or when a rat fell completely off the beam. Rats that fell off the beam were given a maximum time of 60 sec.

4.2.8. Brain tissue preparation and immunohistochemical procedures

For neuropathological examination at 24 hrs, 72 hrs, and 4 weeks after injury, animals were anesthetized (2.5 g/kg urethane) and perfused transcardially with PBS, followed by 4% paraformaldehyde in PBS (pH 7.2–7.4). Brains were removed, post-fixed for 24 hrs at 4°C, cryoprotected in increasing concentrations of sucrose, and sectioned into 35 μm cross-sections for immunohistochemical staining.

The brains from six rats per treatment group were randomly selected for immunohistochemical analysis. Monoclonal mouse anti-ED1 antibody (1:500, Serotec,

Raleigh, NC) and polyclonal rabbit anti-neutrophil antibody (1:20000, gift of Dr. Daniel Anthony, Oxford University, Oxford, UK) were used for immunohistochemical staining. Neutrophil staining was carried out in brains obtained 24 hrs after injury. ED1 staining was carried out in brains obtained 72 hrs and 4 weeks after injury. Randomly selected, representative cross-sections of the injured area (approximately 3 mm posterior to Bregma) from each animal were processed free-floating for staining as described previously (Weaver et al., 2001). Immunoreactivity was revealed with a glucose-diaminobenzidine-nickel solution. The stained sections were rinsed in PBS, mounted on slides, dehydrated through a gradient of ethanol, cleared, and coverslipped with DPX mountant. PBS was substituted for the primary antibody on control sections in each reaction.

Western blotting analysis of NeuN antibody was used to assess gross neuronal loss after lateral fluid percussion injury (see Appendix A).

4.2.9. Immunohistochemical quantification

An experimenter blinded to the treatment groups completed the neutrophil and ED1 immunoreactivity analyses to examine the number of activated inflammatory cells at the site of injury. For each rat, photomicrograph images at 20X magnification were obtained from the coronal cross-section closest to the level of injury (approximately 3 mm posterior to Bregma) using an Olympus BX50 microscope and ImagePro Plus software. Each photo was placed in the same orientation, with the longitudinal fissure oriented horizontally and to the right. To indicate the epicenter of the injury, one line was drawn from the longitudinal fissure to the third ventricle. A second line was drawn at a 45˚ angle from the third ventricle to the surface of the cortex. This angle was pre-

determined by an experimenter that averaged the angles to locate the epicenter of the injured cortex in rats given lateral fluid percussion injury. A circle with an area of 0.2 mm^2 was placed along the 45˚ line, just below the surface of the cerebral cortex. In the event that the greatest immunoreactivity did not appear on the line set at a 45˚ angle, a kidney bean shaped object with an area of 1 mm^2 was centered on the line. This allowed for the area with the greatest immunoreactivity to be used for analysis if the epicenter of the injury had shifted from the standard 45˚ angle. The circle (0.2mm^2 area) was then placed over the region with the greatest immunoreactivity within these set boundaries and used for quantification. A color detection function was used to identify and automatically count the total number of positively-stained cells. The color threshold was adjusted to detect inflammatory cells while excluding the background.

4.2.10. Statistical analyses

Water maze search time and beam traverse time were analyzed by SPSS 17.0 using repeated measures ANOVA with treatment as the between-subjects factor and trial as the within-subjects factor. One-way ANOVAs, with treatment as the between-subjects factor, were used to analyze acute injury measures, elevated-plus maze measures, direct and circle swims, swim speed, slips and falls, and immunohistochemical markers. Fisher's LSD post hoc pair-wise comparisons were carried out when appropriate. Statistical significance was set at $p < .05$.

4.3. Results

4.3.1. Acute injury measures

Apnea time, unconsciousness time, and self-righting reflex time were used as acute measures of injury severity. As shown in Table 4.1, aCD11d- and 1B7-treated rats

displayed significantly longer apnea ($F(2, 112) = 58.733$, $p < .001$; both aCD11d and 1B7 > SHAM, all $ps < .001$), unconsciousness ($F(2, 112)=29.988$, $p < .001$; both aCD11d and 1B7 > SHAM, all $ps < .001$), and self-righting reflex times ($F(2, 112)=75.120$, $p < .001$; both aCD11d and 1B7 > SHAM, all $ps < .001$) compared to the SHAM group.

TREATMENT GROUP	***ACUTE INJURY MEASURES***		
	Apnea (sec)	**Unconsciousness (sec)**	**Self-Righting (sec)**
aCD11d	7.94 ± .84 *	98.05 ± 12.54 *	685.77 ± 202.03 *
1B7	7.64 ± .54 *	98.24 ± 12.45 *	694.84 ± 183.27 *
Sham-Control	0 ± 0	0 ± 0	272.84 ± 17.64

Table 4.1. Acute injury measures. The aCD11d and 1B7 groups displayed significantly longer apnea, unconsciousness, and self-righting reflex times than the SHAM group. * = different from SHAM (all *ps* < .001). For additional statistical detail see Results.

4.3.2. Elevated-plus maze

Time spent in open arm of the elevated-plus maze was used to measure anxiety-like behavior. The number of closed arm entries was used as a measure of locomotion. There were no significant SR group differences in time spent in the open arm or number of closed arm entries (all *ps* > .05; see Fig. 4.1A-B).

During LR elevated-plus maze testing the aCD11d-LR and SHAM-LR groups spent more time in the open arms compared to the 1B7-LR group, as indicated by a significant *treatment* effect ($F(2, 41) = 3.925$, $p < .05$; both aCD11d-LR and SHAM-LR > 1B7-LR, all *ps* < .05; see Fig. 4.1C). There was no significant difference between groups in the number of closed arm entries ($p > .05$; see Fig. 4.1D).

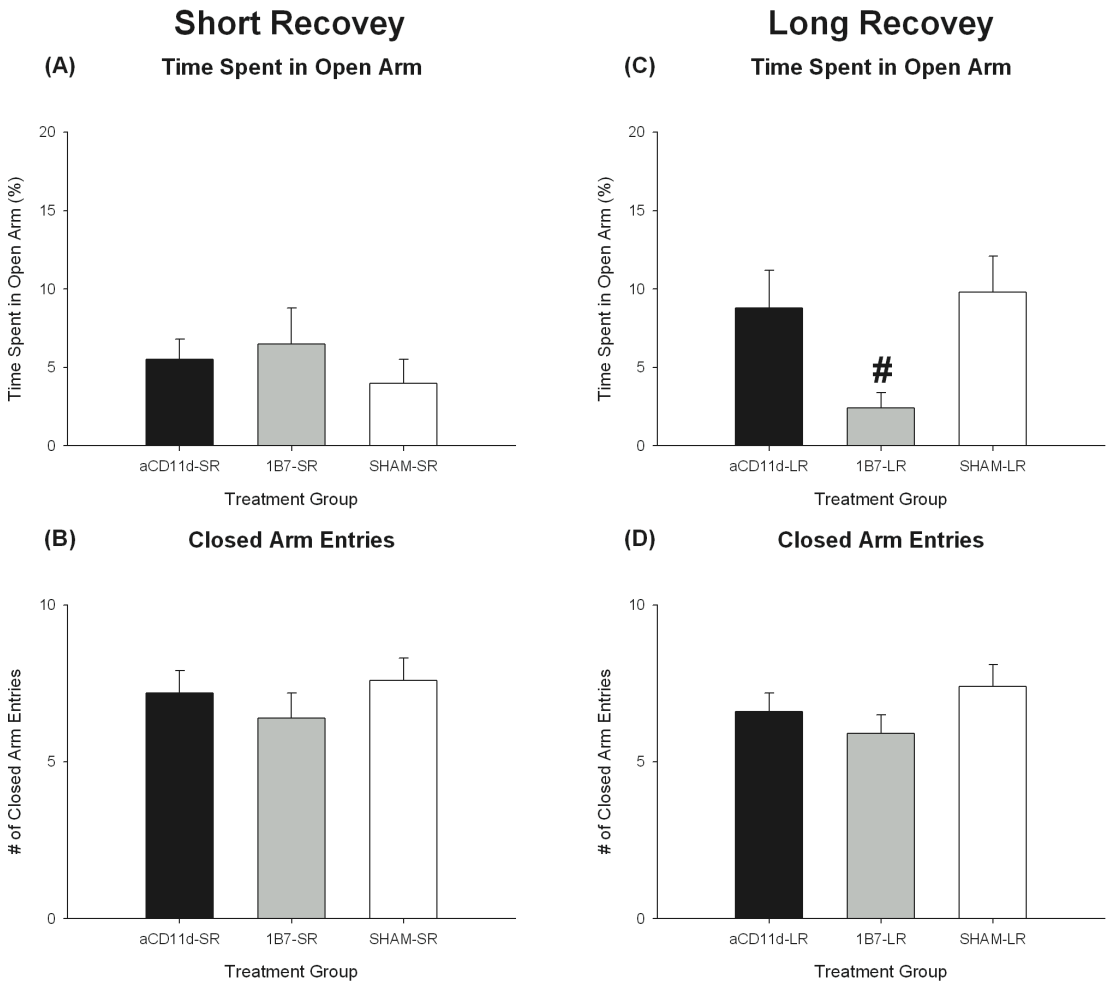

Figure 4.1. Short recovery and long recovery elevated-plus maze results. There were no significant SR group differences on the measures of percent of time in the open arm (A) or closed arm entries (B). (C) The 1B7-LR group spent significantly less time in the open arms than both the aCD11d-LR and SHAM-LR group, suggesting increased anxiety in the 1B7-LR group. (D) There were no significant group differences on the measure of closed arm entries. Histogram bars represent group means and error bars represent ± SEM. # = different from all other groups (all *ps* < .05). For additional statistical detail see Results.

4.3.3. Water maze: short recovery

Search time and direct and circle swims were used as measures of cognitive ability in the water maze. Swim speed was included as a measure of motor ability. During SR acquisition search time decreased as testing progressed, as indicated by a significant effect of *trial* ($F(9, 306) = 5.473, p < .001$; see Fig. 4.2A). Search time decreased less in the 1B7-SR and aCD11d-SR groups than in the SHAM-SR group, as indicated by a significant effect of *treatment* ($F(2, 34) = 7.655, p < .01$; both 1B7-SR and aCD11d-SR > SHAM-SR, all $ps < .05$).

The direct and circle swim data were consistent with the search time data in revealing fewer direct and circle swims in the 1B7-SR group during acquisition compared to SHAM-SR rats, as indicated by a significant effect of *treatment* ($F(2, 36) = 5.616, p < .01$; 1B7-SR < SHAM-SR, $p < .01$; see Fig. 4.2B).

During SR reversal training search time decreased in all groups as testing progressed, as indicated by a significant effect of *trial* ($F(9, 306) = 4.330, p < .001$; see Fig. 4.2C). Search times decreased less in the 1B7-SR group than in the aCD11d-SR and SHAM-SR groups, as indicated by a significant effect of *treatment* ($F(2, 34) = 7.912, p < .001$; 1B7-SR > both aCD11d-SR and SHAM-SR (all $ps < .01$).

The direct and circle swim data were consistent with the search time data in revealing fewer direct and circle swims in the 1B7-SR group during reversal compared to the aCD11d-SR and SHAM-SR groups, as indicated by a significant effect of *treatment* ($F(2, 36) = 4.223, p < .05$; 1B7-SR < both aCD11d-SR and SHAM-SR (all $ps < .05$; see Fig. 4.2D).

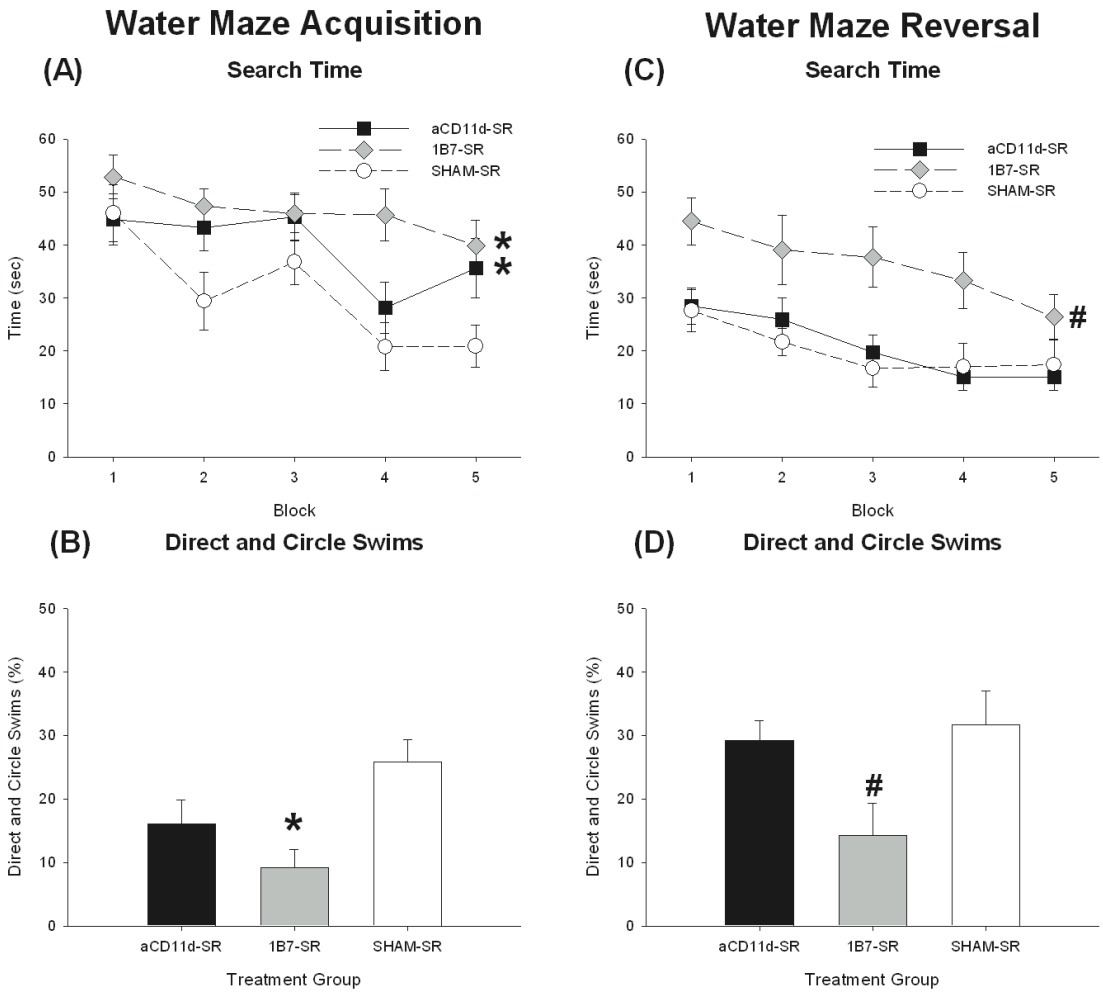

Figure 4.2. Short recovery water maze results. (A) The aCD11d-SR and 1B7-SR groups displayed significantly longer search times than the SHAM-SR group during acquisition training. (B) The 1B7-SR group displayed significantly fewer direct and circle swims during acquisition training than the SHAM-SR group. (C) The 1B7-SR group displayed significantly longer search time than both the aCD11d-SR and the SHAM-SR group during reversal training. (D) The 1B7-SR group displayed significantly fewer direct and circle swims than both the aCD11d-SR group and the SHAM-SR group during reversal training. In panels (A) and (C) data points represent group means of mean search time for each block of two trials (± SEM). In panels (B) and (D) histogram bars represent

means of data collected during the 10 water maze trials (± SEM). # = different from all other groups (all *ps* < .05 or better). * = different from SHAM-SR (all *ps* < .05 or better). For additional statistical detail see Results.

4.3.4. Water maze: long recovery

During LR acquisition the 1B7-LR rats displayed fewer direct and circle swims compared to SHAM-LR rats, as indicated by a significant *treatment* effect ($F(2, 41) = 4.346$, $p < .05$; 1B7-LR < SHAM-LR, $p < .01$; see Fig. 4.3B). There were no significant group differences in search time (see Fig. 4.3A) or swim speed (data not shown) during LR acquisition training (all *ps* > .05). During reversal training there were no significant LR group differences in search time (see Fig. 4.3C), direct and circle swims (see Fig. 4.3D), or swim speed (data not shown; all *ps* > .05).

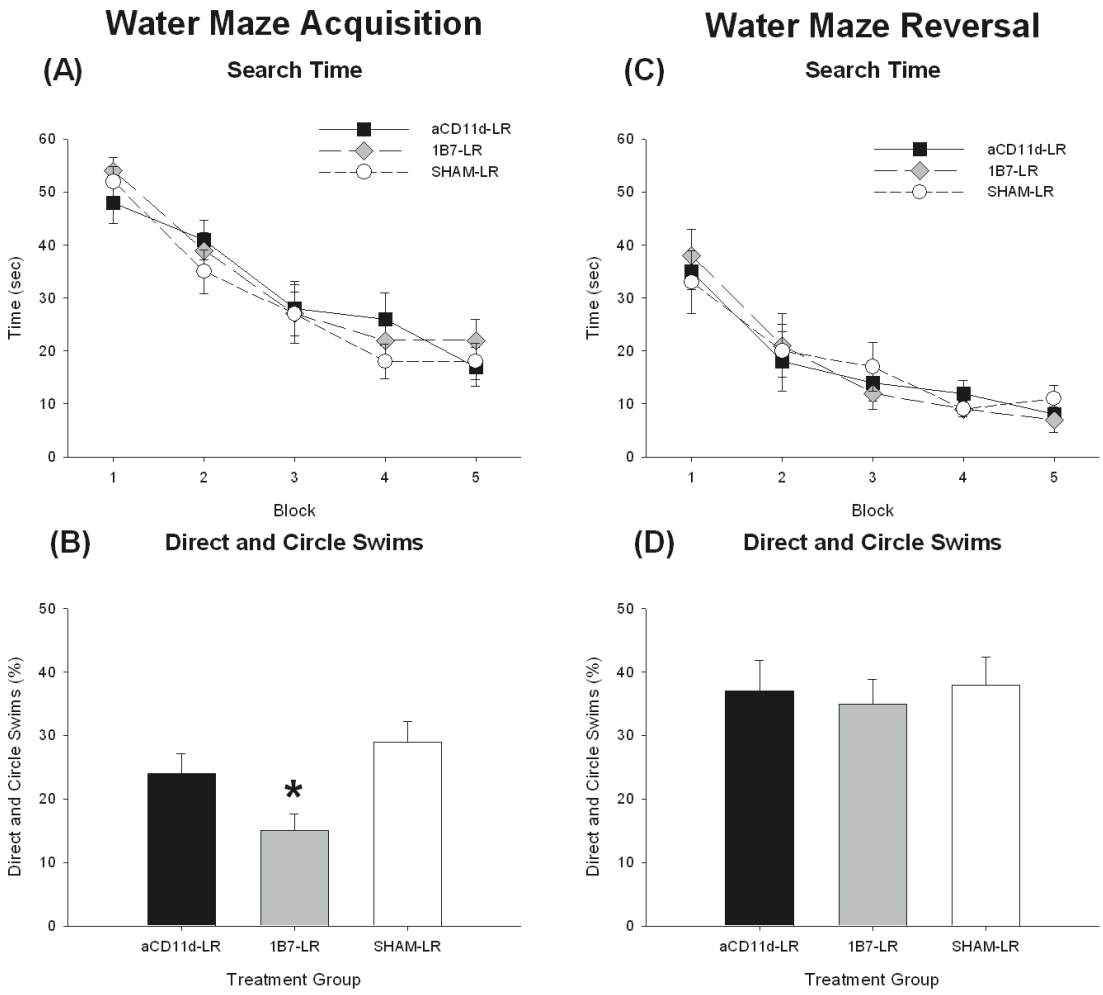

Figure 4.3. Long recovery water maze results. (A) There were no significant group differences on search time during acquisition training. (B) The 1B7-LR group displayed significantly fewer direct and circle swims during acquisition training than the SHAM-LR group. There were no significant group differences on the measures of search time (C) or direct and circle swims (D) during reversal training. In panels (A) and (C) data points represent group means of mean search time for each block of two trials (± SEM). In panels (B) and (D) histogram bars represent means of data collected during the 10 water maze trials (± SEM). * = different from SHAM-LR ($p < .01$). For additional statistical detail see Results.

