The Role of Neuroinflammation in Traumatic Brain Injury-Induced Anxiety Disorders: a possible therapeutic target

by

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ABSTRACT

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Thesis directed by Professor Daniel S. Barth

Traumatic brain injury (TBI) is a rising public health concern, with approximately 1.7 million people in the United States alone sustaining a TBI each year (Vaishnavi et al., 2009; Faul, 2010). In addition to the physical, cognitive and behavioral impairments, the long-term consequences of TBI include increased risk of neuropsychiatric disorders, and anxiety disorders are among the most prevalent (Rao and Lyketsos, 2000; Moore et al., 2006; Vaishnavi et al., 2009). In spite of increasing evidence that anxiety disorders are elevated following TBI, very little is known about the etiology of post-traumatic anxiety.

Chronic neuroinflammation is now thought to contribute to the development of post-traumatic anxiety, the primary injury initiates complex cascades that can lead to secondary injury and worsened functional outcomes. These secondary cascades may contribute to the dysfunction of brain regions and neurotransmitter systems associated with anxiety following TBI. New evidence supports this hypothesis, including peripheral elevations of proinflammatory cytokines in patients with post-traumatic stress disorder (PTSD) and panic disorder (Spivak et al., 1997; Tucker et al.,

2004; von Kanel et al., 2007; Hoge et al., 2009) and anxiety-like behavior (Connor et al., 1998; Cragnolini et al., 2006; Sokolova et al., 2007; Zubareva and Klimenko, 2009) when these cytokines are administered in rats.

The goal of this thesis is to better understand the role of neuroinflammation in post-traumatic anxiety. The development of post-traumatic anxiety involves a number of complex molecular, cellular, neurochemical and physiological changes, and better understanding of these processes will be needed for successful treatment strategies in this population.

DEDICATION

I would like to dedicate this work to my two biggest cheerleaders, my mom and my best friend Patty Flueck. Without their support, laughter, confidence, and encouragement this journey would have never been possible.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

INTRODUCTION

Extensive psychological and neurobehavioral evidence indicates that TBI is a risk factor for the development of neuropsychiatric disorders (van Reekum et al., 1996; van Reekum et al., 2000; Fann et al., 2004; Hoge et al., 2008; Schneiderman et al., 2008; Whelan-Goodinson et al., 2009). TBI has often been described as a silent epidemic because many of the post-injury disabilities are neuropsychiatric in nature, and are not always apparent following primary injury (Rao and Lyketsos, 2000; Moore et al., 2006; Vaishnavi et al., 2009). The risk for development of psychiatric disorders is increased across the spectrum of TBI severity, from mild to severe. A large scale study of over 900 patients diagnosed with TBI found the prevalence of psychiatric illness at 1-year post-injury to be 49% in moderate to severe TBI and 34% in mild TBI, compared to 18% of controls (Fann et al., 2004). The higher incidence of post-traumatic stress disorder (PTSD) in patients with mild TBI has been reported in a number of studies (Bryant and Harvey, 1998; Hoge et al., 2008; Schneiderman et al., 2008).

Commonly reported syndromes are anxiety disorders, psychosis, depression and mood disorders (Rao and Lyketsos, 2000). Post-traumatic anxiety disorders are among the most prevalent, with rates ranging from 10-70% (see Table 1) across studies (Rao and Lyketsos, 2000; Hiott and Labbate, 2002; Vaishnavi et al., 2009). Studies report that both civilian and military patient populations diagnosed with TBI have increased risk for post-

traumatic anxiety, including generalized anxiety disorder (GAD), panic disorder (PD), obsessive/compulsive disorder (OCD) and PTSD. The rates of anxiety disorders reported by patients with TBI are consistently elevated relative to general population rates: 2.5% to 24% for GAD, compared with 5% in general population estimates; 6.7% to 16.7% for PD, compared with 0.8% to 3.5% in the general population; 1.6% to 7.8% for OCD, compared with 1.2% to 2.5% in the general population and 11% to 48% for PTSD, compared 8% in the general population (van Reekum et al., 2000; Hiott and Labbate, 2002; von Kanel et al., 2007; Vaishnavi et al., 2009).

TABLE 1. Prevalence, Core Features, and Treatment of Post-Traumatic Brain Injury (TBI) Psychiatric Disorders

Psychiatric Problems	Prevalence	Core Features	Correlates	First-Line Medication Management
Major depression	25%–50%	Episodes of sadness, negativism, loss of pleasure, feelings of hopelessness, and suicidal thoughts, with or without psychosis.	Lesions to left dorsolateral-frontal and/or basal ganglia regions. Poor pre-TBI psychosocial functioning.	Selective serotonin-reuptake inhibitors (SSRIs) or tricyclic antidepressants (TCAs).
Mania	1%–10%	Episodes of irritability and/or elated mood, increased energy, and impulsivity, with or without psychosis.	Lesions to temporal lobe and right-orbitofrontal cortex.	Valproate, lithium
Psychosis	3%–8%	Loss of touch with reality, disorganized thought process, and presence of hallucinations and/or delusions.	TBI before adolescence. Congenital neurological disorder.	Second-generation neuroleptics
Cognitive impairment	25%–70%	Inattention, difficulty learning new information, and inability to process information and problem-solve; executive dysfunction.	TBI severity	Psychostimulants, cholinesterase inhibitors, amantadine
Anxiety	10%–70%	Feeling of apprehension or dread with or without autonomic signs and symptoms. Anxiety with feelings of re-experiencing trauma, avoidant behavior, emotional numbing, and hypervigiliance is called posttraumatic stress disorder (PTSD).	Lesions to the right hemisphere in anxious depression. PTSD more common with mild TBI.	SSRIs, short-term benzodiazepines
Apathy	10%	Lack of motivation and initiative in the absence of dysphoria.	Damage to the mesial frontal lobe and its subcortical structures.	Psychostimulants, dopamine agonists
Insomnia	30%–70%	Problems falling asleep, staying asleep, or early morning awakenings.	Mild TBI, depression, and pain.	Sleep hygiene before initiation of medications: trazodone, mirtazapine
Aggression	30%	Verbal outbursts, use of profanity, destruction of property, violent attacks on others.	Pre-injury history of substance abuse and aggressive behavior; frontal lobe injuries.	Beta-blockers, valproate, psychostimulants; SSRIs

(Vaishnavi, et al., 2009)

Studies also indicate that prevalence rates continue to increase years after injury (Morton and Wehman, 1995; Deb et al., 1999; Koponen et al., 2002). Evidence for long-term risk in the development of post-traumatic anxiety was well documented in a study of TBI patients that were assessed 30 years following injury, the results indicated that almost half (48.3%) of TBI participants developed a psychiatric disorder following injury, and almost half (23.3%) of the reported disorders were anxiety disorders, showing the importance of psychiatric follow-up and chronic treatment management after injury (Koponen et al., 2002). Higher rates of anxiety-like behaviors have also been seen in recently developed models of post-traumatic anxiety in rodents.

Rat behavior typically associated with anxiety has been reported across a range of injury models including: impact-acceleration, pneumatic cortical contusions, controlled cortical impact and lateral fluid percussion injury (LFPI). Animal models of post-traumatic anxiety have revealed elevations in marble-burying behavior and decreases in social interaction and exploratory behavior in the open field test, following impact-