4.3.5. Beam task

Traverse time and the number of slips and falls on the beam task were used as measures of sensorimotor ability. During SR beam testing the 1B7-SR group displayed more slips and falls than the SHAM-SR group, as indicated by a significant effect of *treatment* ($F(2, 36) = 3.748$, $p < .05$; 1B7-SR > SHAM-SR, $p < .01$; see Fig. 4.4A). There were no significant group differences in beam traverse times (all *ps* > .05; data not shown).

During LR beam testing the 1B7-LR group displayed more slips and falls than both the aCD11d-LR and SHAM-LR groups, as indicated by a significant *treatment* effect ($F(2, 41) = 4.526$, $p < .05$; 1B7-LR > both aCD11d-LR and SHAM-LR, all *ps* < .05; see Fig. 4.4B). There were no significant group differences in beam traverse times (all *ps* > .05; data not shown).

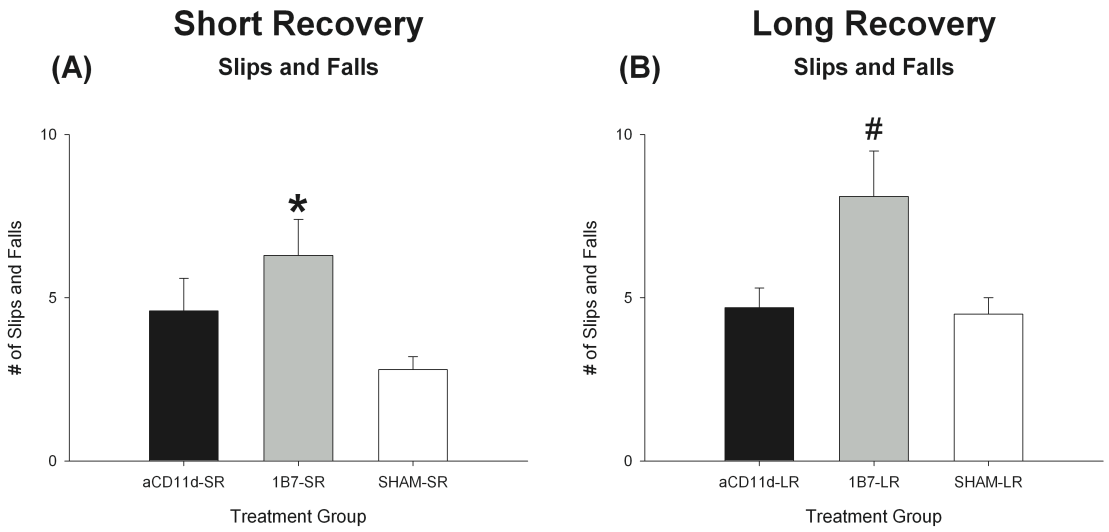

Figure 4.4. Short recovery and long recovery beam task results. (A) The 1B7-SR group displayed significantly more slips and falls during beam testing than the SHAM-SR group. (B) The 1B7-LR group displayed significantly more slips and falls during beam testing than both the aCD11d-LR and SHAM-LR groups. Histogram bars represent means of data collected during the 10 beam task trials (± SEM). # = different from all other groups (all *ps* < .05). * = different from SHAM-LR ($p < .01$). For additional statistical detail see Results.

4.3.6. Immunohistochemistry

The number of neutrophils and ED1-labeled microglia/macrophages in the injured cortex were used to assess neuroinflammation. At 24 hrs post-injury the 1B7-SR rats displayed more neutrophils than both the aCD11d-SR and SHAM-SR groups, as indicated by a significant *treatment* effect ($F(2, 11) = 8.602$, $p < .01$; 1B7-SR > both aCD11d-SR and SHAM-SR, all $ps < .05$; see Fig. 4.5).

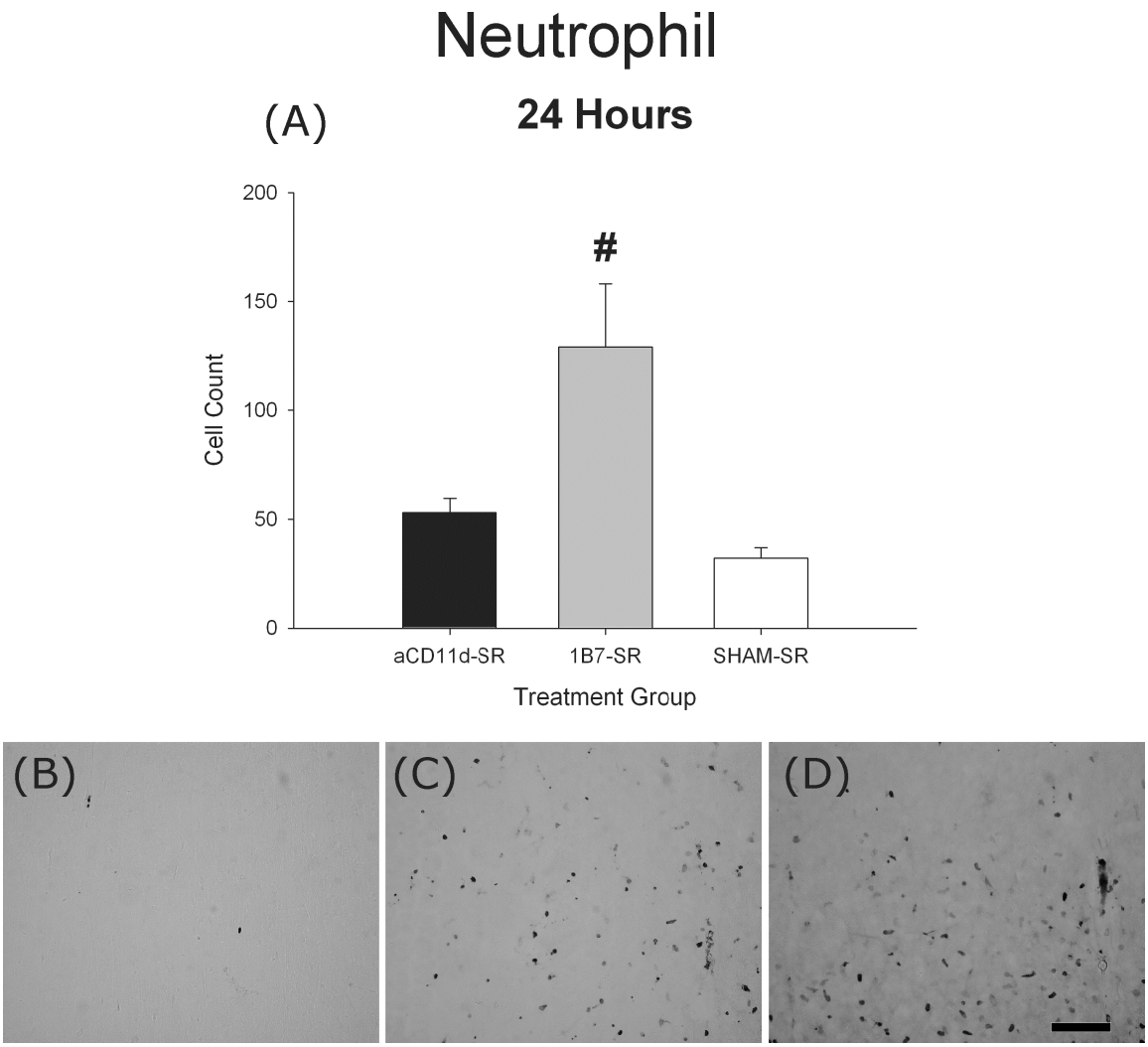

Figure 4.5. 24 hour neutrophil results. (A) The 1B7-SR group displayed significantly more neutrophils in the injured cortex than both the aCD11d-SR and SHAM-SR groups. Histogram bars represent mean neutrophils counted in the injured cortex (± SEM). # = different from the aCD11d-SR and SHAM-SR groups, *ps* < .05. (B-D) Representative photomicrographs showing neutrophil immunoreactivity in the injured cortex from the SHAM-SR (B), aCD11d-SR (C), and 1B7-SR (D) groups at 20X magnification. Scale bar = 100 μm. For additional statistical detail see Results.

At 72 hrs post-injury the 1B7-SR rats displayed more ED1-labeled activated microglia/macrophages than the aCD11d-SR and SHAM-SR groups, as indicated by a significant *treatment* effect ($F(2, 11) = 61.337$, $p < .001$; 1B7-SR > both aCD11d-SR and SHAM-SR, all $ps < .05$; see Fig. 4.6). The aCD11d-SR group displayed more ED1-labeled activated microglia/macrophages than the SHAM-SR group ($p < .05$; see Fig. 4.6).

At 4 weeks post-injury the 1B7-LR rats displayed more ED1-labeled activated microglia/macrophages than the SHAM-LR group, as indicated by a significant *treatment* effect ($F(2, 11) = 7.039$, $p < .01$; 1B7-LR > SHAM-LR, $p < .01$; see Fig. 4.6).

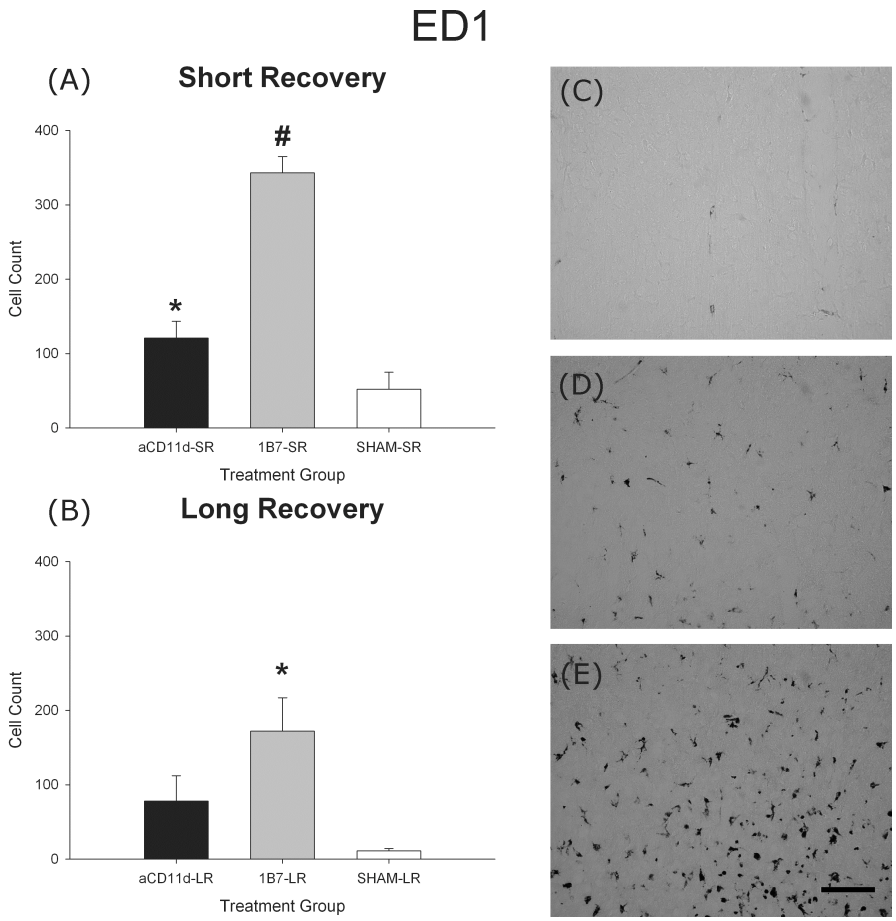

Figure 4.6. Short recovery and long recovery ED1 results. (A) After 72 hrs the 1B7-SR rats displayed significantly more ED1-labeled activated microglia/macrophages in the injured cortex than both the aCD11d-SR and SHAM-SR groups, and the aCD11d-SR group displayed more ED1-labeled activated microglia/macrophages than the SHAM-SR group. (B) After 4 weeks the 1B7-LR rats displayed significantly more ED1-labeled activated microglia/macrophages in the injured cortex than the SHAM-LR group. Histogram bars represent mean ED1 labeled cells (± SEM). # = different from the aCD11d and SHAM groups, *ps* < .05. * = different from the SHAM group, *ps* < .05. (C-E) Representative photomicrographs at 20X magnification showing ED1 immunoreactivity after short recovery from the SHAM-SR (C), aCD11d-SR (D), and 1B7-SR (E) groups. Scale bar = 100 μm. For additional statistical detail see Results.

4.4. Discussion

The findings suggest that anti-CD11d integrin antibody treatment can reduce cognitive, emotional, and motor impairments relative to treatment with 1B7 control antibody after traumatic brain injury in the Long-Evans rat. The anti-CD11d antibody treatment also appeared to decrease neuroinflammation and gross neuronal loss (see Appendix A). The aCD11d-LR and SHAM-LR groups performed similarly during elevated-plus maze testing while the 1B7-LR group displayed an increase in anxiety-like behavior, spending significantly less time in the open arm compared to the other groups. Although the aCD11d-SR group displayed some evidence of short-term cognitive impairment in the water maze, they performed significantly better than the 1B7-SR group on a number of SR water maze measures and did not differ from the SHAM-LR group on any of the LR water maze measures. The aCD11d groups also displayed no evidence of motor impairment, performing similar to SHAM rats and significantly better than 1B7-LR rats on the beam task. Neuropathological analysis revealed increased neutrophils and activated microglia/macrophages in the 1B7-SR group compared to the aCD11d-SR and SHAM-SR groups. The aCD11d-SR group also had increased activated microglia/macrophages compared to the SHAM-SR group. However, only the 1B7-LR group displayed significantly greater neuroinflammation than the SHAM-LR rats at 4 weeks post-injury. As shown in Appendix A, although 1B7 and aCD11d groups both appeared to display gross neuronal loss after lateral fluid percussion injury, NeuN expression was significantly increased in aCD11d groups relative to 1B7 groups. Taken together, these pathological findings suggest that increased peripheral leukocyte

infiltration, neuroinflammation, and gross neuronal loss may be associated with worse behavioral deficits and that anti-CD11d antibody appeared to reduce these effects.

4.4.1. Behavioral outcome

Previous studies that found decreased time spent in the open arm of the elevated-plus maze have consistently interpreted this outcome as representing an increase in anxiety-like behavior (Jones et al, 2008; Walf and Frye, 2007). The current finding that 1B7-LR rats spent less time in the open arm of the elevated-plus maze suggests that 1B7-LR rats experienced heightened levels of anxiety and that the anti-CD11d antibody treatment reduced the anxiogenic effect of lateral fluid percussion injury on this measure.

As locomotor impairments might confound elevated-plus maze results, the finding that 1B7-LR rats displayed impairments on the beam task must be considered in the interpretation of elevated-plus maze findings. Although 1B7-LR rats displayed increased slips and falls on the beam task, they were not impaired on the measures of beam traverse time, the number of closed arm entries in the elevated-plus maze, or swim speed in the water maze. Therefore, it appears that 1B7-LR rats likely experienced fine motor deficits, or possibly impairments on balance mechanisms, but not gross locomotor impairment that would confound elevated-plus maze findings.

Short-term cognitive impairments were present in lateral fluid percussion injury rats regardless of treatment, as both the aCD11d-SR and 1B7-SR groups displayed longer search times than SHAM rats during water maze acquisition. However, during water maze reversal the aCD11d-SR group performed as well as the SHAM-SR group, spending less time locating the platform and displaying more direct and circle swims than 1B7-SR rats. In addition, the aCD11d-LR rats displayed no water maze impairments after

the 4 week recovery period, whereas the 1B7-LR rats displayed cognitive deficits as indicated by significantly fewer direct and circle swims during acquisition compared to SHAM-LR rats. Taken together, these findings indicate that rats treated with anti-CD11d antibody were cognitively impaired only during water maze acquisition after short recovery whereas rats treated with 1B7 control antibody were cognitively impaired after both short and long recovery times.

The finding that 1B7 groups experienced fine motor deficits on the beam task suggests that locomotor ability may need to be considered when interpreting water maze results. However, there were no group differences on the measure of swim speed, which is a direct measure of motor ability in the water maze, and observation of the 1B7 rats during water maze testing indicated no difficulty in swimming or climbing onto the hidden platform. Thus, it appears unlikely that the fine motor impairments experienced by the 1B7 group directly accounted for the deficits observed in the water maze.

The fact that the aCD11d groups performed as well as the SHAM groups on the beam task, the 1B7 groups displayed significantly more slips and falls than the SHAM groups, and the 1B7-LR group displayed significantly more slips and falls than the aCD11d-LR group suggests that anti-CD11d antibody treatment preserved motor function following lateral fluid percussion injury. As discussed earlier, the fact that the 1B7-treated groups showed no impairments on the measures of beam traverse time, swim speed in the water maze, and closed arm entries in the elevated-plus maze suggests that their beam task impairments were a result of fine motor deficits rather than gross motor impairments (Shultz et al., 2009).

4.4.2. Pathology and its relation to behavioral impairments

In the present study rats treated with 1B7 control antibody displayed increased neuroinflammation at 24 hrs, 72 hrs, and 4 weeks after lateral fluid percussion injury while rats treated with anti-CD11d antibody either displayed significantly less neuroinflammation than 1B7-treated rats or did not significantly differ from sham-controls. Along with an increased neuroinflammatory response, 1B7-treated rats also appeared to display significantly more gross neuronal loss than the aCD11d-treated rats as indicated by NeuN expression (see Appendix A). As neuroinflammation has neurotoxic properties that contribute to secondary damage in traumatic brain injury (Dringen, 2005; Morganti-Kossman et al., 2001; Schmidt et al., 2005), it seems possible that the increased neuroinflammation in 1B7 rats contributed to this increased neuronal loss. These findings are consistent with evidence from a previous study suggesting that the infiltration of peripheral leukocytes into the brain in traumatic brain injury increases neuroinflammation and worsens brain damage, and that anti-CD11d antibody treatment can limit this process (Utagawa et al., 2008).

It seems possible that neuroinflammation and gross neuronal loss could have contributed to the behavioral impairments that were induced by lateral fluid percussion injury in the current study. Previous research from our laboratory and others has found cognitive deficits in the water maze in the presence of a neuroinflammatory response (Shultz et al., 2009; Wu et al., 2006, 2010; see Chapter 2 and 3). Other lateral fluid percussion injury studies have also found motor, emotional, and cognitive impairments that occurred in the presence of extensive damage to the hippocampus and the frontal, parietal, and temporal cortices (Jones et al., 2008; Wahl et al., 2000). Although neuronal loss in the current study was not attributed to a specific brain structure or area, it seems

possible that damage to these areas might have contributed the behavioral impairments found in the elevated-plus maze, water maze, and beam task.

Although anti-CD11d antibody appeared to significantly reduce behavioral impairments, neuroinflammation, and gross neuronal loss compared to 1B7-treated rats, rats treated with anti-CD11d antibody still performed significantly worse on some behavioral measures and displayed significantly more gross neuronal loss than sham-controls. Irreversible primary injuries may have contributed to these findings (Graham et al., 2000; Marshall, 2000). However, as aCD11d-SR rats also displayed increased microglia/macrophages compared to SHAM-SR rats it is possible that neuroinflammatory-mediated secondary injuries still occurred. As this is just the second study to use anti-CD11d antibody to treat traumatic brain injury, it is possible that the treatment regimen applied in the current study was not optimal, and for that reason anti-CD11d antibody did not reduce neuroinflammation to its full potential. Alternatively, the increased ED1 staining found in the aCD11d-treated rats might represent microglia and the brains innate neuroinflammatory response (Dringen et al., 2005; Morganti-Kossman et al., 2002). Thus, a treatment targeting the innate neuroinflammatory response may further reduce secondary damage. However, it is important to consider that neuroinflammation also has neuroprotective properties in traumatic brain injury (Ekdahl et al., 2009; Laird et al., 2008; Morganti-Kossman et al., 2007). Overall, while the current findings support the continued investigation of anti-CD11d antibody as a traumatic brain injury treatment, further research is needed to better understand the complex neuroinflammatory cascade in traumatic brain injury, it's role in functional impairments,

and how to optimally target the inflammatory process in the treatment of traumatic brain injury.

4.4.3. Conclusions

A single treatment of anti-CD11d integrin antibody administered two hours after moderate traumatic brain injury reduced cognitive, emotional, and motor impairments in the Long-Evans rat. As the reduction in impairment appeared to be associated with decreased neuroinflammation and gross neuronal loss, anti-CD11d antibody may have improved outcomes by limiting the infiltration of peripheral leukocytes and decreasing the neurotoxic effects of neuroinflammation. Together, the current findings suggest that infiltrating leukocytes increase neuroinflammation, secondary brain damage, and functional impairment following traumatic brain injury, and that the anti-CD11d antibody holds promise as a novel treatment to limit this process and improve traumatic brain injury recovery.

4.5. References

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Chapter 5

5. Treatment with anti-CD11d integrin antibody reduces cognitive, emotional, and motor impairments in an animal model of repeated concussion in the Long-Evans rat[4]

5.1. Introduction

Brain concussion can be defined as a complex pathophysiological process that affects the brain and is induced by traumatic biomechanical forces (Cassidy et al., 2004; McCrory et al., 2009). Although a single concussion often results in transient symptoms with no indication of structural brain damage (Boll and Barth, 1983; Giza and Hovda, 2001; Kushner, 1998; McCrory et al., 2009), evidence suggests that repeated concussion is associated with cumulative and chronic neurological impairments. Repeated concussion may result in long-term cognitive impairments, emotional disturbances, motor abnormalities, dementia, and the neurodegenerative disease chronic traumatic encephalopathy (Cantu, 2007; Collins et al., 2002; Guskiewicz et al., 2003; Guskiewicz et al., 2005; Guskiewicz et al., 2007; Jellinger, 2004; McKee et al., 2009). Pathological studies that have examined post-mortem brain tissue taken from chronic traumatic encephalopathy patients have reported neuronal loss, atrophy, scarring, amyloid and tau deposition, diffuse axonal injury, and neuroinflammation (McKee et al., 2009; Omalu et al., 2005; Omalu et al., 2006).

Given the debilitating effects of repeated concussion and chronic traumatic encephalopathy, there is growing concern regarding the proper medical treatment of concussion, particularly for individuals at high risk of experiencing repeated concussion

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[4] A version of this chapter is currently being prepared for publication. Shultz, S.R., Bao, F., Omana, V., Brown, A., and Cain, D.P.

such as athletes and military personnel (Cantu, 2009; Maroon et al., 2000). As repeated concussion remains poorly understood (Cantu, 2009; McKee et al., 2009), our laboratory has recently developed a rat model of repeated concussion to allow for the investigation of factors and mechanisms that might contribute to the cumulative and long-term effects of these injuries (see Chapter 3). This work has found that rats given repeated mild lateral fluid percussion injury display cumulative and long-term cognitive deficits, anxiety- and depression-like behaviors, and brain damage in the presence of a neuroinflammatory response. These findings are consistent with a number of symptoms and pathologies that can occur in repeated concussion and support the use of a repeated mild lateral fluid percussion injury model.

As a single concussion rarely results in structural brain damage (McCrory et al., 2009), it seems unlikely that the extent of brain damage found in repeated concussion and chronic traumatic encephalopathy is a simple summation of these injuries. Rather, secondary processes may be key contributors to the more severe and chronic effects of repeated concussion. The neuroinflammatory response is an important secondary process that occurs in traumatic brain injury (Graham et al., 2002; Maas et al., 2008). Although the neuroinflammatory response can have neuroprotective properties following traumatic brain injury (Morganti-Kossman et al., 2002), it can also induce secondary brain damage through a number of processes such as apoptosis, free radical formation, and lipid peroxidation (Bao et al., 2004; Bao et al., 2005; Farkas et al., 1998; Hall, 1995; Juurlink and Paterson, 1998; Morganti-Kossman et al., 2002; Schmidt et al., 2005; Taoka and Okajima, 1998). Therefore, recovery from repeated concussion might be improved by a treatment strategy that limits the secondary damage associated with neuroinflammation.

The infiltration of peripheral leukocytes into the central nervous system following injury can further increase the neuroinflammatory response and worsen secondary brain damage (Juurlink and Paterson, 1998; Schoettle et al., 1990; Taoka and Okajima, 1998; Utagawa et al., 2008; Zhuang et al., 1993). Leukocyte infiltration is mediated by the CD11d/CD18 integrin that is expressed on leukocytes and binds to adhesion molecules in both rats (vascular adhesion molecule-1) and humans (vascular adhesion molecule-1 and intercellular adhesion molecule-1; Bevilacqua, 1993; Hogg and Leitinger, 2001). Previous work from our laboratory and others has used anti-CD11d antibody to block the CD11d/CD18 and vascular adhesion molecule-1 interaction and prevent leukocyte infiltration following a single fluid percussion injury in the rat (Utagawa et al., 2008; see Chapter 4). These studies have reported that anti-CD11d antibody treatment improved cognitive, emotional, and sensorimotor recovery, while reducing contusion size and leukocyte infiltration (Utagawa et al., 2008; see Chapter 4).

Considering the benefits of anti-CD11d antibody treatment following a single brain injury and the presence of neuroinflammation after repeated concussion, it seems possible that anti-CD11d antibody may be beneficial in the treatment of repeated concussion. As three concussions have been associated with acute and long-term changes in both humans and rats (Guskiewicz et al., 2003; Guskiewicz et al., 2005; Guskiewicz et al., 2007; Macciocchi et al., 2001; see Chapter 3), the current study investigated the therapeutic potential of anti-CD11d antibody treatment in rats given three mild lateral fluid percussion injuries. Both short- and long-term recovery periods were included to more fully assess anti-CD11d antibody therapy. After recovery, rats were tested on tasks

of cognition, anxiety-like behavior, and sensorimotor ability, and brains were assessed for neuroinflammation and cortical damage.

5.2. Materials and methods

5.2.1. Subjects

Subjects were 87 young adult male Long-Evans hooded rats obtained from Charles River Laboratories (Quebec, Canada). Prior to surgery rats weighed between 250-300 g, were housed in pairs in standard acrylic cages (26 cm x 48 cm x 21 cm) at a 21 ±1.0 °C, and were naïve to all experimental procedures. After surgery rats were housed individually for the remainder of the study under a 12:12 light/dark cycle, with lights on from 7:00 to 19:00 hrs. Animals were allowed access to food and water *ad libitum*. Behavioral test procedures were in accordance with guidelines of the Canadian Council on Animal Care and approved by the University of Western Ontario Animal Use Subcommittee.

5.2.2. Treatment groups

Rats were randomly assigned to one of three groups: three mild lateral fluid percussion injuries + anti-CD11d integrin monoclonal antibody treatment (aCD11d), three mild lateral fluid percussion injuries + isotype matched control 1B7 monoclonal antibody treatment (1B7), or sham-control injury + PBS treatment (SHAM). Rats received their assigned anti-CD11d antibody or 1B7 control antibody treatment both 2 hrs and 24 hrs after each injury via tail vein injection (1.0 mg/kg in PBS). SHAM rats received an equivalent volume of PBS. There was a 5-day inter-injury recovery period between repeated mild lateral fluid percussion injuries. After the final assigned treatment, rats were randomly assigned to one of two recovery groups: short recovery (SR, 24 hrs),

or long recovery (LR, 8 weeks). After mild lateral fluid percussion injury, three rats died and three rats were removed from the study prior to the onset of behavioral testing because of the loss of the injury cap or failure to maintain normal body weight. There were a total of six experimental groups, with final group sizes as follows: aCD11d-SR (n = 15); IB7-SR (n = 15); SHAM-SR (n = 15); aCD11d-LR (n = 12); IB7-LR (n = 12); SHAM-LR (n = 12)[5].

5.2.3. Surgery and injuries

Rats were placed in a sealed Plexiglas box into which 4% isoflurane and 2 L/min oxygen flow was introduced for anesthesia. Rats were then placed in a standard stereotaxic device equipped with a gas anesthesia nose cover to maintain anesthesia throughout surgery with 2% isoflurane and 500 ml/min oxygen flow. Under aseptic conditions rats underwent a craniotomy surgery. All craniotomies were circular windows (3 mm diameter) centered over the following coordinates with reference to Bregma: anterior/posterior -3.0 mm; medial/lateral 6.0 mm (Paxinos and Watson, 1986). A hollow plastic injury cap was sealed over the craniotomy with silicone adhesive, cyanoacrylate, and dental acrylic. Three small stainless steel screws were inserted into the skull surrounding the injury cap to provide anchors for dental acrylic, which attached the injury cap to the skull. After the dental acrylic hardened the scalp was sutured, topical antibiotic ointment was applied, and a removable plug was inserted into the injury cap to seal the craniotomy until, and after, the injury was administered. Immediately post-surgery all rats received a subcutaneous injection of analgesic (Ketoprofin, 5 mg /kg).

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[5] The same group names are used in Chapters 4 and 5. In Chapter 4 rats were given a single moderate lateral fluid percussion injury with a single treatment 2 hrs post-injury. Here rats were given three mild lateral fluid percussion injuries with two treatments at 2 and 24 hrs after each injury.

Repeated mild lateral fluid percussion injury methods were based on previous work from our laboratory (see Chapter 3). Approximately 24 hrs post-surgery rats were placed in a sealed Plexiglas box into which 4% isoflurane and 2 L/min oxygen flow was introduced for anesthesia (Utagawa et al., 2008). After rats were anesthetized the plug was removed from the injury cap, and under aseptic conditions the injury cap was filled with sterile saline and connected to the fluid percussion device. At the first response of hind-limb withdrawal to a toe pinch, rats in the aCD11d and 1B7 groups received a single mild lateral fluid percussion injury with a force of 1 – 1.5 atm. This mild fluid percussion force was chosen based on previous rodent studies that have validated the use of mild lateral fluid percussion injury as a model of concussion (DeRoss et al., 2002; Griesbach et al., 2009; Li et al., 2006; Wu et al., 2010). SHAM rats were treated similarly but were removed from the injury device without receiving mild lateral fluid percussion injury. Rats received tail vein injections of their assigned treatments 2 and 24 hrs post-injury. All rats received a 5-day inter-injury recovery time before undergoing the identical injury procedure as described above for the remaining two injuries. Behavioral testing began after each group's respective recovery time.

Apnea, time of unconsciousness, and self-righting reflex were all monitored immediately following each injury (Griesbach et al., 2004; Griesbach et al., 2009). Apnea times were determined as the time from injury to the return of spontaneous breathing. Time of unconsciousness was determined by the return of hind-limb withdrawal in response to toe pinch. Self-righting was determined as the time from injury to return to an upright position.

5.2.4. Behavioral test apparatus

Anxiety-like behavior was assessed using an elevated-plus maze consisting of two arms intersecting at a 90° angle, thereby creating four individual arms each 55 cm long and 12 cm wide. Two opposing arms were enclosed by 46 cm high walls. The remaining two opposing open arms had no walls. The maze was placed 50 cm above the ground. An overhead video camera recorded all trials. Following testing, the videotape was scored and the number of entries into and amount of time spent on each arm were recorded.

Spatial cognition was assessed using a water maze consisting of a circular pool (1.5 m in diameter, 45 cm deep) filled with tap water at 29 ±1.0 °C. A clear Plexiglas escape platform (9 cm X 9 cm) was hidden approximately 2 cm below the water surface in the center of the south-east quadrant of the pool during acquisition, and in the center of the north-west quadrant during reversal. Polypropylene beads floating on top of the water prevented the rats from seeing the hidden platform (Cain et al., 1993). Doors, cabinets, and posters on the walls provided a variety of distal cues. Behavior was recorded by a video camera mounted to the ceiling above the centre of the pool. The camera was connected to a computer and behavior was objectively analyzed by a tracking system that created a digital record of each swim trial (*Poly-Track*, *San Diego Instruments*, San Diego, CA).

Sensorimotor ability was evaluated using a 1 m long narrow wooden beam that was rigidly suspended at each end 1 m above the floor, with soft padding on the floor underneath in case a rat fell off the beam (Kolb and Whishaw, 1985). One edge of the beam was 4 cm wide and was placed facing up for initial acclimation to the task. The other edge was 2 cm wide and was placed facing up during beam task data collection. The lights in the testing room were off and a halogen lamp was placed above the start end

of the beam to illuminate it and provide incentive for the rats to walk along the beam, which led to a dark goal platform at the far end of the beam. These conditions provide ample incentive for rats to traverse the beam (Beiko et al., 1997; Kolb and Whishaw, 1985; Shultz et al., 2009). Experience with the water maze does not affect performance on the beam task (Beiko et al., 1997).

5.2.5. Experimental procedure: day 1

Behavioral testing began at the end of the assigned recovery period, either 24 hrs or 8 weeks after the last treatment. Rats were placed in the center of the elevated-plus maze facing an open arm and allowed to explore the maze freely for 5 min.

Water maze acquisition training began shortly after completion of elevated-plus maze testing and consisted of 10 training trials, with each trial beginning with the rat being placed gently in the pool adjacent to, and facing, the pool wall, and ending when the rat stood on the hidden platform. Each trial began at one of the four pool wall start locations (North, South, East, or West), with start locations pseudo-randomly ordered to prevent sequential starts from the same location. As this resulted in start locations that varied in distance from the hidden platform, for graphic presentation of search time data in Results, the search time to reach the platform was averaged for each block of 2 trials (e.g. Block 1= (Trial 1 + Trial 2)/2). Rats that failed to reach the hidden platform within 60 sec of the commencement of the trial were placed on the platform by the experimenter. Rats remained on the platform for 15 sec before they were placed in a drying chamber that was heated from above by a lamp. Rats were run in squads of five so that the inter-trial intervals were not more than 6 min.

Training on the beam task took place after water maze acquisition. For acclimation to the beam task, rats were given a training session consisting of 5 trials to traverse the beam with the 4 cm edge facing up, and a further 5 trials with the 2 cm edge of the beam facing up.

5.2.6. Experimental procedure: day 2

Beam testing began approximately 24 hrs after beam task training and consisted of 10 trials. A trial began with the rat being placed on the illuminated end of the beam and ended when the animal successfully reached the dark goal platform. A maximum of 60 sec was allowed for each trial. Rats were run in squads of five so that the inter-trial intervals were not more than 5 min.

Shortly after completion of beam testing rats underwent a second water maze session for reversal training. The procedures for the reversal session were identical to acquisition except that the hidden platform was now located in the opposite quadrant of the pool relative to the location during acquisition.

5.2.7. Behavioral analyses

For the elevated-plus maze, time spent in the open arm of the maze was used to evaluate anxiety-like behavior in rats. All four of the rat's paws had to enter an arm for it to be considered an entry (Walf and Frye, 2007). As time spent in the open arm is decreased in rats that exhibit greater anxiety-related behaviors, a percentage score was calculated for the time spent in the open arm, as follows: time in the open arm/[time in the open arm + time closed arm] (Saucier et al., 2008, Steimer and Driscoll 2003). The number of entries into the closed arm of the maze was also calculated as a measure of locomotion (Walf and Frye, 2007).

For the water maze, search time and direct and circle swims were used as measures of spatial place memory (Morris, 1989; Whishaw and Jarrard, 1995). Search time was defined as the time in sec from release until the rat climbed onto the hidden platform. A maximum of 60 sec was allowed for each trial. Direct and circle swims were measured because they represent efficient swim paths that are normally generated by control rats swimming to a fixed visible or hidden platform (Beiko et al., 2004; Cain and Boon, 2003; Cain et al., 2006; Cain et al., 1996). This measure has the advantage of providing data from each trial, and is not confounded by changes in swim speed. A direct swim was defined as a swim that remained entirely within an 18 cm wide virtual alley from the start point to the hidden platform without crossing over itself. A circle swim was defined as a swim that approximated an arc of a circle without exceeding 360° or crossing over itself (Beiko et al., 2004; Cain and Boon, 2003; Cain et al., 2006; Whishaw and Jarrard, 1995). Direct and circle swims were summed and calculated as a percentage of the total swims for each test session. Swim speed was used as a measure of motor ability and was objectively calculated in cm/sec by the *Poly-track* system.

For the beam task, traverse time and the number of slips and falls were used as measures of sensorimotor function. Traverse time was defined as the time required to traverse the beam, with a maximum allowed time of 60 sec. Slips and falls were scored when one or more paws slipped off the beam or when a rat fell completely off the beam. Rats that fell off the beam were given a maximum time of 60 sec.

5.2.8. Brain tissue preparation and immunohistochemical procedures

For neuropathological examination at 72 hrs and 8 weeks after the final assigned treatment, rats were anesthetized (2.5 g/kg urethane) and perfused transcardially with

PBS, followed by 4% paraformaldehyde in PBS (pH 7.2–7.4). Brains were removed, post-fixed for 24 hrs at 4°C, cryoprotected in increasing concentrations of sucrose, and sectioned into 35 μm cross-sections for immunohistochemical staining.

Randomly selected brains from six rats per treatment group were used for immunohistochemical analysis. Serial 35 μm coronal cross-sections were obtained through the cortex at the level of injury (approximately -3.0 mm posterior to Bregma). This anatomical site was chosen because it allowed reliable quantification of possible changes induced by mild lateral fluid percussion injuries in cortex areas that have been shown to be altered in human concussion (Umile et al., 2002). Monoclonal mouse anti-ED1 antibody (1:500, Serotec, Raleigh, NC), polyclonal rabbit anti-neutrophil antibody (1:20000, gift of Dr. Daniel Anthony, Oxford University, Oxford, UK), and NeuN antibody (1:500, Chemican, Temecular, CA) were used for immunohistochemical staining of microglia/macrophages, neutrophils, and neurons. Randomly selected, representative sections of the injured area from each animal were processed free-floating for staining as described previously (Weaver et al., 2001). Immunoreactivity was revealed with a glucose-diaminobenzidine-nickel solution. The stained sections were rinsed in PBS, mounted on slides, dehydrated through a gradient of ethanol, cleared, and coverslipped with DPX mountant. PBS was substituted for the primary antibody on control sections in each reaction.

5.2.9. Immunohistochemical analyses

An experimenter blinded to the treatment groups completed the neutrophil and ED1 immunoreactivity analyses to assess the number of activated inflammatory cells at the site of injury. Using a standard light microscope, a photomicrograph image of the

injured cortex was taken at 1.25X magnification for each rat (see Fig. 3.1). These photomicrographs were captured from the coronal cross-section closest to the level of injury (approximately -3.0 mm posterior to Bregma) and placed in the same orientation, with the longitudinal fissure oriented vertically (see Fig. 3.1). Using Image Pro Plus software, one line was drawn from the most dorsal point of the longitudinal fissure to the third ventricle (see Fig. 3.1). A second line was drawn from the third ventricle at a 60˚ angle to the surface of the injured cortex. It was known that this 60˚ angle would indicate the approximate epicenter of the injury (Paxinos and Watson, 1986). In the event that the epicenter of the injury had shifted from the standard 60˚ angle, an additional 20˚ was allotted to either side of the 60˚ line and the region of greatest immunoreactivity was located within these boundaries (see Fig. 3.1). A photomicrograph image at 20X magnification was then obtained for quantification. All photomicrographs were captured under fixed microscope illumination settings and exposure times to ensure objective and consistent image quality across all pictures. To count the total number of activated neutrophils and microglia/macrophages, the Image Pro Plus color detection function was used to identify the positively-stained cells. The color threshold was adjusted to detect inflammatory cells while excluding the background.

An experimenter blinded to the injury groups also completed a semi-qualitative analysis that assessed damage in the injured cortex. A 1.25X magnification photomicrograph image of NeuN-stained injured cortex was obtained from each coronal cross-section using a standard light microscope. Each image was placed in the same orientation, with the longitudinal fissure oriented vertically, and spanned to the parietal/temporal cortex (Paxinos and Watson, 1986). A score of 1 was given for no

damage or mild (e.g. slight depression) cortical damage. A score of 2 was given for moderate cortical damage (e.g. slight cavitation/cortical loss). A score of 3 was given for severe cortical damage (e.g. obvious cavitation/cortical loss). This rating scale was adapted from previous studies (Braak and Braak, 1991; Lemstra et al., 2007; Li et al., 2006; see Chapter 3).

5.2.10. Statistical analyses

Acute injury measures were analyzed by SPSS 17.0 using mixed design ANOVA with treatment group as the between-subjects factor and injury number as the within-subjects factor. Simple effects post hoc *F*-tests were carried out when appropriate. Water maze search time and beam traverse time were analyzed using mixed design ANOVA with treatment group as the between-subjects factor and trial as the within-subjects factor. One-way ANOVAs, with treatment group as the between-subjects factor, were used to analyze percent of time in open arm, closed arm entries, direct and circle swims, swim speed, slips and falls, and immunohistochemistry quantification. Fisher's LSD post hoc pair-wise comparisons were carried out when appropriate. Statistical significance was set at $p < .05$.

5.3. Results

5.3.1. Acute injury measures

Apnea time, unconsciousness time, and self-righting reflex time were used as acute measures of injury severity. Apnea time increased with additional injuries, as indicated by a significant effect of *injury number* ($F(2, 156) = 4.273$, $p < .005$; 3 injuries > 1 injury, $p < .05$; see Fig. 5.1A). There were no significant between-group differences for apnea time ($p > .05$).

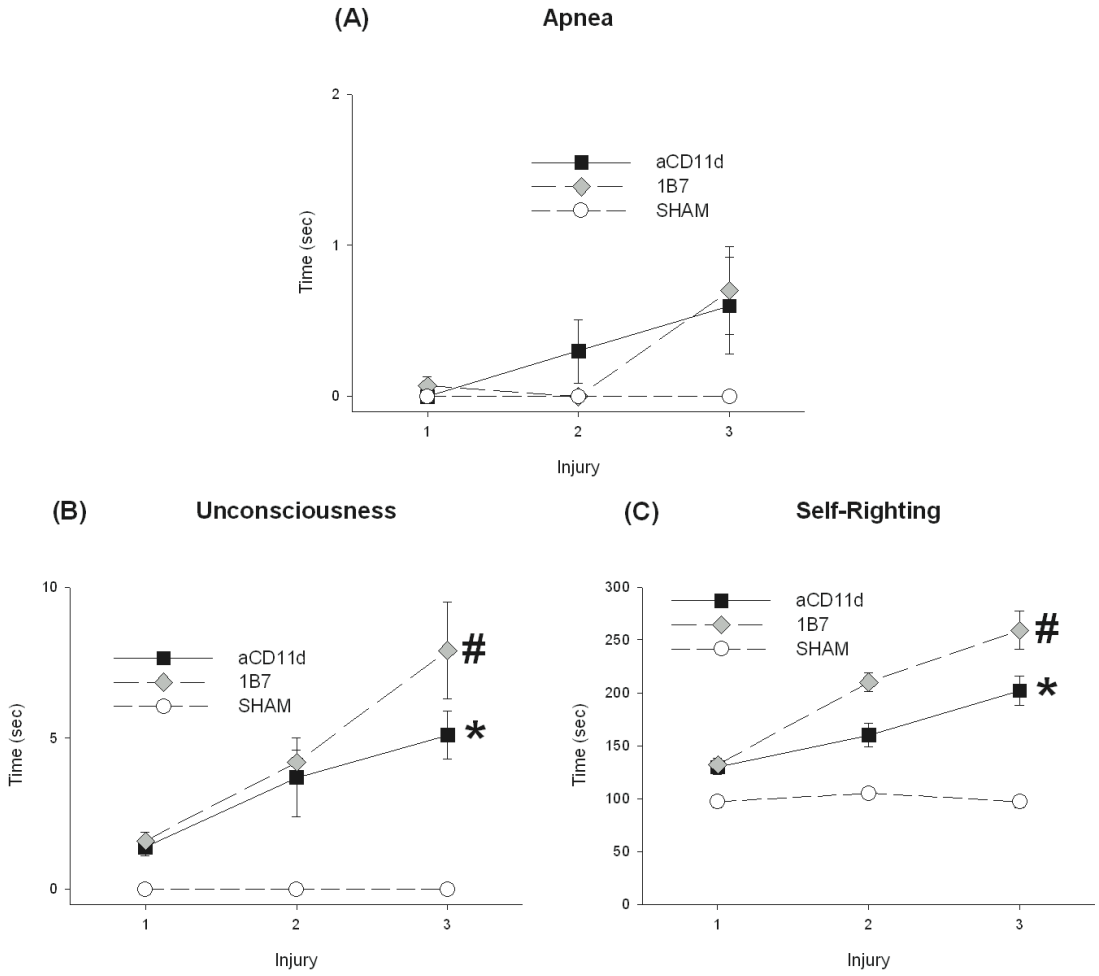

Figure 5.1. Acute injury measures. (A) Apnea time increased with additional injuries. (B) Unconsciousness time increased with additional injuries. The 1B7 and aCD11d groups displayed longer unconsciousness time than the SHAM group. The third injury in the 1B7 group induced significantly longer unconsciousness time than the third injury in the aCD11d and SHAM groups. (C) Self-righting reflex time increased with additional injuries. The 1B7 group displayed longer self-righting reflex time than the aCD11d and SHAM groups. # = different from all other groups; * = different from SHAM group; all *ps* < .05. Histogram bars represent group means (± SEM). For additional statistical detail see Results.

Unconsciousness time increased with additional injuries, as indicated by a significant effect of *injury number* ($F(2, 156) = 14.322$, $p < .001$; 3 injuries > 2 injuries > 1 injury, all *ps* < .05; see Fig 5.1B). The 1B7 and aCD11d groups displayed longer unconsciousness time than the SHAM group, as indicated by a significant effect of *treatment group* ($F(2, 78) = 30.464$, $p < .001$; aCD11d and 1B7 > SHAM, all *ps* < .01; see Fig. 5.1B). The third injury induced significantly longer unconsciousness time in the 1B7 group than the aCD11d and SHAM groups, as indicated by a *treatment group x injury number* interaction ($F(4, 156) = 4.424$, $p < .01$; 1B7 > aCD11d > SHAM, all *ps* < .05; see Fig. 5.1B).

Self-righting reflex time increased with additional injuries, as indicated by a significant effect of *injury number* ($F(2, 156) = 35.154$, $p < .001$; 3 injuries > 2 injuries > 1 injury, all *ps* < .01; see Fig. 5.1C). The 1B7 group displayed longer self-righting reflex time than the aCD11d and SHAM groups, as indicated by a significant effect of *treatment group* ($F(2, 78) = 67.114$, $p < .001$; 1B7 > aCD11d > SHAM, all *ps* < .001; see Fig. 5.1C). The second and third injuries induced significantly longer self-righting reflex time in the 1B7 group, as indicated by a *treatment group x injury number* interaction ($F(4, 156) = 4.424$, $p < .01$; 1B7 > aCD11d > SHAM, all *ps* < .05; see Fig. 5.1C).

5.3.2. Elevated-plus maze

Time spent in open arms of the elevated-plus maze was used to measure anxiety-like behavior. The number of closed arm entries was used as a measure of locomotion. During SR elevated-plus maze testing the 1B7-SR group spent less time in the open arms compared to the SHAM-SR group, as indicated by a significant *treatment* effect ($F(2, 43) = 3.283$, $p < .05$; 1B7-SR < SHAM-SR, $p < .05$; see Fig. 5.2A). There was no significant

difference between groups in the number of closed arm entries ($p > .05$; see Fig. 5.2B).

During LR elevated-plus maze testing the 1B7-LR group spent less time in the open arms compared to the SHAM-LR group, as indicated by a significant *treatment* effect ($F(2, 35) = 3.459, p < .05$; 1B7-LR < SHAM-LR, $p < .05$; see Fig. 5.2C). There was no significant difference between groups in the number of closed arm entries ($p > .05$; see Fig. 5.2D).

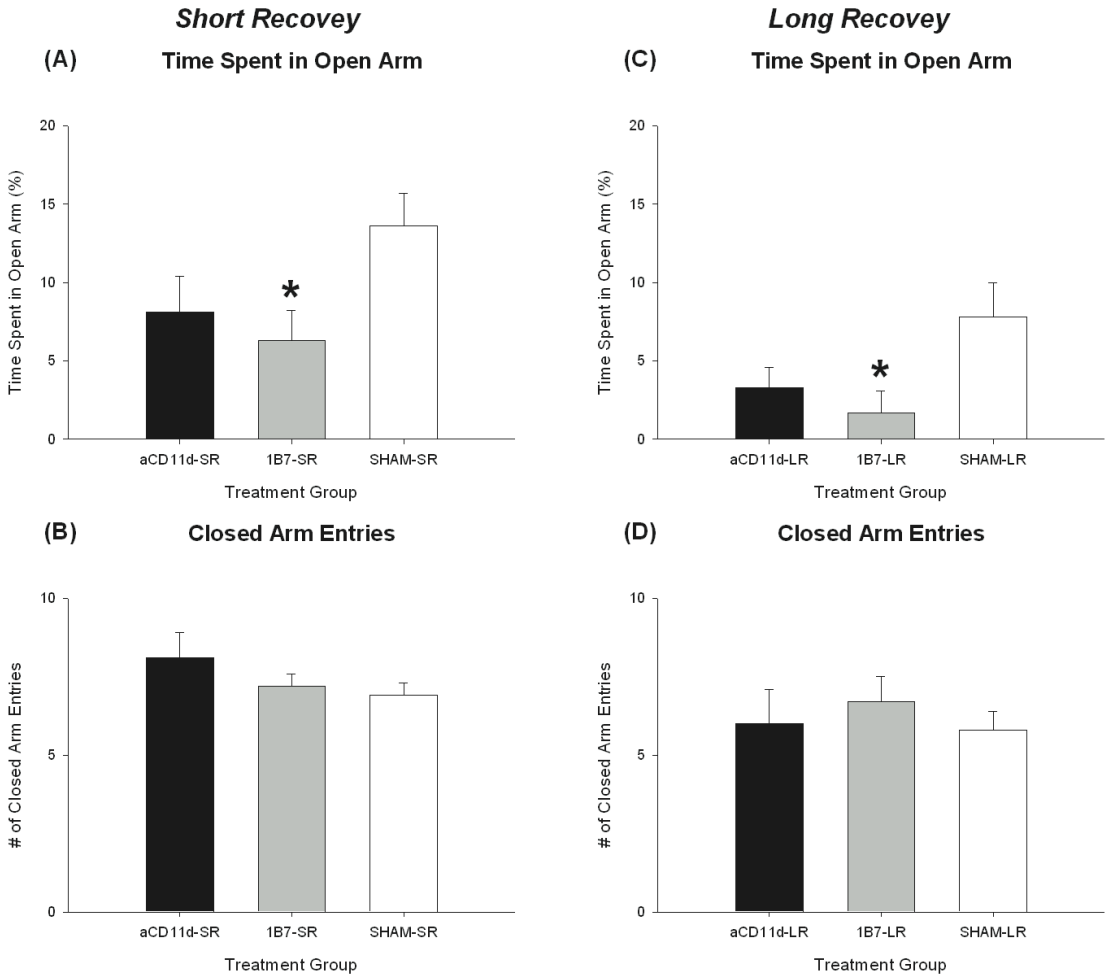

Figure 5.2. Short recovery and long recovery elevated-plus maze results. (A) The 1B7-SR group spent significantly less time in the open arm than the SHAM-SR group. (B) The SR groups did not differ in the number of entries into the closed arm. (C) The 1B7-LR group spent significantly less time in the open arm than the SHAM-LR group. (D) The LR groups did not differ in the number of entries into the closed arm. Histogram bars represent group means (± SEM). * = different from the SHAM group; all *ps* < .05. For additional statistical detail see Results.

5.3.3. Water maze: short recovery

Search time and direct and circle swims were used as measures of cognitive ability in the water maze. Swim speed was included as a measure of motor ability. During SR acquisition search time decreased as testing progressed, as indicated by a significant effect of *trial* ($F(9, 369) = 22.034, p < .001$; see Fig. 5.3A). Search time decreased less in the 1B7-SR than in the aCD11d-SR and SHAM-SR groups, as indicated by a significant effect of *treatment* ($F(2, 43) = 5.795, p < .01$; 1B7-SR > aCD11d-SR and SHAM-SR, all $ps < .05$; see Fig. 5.3A).

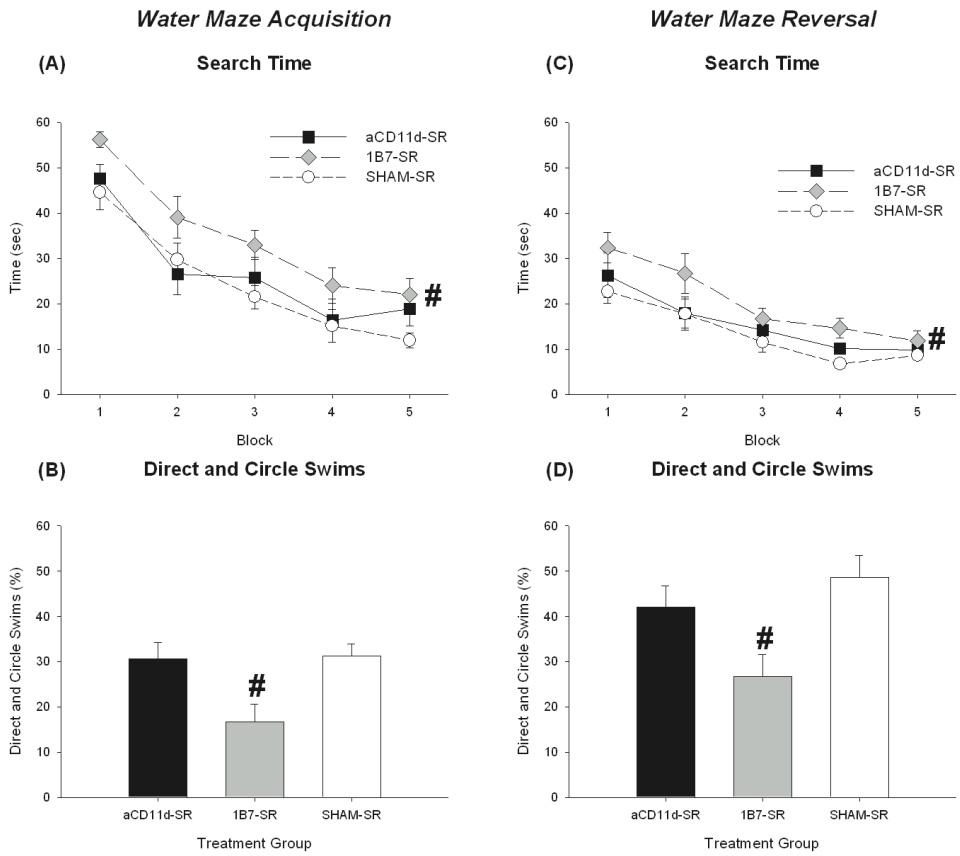

Figure 5.3. Short recovery water maze results. (A) The 1B7-SR group displayed significantly longer search time during acquisition training than the aCD11d-SR and SHAM-SR groups. (B) The 1B7-SR group displayed significantly fewer direct and circle swims during acquisition training than the aCD11d-SR and SHAM-SR groups. (C) The 1B7-SR group displayed significantly longer search time during reversal training than the aCD11d-SR and SHAM-SR groups. (D) The 1B7 group displayed significantly more direct and circle swims during reversal training than the aCD11d-SR and SHAM-SR groups. In panels (A) and (C) data points represent means of data collected for each block of two trials (± SEM). In panels (B) and (D) histogram bars represent group means of data collected during the 10 water maze trials (± SEM). # = different from all other groups; all *ps* < .05 or better. For additional statistical detail see Results.

The direct and circle swim data were consistent with the search time data in revealing fewer direct and circle swims in the 1B7-SR group during acquisition compared to aCD11d-SR and SHAM-SR rats, as indicated by a significant effect of *treatment* ($F(2, 43) = 6.161$, $p < .01$; 1B7-SR < aCD11d-SR and SHAM-SR, all *ps* < .01; see Fig. 5.3B). There were no significant group differences in swim speed during acquisition training ($p > .05$; data not shown).

During SR reversal training search time decreased in all groups as testing progressed, as indicated by a significant effect of *trial* ($F(9, 369) = 13.799$, $p < .001$; see Fig. 5.3C). Search time decreased less in the 1B7-SR than in the aCD11d-SR and SHAM-SR groups, as indicated by a significant effect of *treatment* ($F(2, 43) = 6.016$, $p < .01$; 1B7-SR > aCD11d-SR and SHAM-SR, all *ps* < .05; see Fig. 5.3C).

The direct and circle swim data were consistent with the search time data in revealing fewer direct and circle swims in the 1B7-SR group during reversal compared to aCD11d-SR and SHAM-SR rats, as indicated by a significant effect of *treatment* ($F(2, 43) = 5.727$, $p < .01$; 1B7-SR < aCD11d-SR and SHAM-SR, all *ps* < .05; see Fig. 5.3D). There were no group differences in swim speed during reversal ($p > .05$; data not shown).

5.3.4. Water maze: long recovery

During LR acquisition search time decreased as testing progressed, as indicated by a significant effect of *trial* ($F(9, 297) = 14.962$, $p < .001$; see Fig. 5.4A). Search time decreased less in the 1B7-LR group than the aCD11d-LR and SHAM-LR groups, as indicated by a significant effect of *treatment* ($F(2, 33) = 9.057$, $p < .001$; 1B7-LR > aCD11d-LR > SHAM-LR, all *ps* < .05; see Fig. 5.4A). There were no significant group differences in percent direct and circle swims ($p > .05$; see Fig. 5.4B) or swim speed ($p >$

.05; data not shown) during acquisition training.

During LR reversal search time decreased as testing progressed, as indicated by a significant effect of *trial* ($F(9, 297) = 7.198, p < .001$; see Fig. 5.4C). Search time decreased less in the 1B7-LR group than the aCD11d-LR and SHAM-LR groups, as indicated by a significant effect of *treatment* ($F(2, 33) = 7.415, p < .01$; 1B7-LR > aCD11d-LR and SHAM-LR, all *ps* < .05; see Fig. 5.4C).

1B7-LR and aCD11d-LR rats displayed fewer direct and circle swims during reversal compared to SHAM-LR rats, as indicated by a significant *treatment* effect ($F(2, 35) = 9.680, p < .001$; 1B7-LR and aCD11d-LR < SHAM-LR, all *ps* < .01; see Fig. 5.4D). There were no significant group differences in swim speed during reversal training ($p > .05$; data not shown).

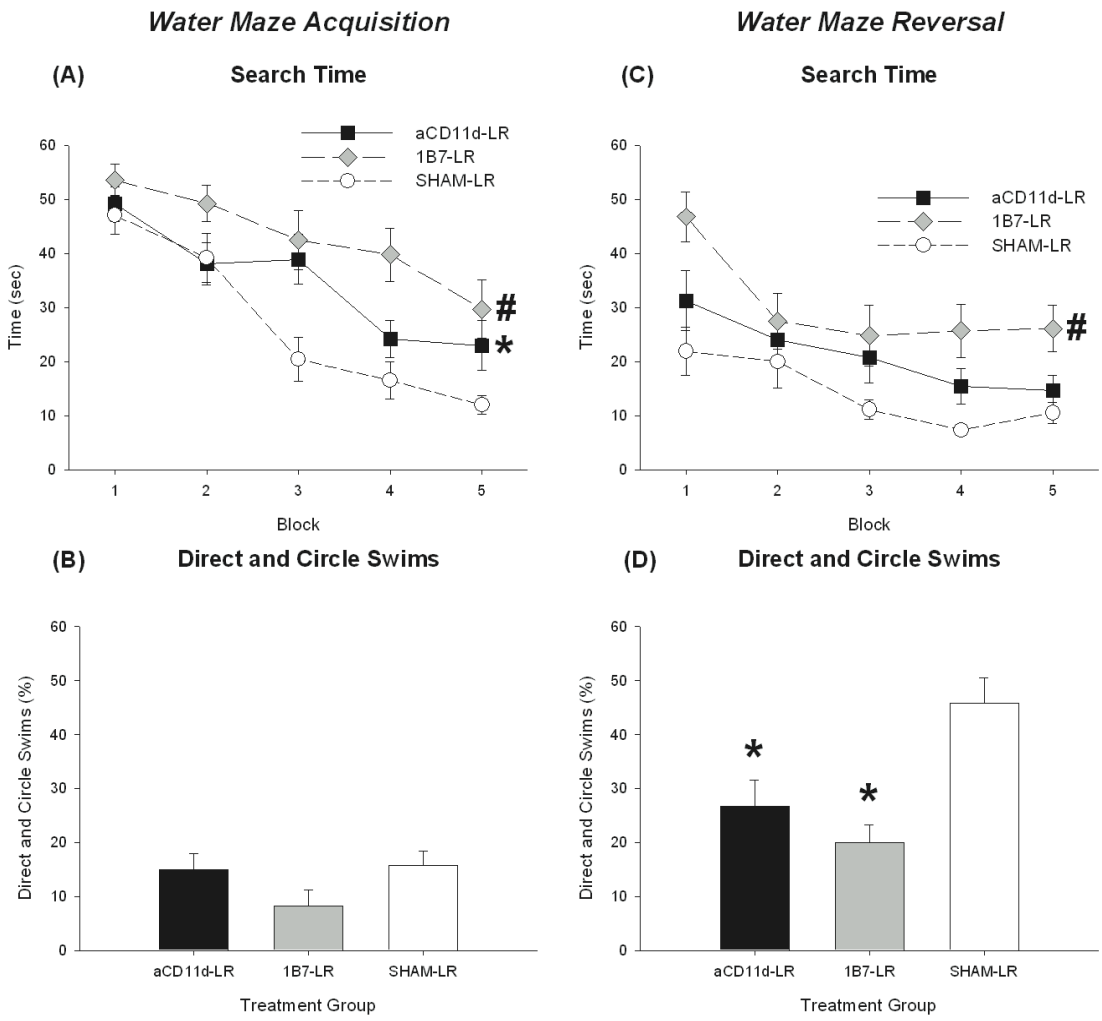

Figure 5.4. Long recovery water maze results. (A) The 1B7-LR group displayed significantly longer search time than both the aCD11d-LR group and the SHAM-LR group, and the aCD11d-LR group displayed significantly longer search time during acquisition training than the SHAM-LR group. (B) The LR groups did not differ in the percent of direct and circle swims during acquisition training. (C) The 1B7-LR group displayed significantly longer search time during reversal training than the aCD11d-LR and SHAM-LR groups. (D) The 1B7-LR and aCD11d-LR groups displayed significantly fewer direct and circle swims during reversal training than the SHAM-LR group. In panels (A) and (C) data points represent group means of data collected for each block of

two trials (± SEM). In panels (B) and (D) histogram bars represent group means of data collected during the 10 water maze trials (± SEM). # = different from all other groups; * = different from SHAM-LR group; all *ps* < .05 or better. For additional statistical detail see Results.

5.3.5. Beam task

Traverse time and the number of slips and falls on the beam task were used as measures of sensorimotor ability There were no significant group differences in SR beam traverse time ($p > .05$; data not shown), or slips and falls ($p > .05$; see Fig. 5.5A).

During LR beam testing the 1B7-LR group displayed more slips and falls than SHAM-LR group, as indicated by a significant *treatment* effect ($F(2, 35) = 4.861$, $p < .05$; 1B7-LR > SHAM-LR, $p < .01$; See Fig. 5.5B). There were no significant LR group differences in beam traverse time ($p > .05$; data not shown).

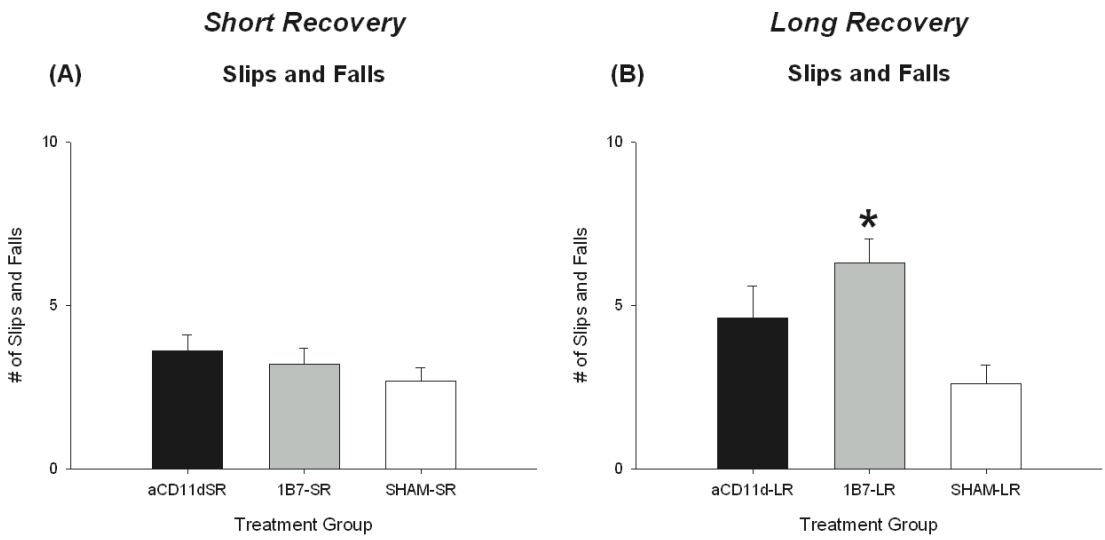

Figure 5.5. Short and long recovery beam task slips and falls results. (A) The SR groups did not differ in the number of slips and falls on the beam task. (B) The 1B7-LR group displayed more slips and falls on the beam task than the SHAM-LR group. Histogram bars represent group means of data collected during the 10 beam task trials (± SEM). * = different from SHAM-LR group; $p < .05$. For additional statistical detail see Results.

5.3.6. Neuroinflammation

The number of neutrophils and ED1-labeled microglia/macrophages in the injured cortex were used to assess neuroinflammation. The 1B7-SR group displayed more neutrophils than the aCD11d-SR and SHAM-SR groups, as indicated by a significant *treatment* effect ($F(2, 17) = 3.846$, $p < .05$; 1B7-SR > both aCD11d-SR and SHAM-SR, all $ps < .05$; see Fig. 5.6).

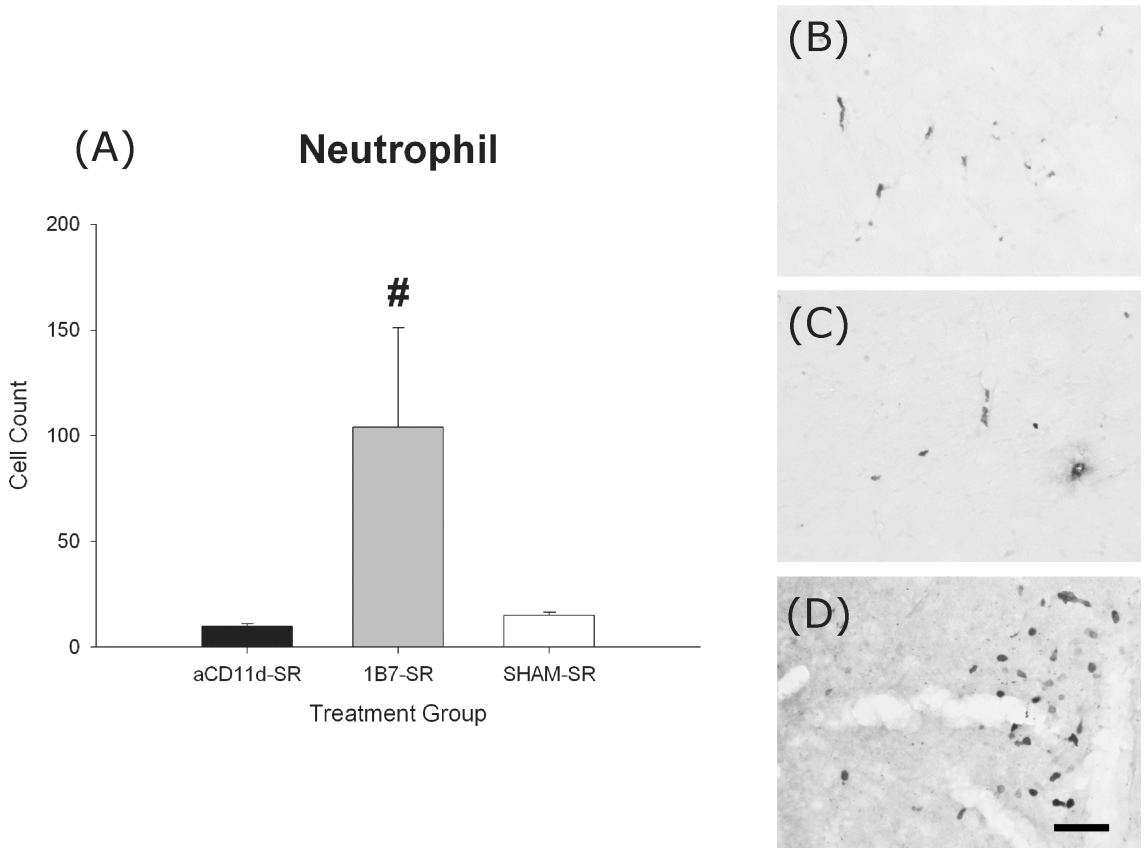

Figure 5.6. Neutrophil results. (A) The 1B7-SR group displayed significantly more neutrophils in the injured cortex than both the aCD11d-SR and SHAM-SR groups. Histogram bars represent mean neutrophils counted in the injured cortex (± SEM). # = different from the aCD11d-SR and SHAM-SR groups, *ps* < .05. (B-D) Representative photomicrographs showing neutrophil immunoreactivity in the injured cortex from the SHAM-SR (B), aCD11d (C), and 1B7-SR (D) groups at 20X magnification. Scale bar = 100 μm. For additional statistical detail see Results.

The 1B7-SR group displayed more ED1-labeled activated microglia/macrophages than the aCD11d-SR and SHAM-SR groups, as indicated by a significant *treatment* effect ($F(2, 17) = 5.699, p < .05$; 1B7-SR > both aCD11d-SR and SHAM-SR, all $ps < .05$; see Fig. 5.7).

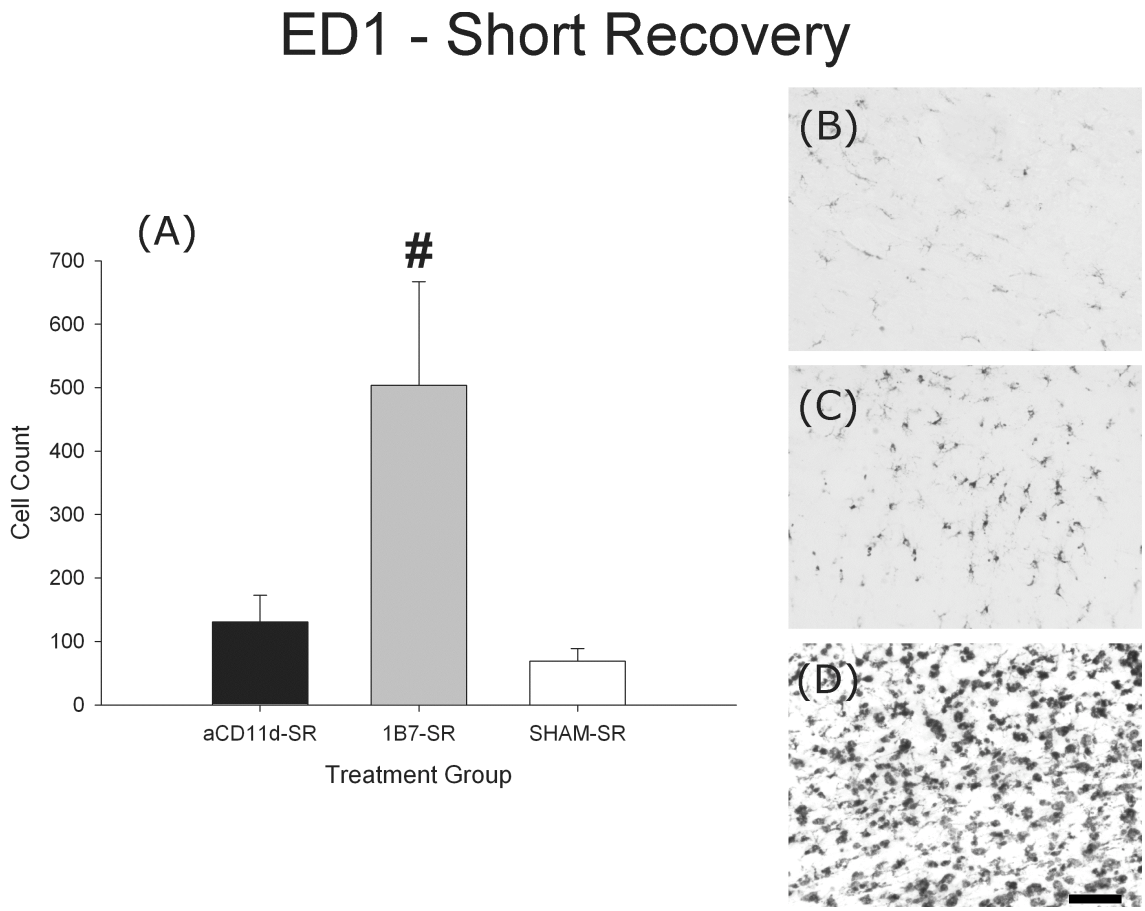

Figure 5.7. Short recovery ED1 results. (A) The 1B7-SR group displayed significantly more ED1-labeled activated microglia/macrophages in the injured cortex than both the aCD11d-SR and SHAM-SR groups. Histogram bars represent mean ED1 labeled cells (± SEM). # = different from the aCD11d and SHAM groups, *ps* < .05. (B-D) Representative photomicrographs at 20X magnification showing ED1 immunoreactivity from the SHAM-SR (B), aCD11d-SR (C), and 1B7-SR (D) groups. Scale bar = 100 μm. For additional statistical detail see Results.

The 1B7-LR and aCD11d-LR groups displayed more ED1-labeled activated microglia/macrophages than the SHAM-LR group, as indicated by a significant *treatment* effect ($F(2, 17) = 1.656$, $p < .001$; 1B7-LR and aCD11d > SHAM-LR, $ps < .05$; see Fig. 5.8).

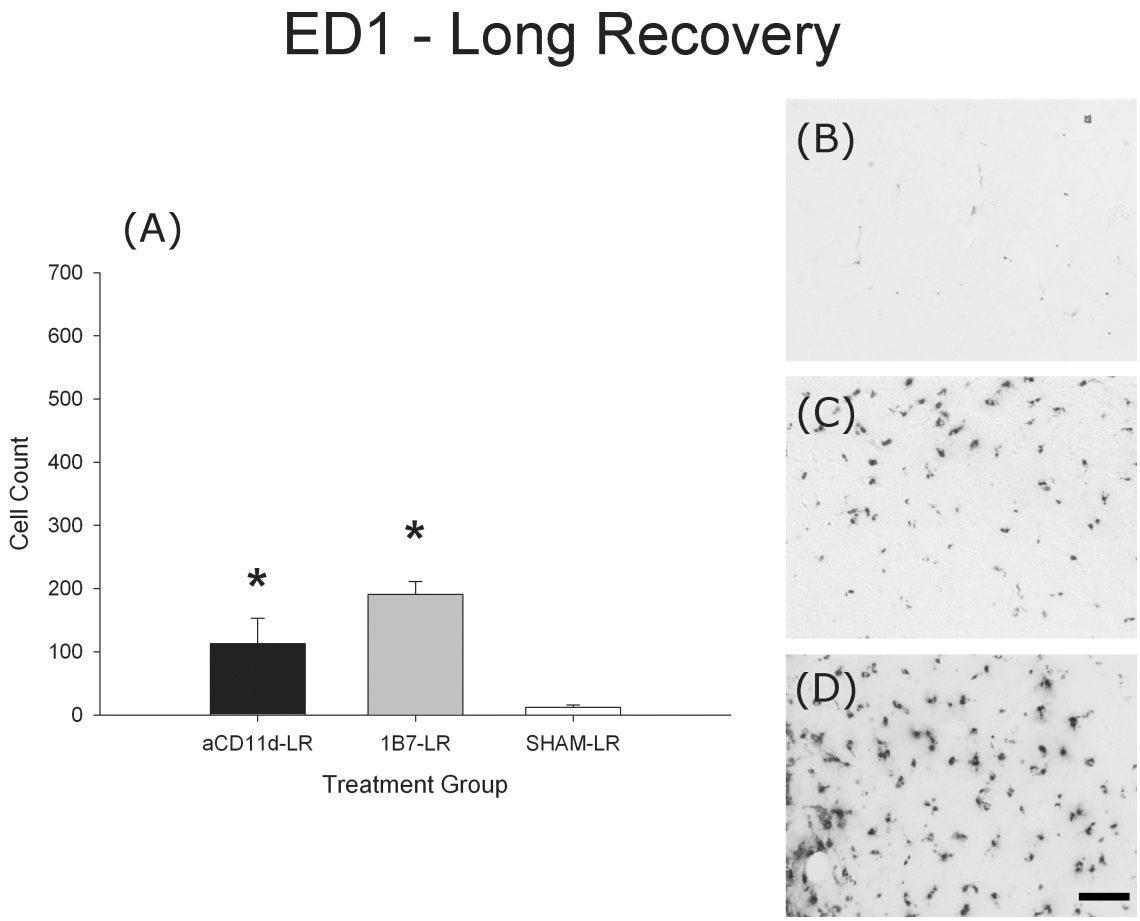

Figure 5.8. Long recovery ED1 results. (A) The 1B7-LR and aCD11d-LR groups displayed significantly more ED1-labeled activated microglia/macrophages in the injured cortex than the SHAM-LR group. Histogram bars represent mean ED1 labeled cells (± SEM). * = different from the SHAM group, *ps* < .05. (B-D) Representative photomicrographs at 20X magnification showing ED1 immunoreactivity from the SHAM-LR (B), aCD11d-LR (C), and 1B7-LR (D) groups. Scale bar = 100 μm. For additional statistical detail see Results.

5.3.7. Cortical Damage

Cortical damage was assessed using a semi-qualitative analysis, with three levels: no/mild damage; moderate damage; severe damage. The majority of 1B7-SR rats displayed moderate or severe cortical damage (see Fig. 5.9). The aCD11d-SR group displayed no damage or moderate cortical damage with no cases of severe damage. There was no indication of moderate or severe damage in the SHAM-SR group.

All 1B7-LR rats displayed moderate or severe cortical damage, with 84% displaying severe damage. The majority of aCD11d-LR rats now displayed moderate or severe cortical damage. There was no indication of moderate or severe damage in the SHAM-LR group.

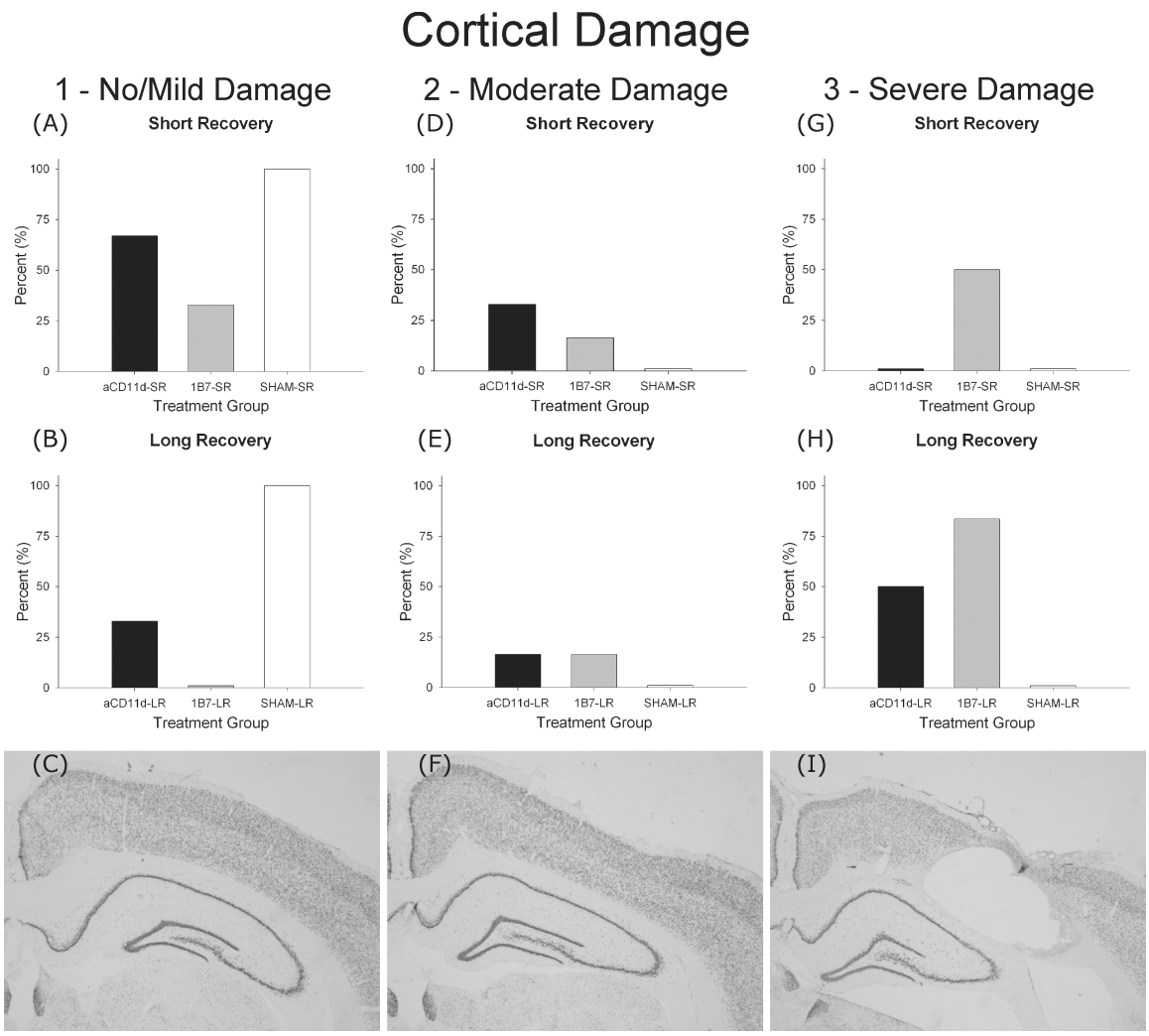

Figure 5.9. Semi-qualitative analysis of cortical damage. The histogram bars in panels (A), (D), and (G) show the percent of each short recovery (SR) injury group scoring 1 (A), 2 (D), or 3 (G) on the semi-qualitative scale of cortical damage. The histogram bars in panels (B), (E), and (H) show the percent of each long recovery (LR) injury group scoring 1 (B), 2 (E), or 3 (H) on the semi-qualitative scale of cortical damage. Panels (C), (F), and (I) show representative photomicrographs at 1.25X magnification of cortical damage scored 1-no/mild damage (C), 2-moderate damage (F), or 3-severe damage (I). For additional detail see Results.

5.4. Discussion

The current findings suggest that anti-CD11d integrin antibody treatment can reduce behavioral impairments, neuroinflammation, and cortical damage relative to treatment with control 1B7 antibody in a model of repeated concussion in the rat. The aCD11d groups displayed no short-term cognitive impairments and, although cognitively impaired on some water maze measures after long-term recovery, in most cases the cognitive impairments were significantly smaller than those displayed by the 1B7-LR group. There was also no evidence that rats treated with anti-CD11d antibody experienced significant emotional or motor impairments during elevated-plus maze and beam task testing, whereas the 1B7 groups displayed increased anxiety-like behavior and LR motor impairments compared to the SHAM groups. Acute injury measures also suggest benefits of anti-CD11d antibody treatment in terms of shorter periods of unconsciousness and shorter self-righting reflex times after repeated mild lateral fluid percussion injury compared to 1B7-treated rats. Neuropathological analyses revealed decreased neutrophils and microglia/macrophages in the injured cortex of rats treated with anti-CD11d antibody after repeated mild lateral fluid percussion injury compared to 1B7-treated rats. There also appeared to be less cortical damage in the anti-CD11d antibody treated rats compared to the 1B7-treated rats.

5.4.1. Behavioral outcomes

Rats treated with anti-CD11d antibody displayed significantly reduced cognitive impairments in the water maze compared to concussed rats treated with control 1B7 antibody. The anti-CD11d antibody groups also displayed no significant anxiety- or motor-related impairments on the elevated-plus maze or beam task relative to sham

controls, whereas 1B7 groups displayed deficits on both of these tasks. The finding that three mild lateral fluid percussion injuries induced short- and long-term cognitive impairments is consistent with previous results (DeRoss et al., 2002; see Chapter 3).

The fact that the 1B7-LR group displayed deficits on the beam task suggests that motor ability should be considered when interpreting 1B7-LR impairments in the water maze and elevated-plus maze. However, there were no group differences on the measures of swim speed in the water maze and closed-arm entries in the elevated-plus maze, both of which are locomotor measures taken directly from each of the tasks. In addition, 1B7-SR rats displayed deficits in the water maze and elevated-plus maze, but displayed no impairment on the beam task. Therefore, it appears unlikely that any motor impairments experienced by the 1B7-LR group directly accounted for the other behavioral findings.

5.4.2. Pathology and relation to behavioral findings

1B7-treated rats displayed increased neuroinflammation that was often accompanied by moderate or severe cortical damage. As a single mild lateral fluid percussion injury has been found to result in little or no cortical damage (Gurkoff et al., 2006; see Chapter 3), it seems possible that secondary injury processes, such as neuroinflammation, may have contributed to the cortical damage observed in 1B7-treated rats. The finding of long-term neuroinflammation in rats given three mild lateral fluid percussion injuries together with the finding that LR injured rats appeared to display more cortical damage than SR injured rats further suggests that neuroinflammation may be involved in neurodegeneration (Lee et al., 2002; Nandoe et al., 2002; O'Sullivan et al., 2009; Stoll and Jander, 1999; Whitton, 2007; Zilka, 2006; see Chapter 3).

Previous research from our laboratory and others has found evidence of cognitive deficits in the presence of a neuroinflammatory response in cortex (Shultz et al., 2009; Wu et al., 2006, 2010; see Chapters 2, 3, and 4), and other studies of lateral fluid percussion injury in the rat have also found cognitive, emotional, and motor impairments associated with damage to the same cortex areas that were examined in the current study (Jones et al., 2008; Wahl et al., 2000; see Chapters 3 and 4). This suggests that the behavioral deficits found in rats given three mild lateral fluid percussion injuries in the current study may have resulted from the neuroinflammation and cortical damage induced by mild lateral fluid percussion injuries.

The finding that anti-CD11d antibody treatment reduced neuroinflammation, cortical damage, and behavioral impairments suggests that infiltrating peripheral leukocytes contribute to neuroinflammation, secondary brain damage, and behavioral impairment in repeated concussion, and supports the use of anti-CD11d antibody to reduce these negative effects. However, the anti-CD11d antibody treatment schedule used in the current study failed to completely prevent long-term cognitive deficits in the water maze task. To date only two other studies have examined the use of anti-CD11d antibody to treat traumatic brain injury (Utagawa et al., 2008; see Chapter 4), and different anti-CD11d antibody treatment schedules were used in each study. The fact that the current study was the first to assess anti-CD11d antibody treatment in a repeated concussion animal model further complicates the issue of treatment schedule. Further research with anti-CD11d antibody in a repeated concussion animal model would be useful for optimizing treatment doses and administration schedules.

Although neuroinflammation is an important process after traumatic brain injury,

other underlying mechanisms must also be considered in the aftermath of concussion. Evidence indicates that single concussion can induce axonal injury (Arfanakis et al., 2002; Benson et al., 2007; Kraus et al., 2007), and in the case of repeated concussion axonal injuries might contribute to cumulative and chronic neurological disturbances (McKee et al., 2009; Omalu et al., 2005; Omalu et al., 2006). Excitatory neurotransmitter release, calcium-mediated damage, and mitochondrial dysfunction are other processes that might occur in traumatic brain injury and contribute to secondary brain damage (Graham et al., 2000; Maas et al., 2008). Tau and amyloid pathologies are poorly understood mechanisms that have also been associated with repeated concussion and related neurological disease (McKee et al., 2009). Further research is needed to investigate the involvement of these mechanisms in repeated concussion to determine optimal management and treatment strategies.

5.4.3. Conclusions

Rats treated with anti-CD11d integrin antibody 2 and 24 hrs following each of three repeated mild lateral fluid percussion injuries displayed no significant anxiety- or motor-related impairments relative to sham controls, and displayed significantly reduced cognitive impairments compared to concussed rats treated with a control antibody. As the beneficial effects of anti-CD11d antibody were associated with decreased neuroinflammation and cortical damage, it appears that the beneficial effects of anti-CD11d antibody might have been mediated by limiting the infiltration of peripheral leukocytes into the brain, thus decreasing the neurotoxic effects of neuroinflammation. Although other underlying mechanisms are likely involved, the current findings suggest that infiltrating leukocytes contribute to both brain damage and behavioral impairments

after repeated concussion. These results support the use of the anti-CD11d antibody to treat the cumulative effects of repeated concussion.

5.5. References

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Chapter 6

6. General discussion

Traumatic brain injury is recognized as an international health concern and a growing socioeconomic problem (Maas et al., 2008). Due to the complex pathophysiology associated with traumatic brain injury, there is currently no safe and effective pharmaceutical treatment available for widespread clinical use (Atif et al., 2009; Stein and Wright, 2010). Although concussion, or mild traumatic brain injury, accounts for the large majority of traumatic brain injury cases, there is little known regarding what occurs in the brain following concussion (Anderson et al., 2006; Cantu, 2009). Even less is understood about the factors and mechanisms involved in repeated concussion (Cantu, 2009; McKee et al., 2009), which can result in cumulative brain damage and neurodegenerative disease (McKee et al., 2009).

Given the current lack of understanding, the present thesis used a rat model of fluid percussion injury to study single and repeated concussion and assess a novel traumatic brain injury treatment. This General Discussion will begin by summarizing the main objectives and findings from each of the studies, and will include discussion on their inconsistencies and implications. The sections that follow will focus on the further development of traumatic brain injury treatments and the future use of the mild lateral fluid percussion injury model to study repeated concussion.

6.1. Summary of current studies

In Studies 1 and 2 (Chapters 2 and 3), the behavioral and pathological effects of single and repeated mild lateral fluid percussion injury in the rat were assessed to validate their use as animal models of single and repeated concussion. In Study 1, a single mild

lateral fluid percussion injury induced short-term behavioral deficits, neuroinflammation, and axonal injury. These findings were consistent with key features of concussion (Arfanakis et al., 2002; Boll and Barth, 1983; Kim, 2002). In Study 2, a novel repeated mild lateral fluid percussion injury schedule resulted in cumulative and long-term cognitive deficits, anxiety, and depression, all of which were consistent with symptoms of repeated concussion (Guskiewicz et al., 2003; Guskiewicz et al., 2005; Guskiewicz et al., 2007; Macciocchi et al., 2001; McKee et al., 2009). This study also provided evidence that neuroinflammation may have been involved in the behavioral deficits and extensive brain damage that occurred in rats given repeated mild lateral fluid percussion injuries. Similar pathologies have also been identified in the brains of athletes who have endured repeated concussion (McKee et al., 2009; Omalu et al., 2005; Omalu et al., 2006).

Neuroinflammation is present following traumatic brain injury in humans and rats (Morganti-Kossman et al., 2007; Thompson et al., 2005). This process has been shown to have both neuroprotective and neurotoxic properties (Ekdahl et al., 2009; Laird et al., 2008; Morganti-Kossman et al., 2007). The infiltration of peripheral leukocytes into the brain during traumatic brain injury might worsen the neuroinflammatory response and associated secondary brain damage (Lu et al., 2009; Utagawa et al., 2008). Studies 3 and 4 (Chapters 4 and 5) were designed to assess the effects of anti-CD11d integrin monoclonal antibody, a novel pharmacological traumatic brain injury treatment that blocks the infiltration of peripheral leukocytes into the brain following injury (Utagawa et al., 2008). In Study 3, anti-CD11d antibody treatment was administered following a single moderate lateral fluid percussion injury. A moderate lateral fluid percussion injury

was used instead of mild lateral fluid percussion injury to induce a broader range of behavioral and pathological change and to allow for a more complete assessment of the therapeutic effects of anti-CD11d antibody. This study found that anti-CD11d antibody treatment reduced cognitive, emotional, and motor impairments while decreasing neuroinflammation and neuronal loss. Study 4 used the repeated concussion model developed in Study 2 to investigate the effectiveness of anti-CD11d antibody treatment administered after each of three mild lateral fluid percussion injuries. Rats treated with anti-CD11d antibody performed significantly better on cognitive measures than injured rats given a control monoclonal antibody treatment. Rats treated with anti-CD11d antibody also performed similar to sham–control rats on measures of motor ability and anxiety-like behavior. In addition to these behavioral changes, the anti-CD11d antibody also reduced neuroinflammation and cortical damage.

6.1.1. Inconsistencies

A number of inconsistencies occurred in the current studies that should be addressed. In Study 1 it was found that rats given a single mild lateral fluid percussion injury spent less time in the open arm of the elevated-plus maze, suggesting a decrease in anxiety-like behavior. This finding was not replicated in Study 2, and this difference in behavioral outcomes might have resulted from methodological differences between the studies. In Study 1 rats were tested after a single mild lateral fluid percussion injury treatment, whereas in Study 2 the experimental design required that the relevant group of rats receive four sham treatments prior to mild lateral fluid percussion injury. The additional sham treatments involved in Study 2 may have increased stress levels and offset any reductions of anxiety induced by a single mild lateral fluid percussion injury.

Although this methodological difference might account for the inconsistent elevated-plus maze findings associated with a single mild lateral fluid percussion injury, this issue should be further investigated given the novelty of the reduced anxiety-like behavior in Study 1.

There was also an inconsistency between these studies regarding the nature of cognitive impairment associated with a single mild lateral fluid percussion injury. Study 1 found that a single mild lateral fluid percussion injury induced cognitive impairments during reversal but not acquisition training in the water maze. Study 2 found that a single mild lateral fluid percussion injury induced significant impairments during acquisition but not reversal. Similar to the issue of anxiety discussed above, the acquisition impairments in Study 2 might also be explained by the increased stress levels associated with the repeated treatment design, as previous research has found that stress can affect water maze performance (Hölscher, 1999). Furthermore, while no significant differences were found on reversal measures in Study 2, it should be noted that there was a non-significant trend suggesting a group difference in reversal search time. Despite the minor inconsistencies on cognitive measures, it is important to note that in both studies a single mild lateral fluid percussion injury successfully induced short-term cognitive impairments similar to those that occur after concussion. Nonetheless, further research is needed to investigate the exact nature of cognitive impairment induced by a single mild lateral fluid percussion injury.

In Study 4, control treated rats given three mild lateral fluid percussion injuries displayed motor deficits and increased anxiety-like behavior. Although Study 2 reported a non-significant trend that suggested increased slips and falls on the beam task, these

rats failed to display significant motor impairments on the beam task, or increased anxiety-like behavior in the elevated-plus maze. There are a number of methodological differences that might account for these inconsistencies. In Study 4 rats received a total of six tail vein injections of control antibody treatment during the administration of the mild lateral fluid percussion injuries. These injections may have increased stress levels and affected elevated-plus maze performance. It must also be considered that the control antibody treatment itself might have affected outcomes in the rats given three mild lateral fluid percussion injuries in Study 4. Due to the limited clinical relevance of uninjured animals given a control treatment, the sham injury + control antibody treatment group necessary to rule out this possibility was not included in the study. However, previous studies that have used the same control treatment have reported no negative effects (Gris et al., 2004; Saville et al., 2004; Utagawa et al., 2008), and rats from both studies given three mild lateral fluid percussion injuries performed similarly in the water maze. Last, although there were no significant elevated-plus maze or beam task differences associated with three mild lateral fluid percussion injuries in Study 2, the figures often illustrate a dose-like response among the sham, 1-, 3-, and 5-concussion groups. Given the additional injury groups used in Study 2, and consequent increase in variability, it seems possible that Study 2 was limited in identifying 3-concussion injury impairments due to statistical reasons that did not affect Study 4.

6.1.2. Implications

Studies 1 and 2 were designed to further the understanding of single and repeated concussion through the validation and development of the rat mild lateral fluid percussion injury model. Although these studies had minor inconsistencies, they largely succeeded

in reaching their objectives, and hold important implications in the understanding of concussion. In Study 1 the short-term behavioral and pathological changes after a single mild lateral fluid percussion injury were consistent with key features of a single concussion in humans. These findings further validated the mild lateral fluid percussion injury concussion model and provided rationale for the evaluation of repeated mild lateral fluid percussion injury as a model of repeated concussion that was carried out in Study 2. There it was found that repeated mild lateral fluid percussion injury induced behavioral and pathological changes consistent with those that occur in repeated concussion in humans, providing further evidence for the cumulative and long-term effects of repeated concussion and support for the use of the repeated mild lateral fluid percussion injury model to study repeated concussion.

It is difficult to identify and study the pathological mechanisms of concussion in humans. As concussion rarely results in death it is difficult to obtain post-mortem pathological information that is not confounded by factors such as aging due to the passage of time, effects of other disorders, consumption of toxic agents, etc. Concussion also fails to induce brain damage that is identifiable using standard clinical imaging techniques (McCrory et al., 2009). Therefore, the model used in Studies 1 and 2 holds important implications for the examination of pathological mechanisms that occur in single and repeated concussion. The fact that a single mild lateral fluid percussion injury induced axonal injury supports evidence that concussion can result in axonal injuries that can contribute to consequent neurological disturbances (e.g. Benson et al., 2007). Studies 1 and 2 also identified neuroinflammation as a possible mechanism contributing to the effects of single and repeated concussion. Overall, the mild lateral fluid percussion injury

models validated and developed in the present thesis provide unique tools for future studies that will have important implications for the management and treatment of concussions through the investigation of the factors and mechanisms involved in these injuries.

Studies 3 and 4 were designed to assess a novel traumatic brain injury treatment and further investigate the role of peripheral leukocytes and neuroinflammation in traumatic brain injury. Study 3 found that anti-CD11d antibody successfully reduced cognitive, emotional, and motor impairments while decreasing neuroinflammation and neuronal loss after a single moderate lateral fluid percussion injury. These findings further implicate neuroinflammation as a key contributor to traumatic brain injury and support the further investigation of anti-CD11d antibody in the treatment of traumatic brain injury.

These findings, coupled with the previous identification of neuroinflammation after repeated mild lateral fluid percussion injury (Study 2), provided the rationale to administer anti-CD11d antibody treatment to rats given repeated mild lateral fluid percussion injuries in Study 4. It was found that anti-CD11d antibody treatment given after each of three repeated mild lateral fluid percussion injuries resulted in reduced behavioral impairments, and reduced neuroinflammation and cortical loss. These findings further implicate neuroinflammation as a factor in the cumulative properties of repeated concussion and support the use of anti-CD11d antibody to potentially combat these negative effects. In addition, Studies 3 and 4 both suggest that the infiltration of peripheral leukocytes increase neuroinflammation and contribute to secondary brain damage (see Lu et al., 2009; Morganti-Kossman et al., 2007). Taken together, the

findings from Studies 3 and 4 support the notion that traumatic brain injury should be regarded as a systemic response (Lu et al., 2009), and hold important implications for the management and treatment of traumatic brain injury in humans.

6.2. Future direction of traumatic brain injury treatment

6.2.1. Neuroinflammation

It is well established that the neuroinflammatory process is an important mechanism that contributes to damage in traumatic brain injury (Morganti-Kossman et al., 2007). As a result, traumatic brain injury treatments with anti-inflammatory properties have been tested in pre-clinical and clinical studies (Gomes et al., 2005; Hatton et al., 2008; Thompson et al., 2005; Vink and Van Den Heuvel, 2004). At best, these agents have resulted in limited benefits in traumatic brain injury patients, and are not used in widespread clinical practice (Hatton et al., 2008; Thompson et al., 2005; Stein and Wright, 2010). The lack of a successful anti-inflammatory agent may be a result of the complex neuroinflammatory cascade in traumatic brain injury. For example, although the acute neuroinflammatory response is considered detrimental (Morganti-Kossman et al., 2007; Thompson et al., 2005), neuroinflammation may be crucial for regenerative processes in the days and weeks that follow injury (Morganti-Kossman et al., 2007; Thompson et al., 2005), though chronic neuroinflammation might contribute to neurodegeneration (Nandoe et al., 2002; Stoll and Jander, 1999; Whitton, 2007; Zilka et al., 2006). Clearly this temporal complexity poses a significant challenge in the treatment of traumatic brain injury (Thompson et al., 2005). A pharmaceutical intervention that targets the infiltration of peripheral leukocytes may provide a unique opportunity to balance or alter the dual properties of neuroinflammation in a therapeutic manner. The

early blockage of infiltrating leukocytes would decrease the detrimental effects of acute neuroinflammation. However, this early blockage would not necessarily prevent the innate neuroinflammatory response involving microglia and astrocytes, which would possibly allow the neuroprotective properties of neuroinflammation to occur. Together, the findings from Studies 3 and 4, as well as evidence from other laboratories (Grady et al., 1999; Utagawa et al., 2008), support the use of pharmaceutical interventions that target the infiltration of peripheral leukocytes in traumatic brain injury.

Although the current findings implicate peripheral leukocytes and neuroinflammation in brain damage that occurs in repeated concussion and moderate traumatic brain injury, anti-CD11d antibody treated rats failed to make full recovery in Studies 3 and 4. There are several possibilities that might contribute to these findings. As mentioned above, the neuroinflammatory response in traumatic brain injury is complex. Therefore, it is possible that infiltrating peripheral leukocytes contribute to the signaling of regenerative processes (Stirling et al., 2009). In addition, the ideal anti-CD11d antibody treatment regimen has yet to be determined. To date, only three studies have examined the use of anti-CD11d antibody as a traumatic brain injury treatment, and each of these studies used different injection numbers and treatment windows (Utagawa et al., 2008; see Chapters 4 and 5). Future research is required to clarify the role of infiltrating leukocytes in the neuroinflammatory cascade and determine the most effective anti-CD11d antibody treatment strategy. However, while an optimal anti-CD11d antibody treatment schedule might have further improved recovery in the current studies, it is unlikely that neuroinflammation alone accounted for the remaining damage given the number of other mechanisms involved in traumatic brain injury.

6.2.2. Primary injuries

Irreversible primary injuries are induced at the moment of impact and commonly occur in moderate and severe traumatic brain injury (Graham et al., 2000; Maas et al., 2008; Marshall, 2000). Such injuries might account for the impairments in rats treated with anti-CD11d antibody after a single moderate lateral fluid percussion injury in Study 3. Primary injuries may have also contributed to the lack of full recovery in rats given repeated mild lateral fluid percussion injury and treated with anti-CD11d antibody in Study 4. Although a single concussion usually results in little structural damage or neuronal loss (McCrory et al., 2009), in the case of repeated concussion even slight damage might accumulate and ultimately contribute to long-term effects. Axonal injuries have also been identified after a single concussion (Arfanakis et al., 2002; Benson et al., 2007), and can accumulate and contribute to the effects of repeated concussion (McKee et al., 2009). However, given the extent of brain damage observed in rats given repeated mild lateral fluid percussion injuries, as well as repeated concussion and chronic traumatic encephalopathy patients, other injury mechanisms are likely involved.

6.2.3. Other mechanisms involved in traumatic brain injury

At the onset of traumatic brain injury the initial biomechanical impact to the brain typically results in the indiscriminant depolarization of neurons, the initiation of action potentials, and consequent release of excitatory neurotransmitters (Giza and Hovda, 2001). Excitatory neurotransmitters, such as glutamate, can activate surrounding neuronal excitatory amino acid receptors and further increase neurotransmitter release (Giza and Hovda, 2001). Activation of NMDA receptors through the binding of excitatory neurotransmitters results in the opening of channels that allows calcium to enter the

neuron (Giza and Hovda, 2001). Excess intracellular calcium may sequester in the mitochondria and result in impaired oxidative metabolism or complete energy failure (Giza and Hovda, 2001). In concussion, intracellular calcium accumulation typically induces temporary metabolic abnormalities that resolve in a number of days and result in little or no neuronal loss (Giza and Hovda, 2001). In more severe traumatic brain injury, calcium accumulation and subsequent mitochondrial dysfunction may last for weeks, resulting in free radical overproduction and the signaling of cell death through a number of necrotic and apoptotic pathways (Giza and Hovda, 2001). The mechanical stretching of axons during traumatic brain injury can also result in increased axolemma permeability and consequent calcium influx. Increased intraaxonal calcium may result in microtubule breakdown, the accumulation of organelles, axonal swelling, and secondary axotomy (Giza and Hovda, 2001).

The mechanical disruption of neuronal membranes and axons can also open other ion channels and result in fluctuations such as an increased efflux of neuronal potassium into extracellular space (Giza and Hovda, 2001; Maas et al., 2008). The simultaneous activation of surrounding neurons by excitatory neurotransmitters can further increase these fluctuations. Neuronal membrane pumps are activated in an attempt to restore ionic homeostasis (Giza and Hovda, 2001). To meet this energy demand, the cell enters a state of hyperglycolysis that results in increased lactate production (Giza and Hovda, 2001). Unfortunately, this lactate increase occurs at a time of mitochondrial dysfunction and is not metabolized efficiently. Lactate accumulation can negatively affect neurons by inducing acidosis, membrane damage, and altering blood-brain barrier permeability (Giza and Hovda, 2001).

Given the complexity of the secondary injury cascade in traumatic brain injury, it seems unlikely that any treatment targeting a single factor will attenuate all of the behavioral and pathological consequences. Rather, maximum recovery would be further facilitated by multifactorial or combination therapies (Vink and Nimmo, 2009; Vink and Van Den Heuvel, 2004). For example, future research might combine anti-CD11d antibody with another promising neuroprotective agent, such as progesterone (Stein and Wright, 2010), in an attempt to maximize recovery from traumatic brain injury. In the case of repeated concussion, although anti-CD11d antibody reduced impairments in the current study, pharmaceutical intervention may not be necessary to reduce cumulative effects. Since a single concussion often appears to induce no long-term impairments or structural damage, repeated concussion might be managed through natural recovery processes. However, a better understanding of the factors and mechanism involved in repeated concussion is needed to determine whether this can occur.

6.3. Future direction of mild lateral fluid percussion injury model

6.3.1. Management of concussion

The study of concussive brain injury in humans is scientifically challenging. In light of evidence for cumulative and chronic neurological consequences after repeated concussion (McKee et al., 2009), there is increased medical concern for individuals, such as athletes, who are at risk of suffering multiple concussions (Bailes and Cantu, 2001; Cantu, 2009; Maroon et al., 2000). The current mild lateral fluid percussion injury model provides a means to identify and examine factors involved in concussion and provide insight into their treatment.

A single concussion typically results in no long-term impairments or structural damage (McCrory et al., 2009), and mechanisms that have been implicated in concussion, such as axonal injury, neuroinflammation, and metabolic dysfunction, may be transient in nature (Arfanakis et al., 2002; Giza and Hovda, 2001; Lovell et al., 2007). As a result, the cumulative effects of repeated concussion might be prevented through natural recovery. Unfortunately, little is known about the underlying mechanisms involved in such cumulative injuries and how long they might persist. Currently, most return-to-play decisions in athletes are based on the assumption that an asymptomatic patient is a fully-recovered patient (Maroon et al., 2000). However, the underlying mechanisms involved in the cumulative nature of repeated concussion may still be present. Future research using mild lateral fluid percussion injury could manipulate recovery times based on the presence or absence of a mechanism of interest to provide insight into the use of these mechanisms and their biomarkers as indicators of damage and recovery. For example, Study 1 found that neuroinflammation induced by mild lateral fluid percussion injury returned to control levels by 4 weeks post-injury. Therefore, a future study could assess whether a 4 week between-injury recovery period, rather than the 5 day period used in the current studies, could prevent the cumulative effects of repeated mild lateral fluid percussion injury. Overall, the identification of underlying mechanisms and their associated recovery times with the mild lateral fluid percussion injury model might have implications for important clinical issues such as return-to-play decisions in athletes after concussion.

Although the detrimental effects associated with repeated concussion might be prevented through natural recovery processes, a safe and effective pharmaceutical

intervention probably represents the most certain means of concussion management. Potential mechanisms of interest in concussion might be studied in future work by pharmaceuticals that target the mechanism in the mild lateral fluid percussion injury model. Such studies might increase our understanding of the targeted mechanism, assess potential concussion therapies, and hold important implications for the pharmaceutical treatment of concussion in human patients.

6.3.2. Improving the mild lateral fluid percussion injury model

Although mild lateral fluid percussion injury research has the potential to advance our understanding of concussion and provide important insight into its management, the current mild lateral fluid percussion injury model can still be improved. As previously discussed, future work that incorporates various measures of cognition, impulsivity, social behavior, anxiety, and depression is still needed to better characterize the nature of impairments that occur in the current model.

Given that the lateral fluid percussion injury is the most commonly used and well-characterized animal model of traumatic brain injury (Thompson et al., 2005), the temporal and parietal lobes are the most common concussive impact locations and typically show functional impairments after concussion (Guskiewisz et al., 2007; Umile et al., 2002), and evidence suggests that the location of concussive impact is unrelated to symptoms (Guskiewisz et al., 2007), the current studies all made use of a lateral injury location. However, other mild fluid percussion injury locations should still be characterized to determine their ability to model features of concussion. For example, a frontal mild fluid percussion injury might induce social abnormalities that did not occur in the current mild lateral fluid percussion injury studies. Such research would not only

further validate the mild fluid percussion injury model, but might also identify brain regions associated with specific symptoms of concussion. Similarly, repeated mild lateral fluid percussion injuries in the current studies were all administered through the same lateral craniotomy. Future research using various other craniotomy locations for repeated mild lateral fluid percussion injuries might help to model the mechanism of repeated concussion.

The present repeated mild lateral fluid percussion injury model involved a maximum of five injuries, with repeated injuries spaced 5 days apart. This schedule was used because evidence suggests that outcomes in the rat brain 5 days post-concussion are comparable to outcomes in the human brain 2-4 weeks post-concussion (Giza and Hovda, 2001). Thus, the between-injury recovery period used in the current thesis is comparable to what might occur in athletes under concussion management guidelines (Maroon et al., 2000). Although these guidelines permit athletes to experience three concussions in a single playing season (Maroon et al., 2000; Quality of Standards Subcommittee, 1997), it would be rare that any individual would suffer five concussions over the time span used in Study 2. Future research that used a more clinically relevant recovery time between injuries in cases of three or more mild lateral fluid percussion injuries might provide a more valid model. Furthermore, as it is not unusual for athletes to experience more than five concussions in a career (Chen et al., 2004), these studies might also incorporate more than five mild lateral fluid percussion injuries.

Chronic traumatic encephalopathy is a neurodegenerative disorder that is caused by repeated mild brain injury. Many of the behavioral and pathological findings from the current repeated mild lateral fluid percussion injury studies resemble those that can occur

in chronic traumatic encephalopathy patients, and may have implications regarding the development and neurodegenerative properties of the disorder. Despite these similarities, there are a number of reasons why repeated mild lateral fluid percussion injury should not yet be considered a model of chronic traumatic encephalopathy. The current repeated mild lateral fluid percussion injury studies used a maximum recovery time of 8 weeks after the final injury, and pathological evidence indicated a long-term neuroinflammatory response and brain damage at this time point. Although chronic traumatic encephalopathy has occurred in patients under the age of 25 and involves a long-term neuroinflammatory response and brain damage, chronic traumatic encephalopathy typically occurs in middle-aged individuals and includes other common pathologies such as tau and β-amyloid deposition (McKee et al., 2009; Omalu et al., 2005; Omalu et al., 2006). Therefore, future repeated mild lateral fluid percussion injury research should use longer recovery periods and investigate tau and β-amyloid abnormalities to evaluate repeated mild lateral fluid percussion injury as a model of chronic traumatic encephalopathy.

As young adult males are most commonly affected by traumatic brain injury, the current studies used only male rats. However, there is conflicting evidence regarding a sex difference in the susceptibility to concussion. Overall, males experience twice as many concussions as females, and chronic traumatic encephalopathy primarily affects males (Anderson et al., 2006; McKee et al., 2009). There is also growing evidence that female sex hormones may have neuroprotective properties following brain insult (Vagnerova et al., 2008). Despite this evidence, epidemiological studies indicate that female athletes suffer higher rates of concussion compared to male athletes (Gessel et al., 2007), a finding that contradicts the belief that females are biologically protected against

traumatic brain injury. While this finding may be due to other factors, such as a greater likelihood that females report their concussions (Gessel et al., 2007), future mild lateral fluid percussion injury research that involved female rats might help clarify this possible sex difference and further characterize the mild lateral fluid percussion injury model.

6.4. Conclusions

Animal models of traumatic brain injury can complement human traumatic brain injury research. Concussion, or mild traumatic brain injury, is a very common but poorly understood injury. Studies 1 and 2 of this thesis validated and developed single and repeated mild lateral fluid percussion injury in the rat as models of single and repeated concussion. These models provide helpful tools to further our understanding of concussion through the investigation of factors and mechanisms involved in these injuries using experiments that cannot be carried out with human patients. The potential of these models is highlighted in the current thesis by the identification and treatment of neuroinflammation as a mechanism of secondary injury in concussion.

There is currently no effective pharmacological traumatic brain injury treatment available for widespread clinical practice. Animal models allow for the assessment of novel traumatic brain injury therapies that cannot be conducted in human patients. Studies 3 and 4 of this thesis examined the effects of anti-CD11d integrin monoclonal antibody, a treatment targeting infiltrating peripheral leukocytes, in models of moderate traumatic brain injury and repeated concussion. The finding that anti-CD11d antibody reduced behavioral impairments, neuroinflammation, and brain damage in these studies indicates the involvement of infiltrating peripheral leukocytes in traumatic brain injury and supports the use of anti-CD11d antibody for the treatment of these injuries.

Although a great deal of work is still needed to address the important problem of concussion and develop effective traumatic brain injury therapies, the current thesis provides a foundation for future research and holds important implications for traumatic brain injury in humans.

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Appendix A[6]

Five brains were randomly selected from each treatment group studied in Chapter 4 for assessment of NeuN expression using western blotting analysis to assess gross neuronal loss after lateral fluid percussion injury. After the completion of behavioral testing, animals were anesthetized (2.5 g/kg urethane) at 24 hrs, 72 hrs, or 4 weeks post-injury and perfused with cold 0.9% NaCl. Brains were removed and stored at -80ºC until they were used. To obtain the tissue sample for the western blot assay, injured hemispheres were sectioned by slicing the brain along the longitudinal fissure. To further isolate the injured region of the ipsilateral hemisphere, the cerebellum (-8 mm relative to Bregma; Paxinos and Watson, 1986) and frontal lobe (0 mm relative to Bregma; Paxinos and Watson, 1986) were cut away and discarded. Tissue sample preparation and protein determination were performed as described previously (Bao et al, 2004). Proteins were loaded onto a 10% polyacrylamide gel, separated by SDS/PAGE using a Bio-Rad Mini-Protean 3 apparatus (Bio-Rad, Hercules, CA), and transferred to polyvinylidene difluoride membranes (0.45 μm pore size, Millipore, Mississauga, ON). The membranes were first blocked with 5% non-fat powdered milk and then incubated with a primary antibody. NeuN (1:5000, Chemicon, Temecular, CA, USA) and anti-β-actin (1:10000, Sigma, St. Louis, MO, USA) antibodies were used. This incubation was followed by incubation with horseradish peroxidase-conjugated donkey anti-mouse antibody (1:10000). Signal detection was facilitated with enhanced chemiluminescence (ECL kit, Amersham, Oakville, ON). Immunoreactive bands were measured using Lab Works software (UVP, Upland, CA) and the densitometric values of NeuN were normalized

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[6] Dr. Feng Bao completed the experimental work and western blot analysis included in Appendix A. This work has been included in the present thesis with his permission.

against β-actin to control for variation in protein loading. Molecular weights of the proteins were determined using known molecular weight protein standards (BioRad Prestained Precision Protein Standards).

One-way ANOVAs, with treatment group as the between-subjects factor, were used to analyze NeuN expression at 24 hrs, 72 hrs, and 4 weeks post-injury. Fisher's LSD post hoc pair-wise comparisons were carried out when appropriate. Statistical significance was set at $p < .05$.

At 24 hrs post-injury the 1B7 group displayed less NeuN expression than the aCD11d and SHAM groups, as indicated by a significant *treatment* effect ($F(2, 14) = 10.424$, $p < .01$; 1B7 < both aCD11d and SHAM, all *ps* < .05; see Fig. A.1). The aCD11d group also displayed significantly less NeuN expression than the SHAM group ($p < .05$).

At 72 hrs post-injury the 1B7 group displayed less NeuN expression than the aCD11d and SHAM groups, as indicated by a significant *treatment* effect ($F(2, 14) = 28.959$, $p < .001$; 1B7 < both aCD11d and SHAM, all *ps* < .01; see Fig. A.1). The aCD11d group also displayed less NeuN expression than the SHAM group ($p < .01$).

At 4 weeks the 1B7 rats displayed less NeuN expression than the aCD11d and SHAM groups, as indicated by a significant *treatment* effect ($F(2, 14) = 12.519$, $p < .001$; 1B7 < both aCD11d and SHAM, all *ps* < .05; see Fig. A.1). The aCD11d group also displayed less NeuN expression than the SHAM group ($p < .05$).

These results suggest that the aCD11d group experienced significantly less neuronal loss than the 1B7 group and significantly more neuronal loss than the SHAM group at 24hr, 72 hrs, and 4 weeks after lateral fluid percussion injury.

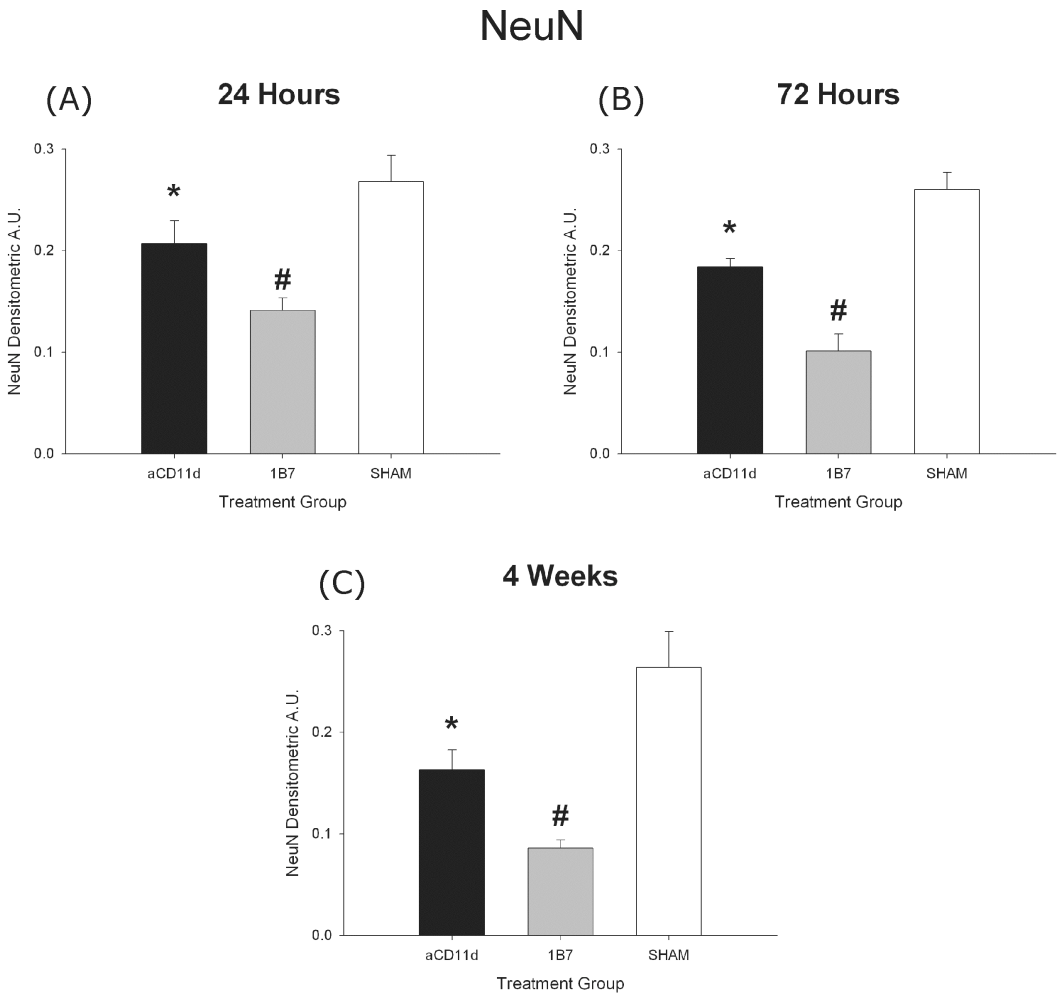

Figure A.1. Western blotting analysis of NeuN expression. (A) 24 hrs after injury the aCD11d group displayed more NeuN expression than the 1B7 group, and less NeuN expression than the SHAM group. (B) 72 hrs after injury the aCD11d group displayed more NeuN expression than the 1B7 group, and less NeuN expression than the SHAM group. (C) 4 weeks after injury the aCD11d group displayed more NeuN expression than the 1B7 group, and less NeuN expression than the SHAM group. Histogram bars represent mean percentage of β-actin densities (± SEM). # = different from the aCD11d and SHAM groups, *ps* < .05. * = different from the SHAM group, $p < .05$.

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Appendix B

Western

September 13, 2007

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2011

Dear Dr. Cain:

Your Animal Use Protocol form entitled:
Studies in Neural Plasticity
Funding Agency NSERC - Grant #A0669

has been approved by the University Council on Animal Care. This approval is valid from **September 13, 2007** to **September 30, 2008.** The protocol number for this project is **2007-082-09 and replaces 2003-073-09**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 1 YR.

Species	Strain	Other Detail	Pain Level	Animal # Total for 1 Year
Rat	Long-Evans Hooded	350 gm Male	D	60
Rat	Long-Evans Hooded	250-350 gm M/F	D	6
Mouse	PRODH	35 gm Male	D	24
Mouse	PRODH	35 gm M/F	D	60
Mouse	C57/BL 6	35 gm Male	D	24
Mouse	M-1836	35 gm Male	D	24
Mouse	M-1841	35 gm Male	D	24
Mouse	M-1836	35 gm M/F	D	60
Mouse	M-1841	35 gm M/F	D	60

STANDARD OPERATING PROCEDURES
Procedures in this protocol should be carried out according to the following SOPs. Please contact the Animal Use Subcommittee office (661-2111 ext. 86770) in case of difficulties or if you require copies.
SOP's are also available at http://www.uwo.ca/animal/acvs
310 Holding Period Post-Admission
320 Euthanasia
321 Criteria for Early Euthanasia/Rodents
330 Post-Operative Care/Rodent
343 Surgical Prep/Rodent/Recovery Surgery

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
1. As per recent requirements by the CCAC, please ensure that any electrical shock equipment previously used in psychology animal experiments have been removed or made non-functional.

c.c. Approved Protocol - P. Cain, J. Majewski
Approval Letter - J. Majewski

The University of Western Ontario
Animal Use Subcommittee/University Council on Animal Care
Health Sciences Centre • London, Ontario • CANADA - N6A 5C1
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Curriculum Vitae

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Post-secondary Education and Degrees:

The University of Saskatchewan
Saskatoon, Saskatchewan, Canada
2000 – 2005 B.A. Psychology

The University of Western Ontario
London, Ontario, Canada
2005-2007 M.Sc. Neuroscience

The University of Western Ontario
London, Ontario, Canada
2007-present Ph.D. Neuroscience

Honours and Awards:

NSERC Post-Graduate Scholarship
2008-2011

Western Research Scholarship
2005-2011

IBNS Travel Award
2010

Margaret Moffat Research Competition Winner
2010

Ontario Graduate Scholarship
2006-2008

Autism Ontario Scholarship
2007

IMFAR Scholarship
2007

CPA Certificate of Academic Excellence (Honours Thesis)
2005

NSERC USRA
2004-2005

Related Work Experience:

Honours Thesis Supervisor
The University of Western Ontario
2005-2010

Research Assistant
The Kilee Patchell-Evans Autism Research Group
2006-2008

Teaching Assistant
The University of Western Ontario
2005-2008

Teaching Assistant
The University of Saskatchewan
2004-2005

Research Assistant
Dr. Deborah Saucier, The University of Saskatchewan
2003-2005

Articles Published or Submitted:

Shultz, S.R., MacFabe, D.F., Foley, K.A., Taylor, R. and Cain, D.P. (2011). A single mild fluid percussion injury induces short-term behavioral and neuropathological changes in the Long-Evans rat: support for an animal model of concussion. Under revision for *Behavioural Brain Research*.

Shultz, S.R., MacFabe, D.F., Martin, S., Jackson, J., Taylor, R., Boon, F., Ossenkopp, K-P., Cain, D.P. (2009). Intracerebroventricular injections of the enteric bacterial metabolic product propionic acid impair cognition and sensorimotor ability in the Long-Evans rat: further development of a rodent model of autism. *Behav Brain Res, 200,* 33-41.

Shultz, S.R., MacFabe, D.F., Ossenkopp, K-P., Scratch, S., Whelan, J., Taylor, R., Cain, D.P. (2008). Intracerebroventricular injection of propionic acid, an enteric bacterial metabolic end-product, impairs social behavior in the rat: implications for an animal model of autism. *Neuropharmacology, 54,* 901–11.

Saucier, D.M., **Shultz, S.R.,** Keller, A.J., Cook, C.M., Binsted, G. (2008). Sex differences in object location memory and spatial navigation in Long-Evans rats. *Animal Cognition, 11,* 129-137.

Saucier, D.M., Yager, J.Y., Armstrong, E.A., Keller, A., **Shultz, S.R.** (2007). Enriched environment and the effect of age on ischemic brain damage. *Brain Research, 1170*, 31-38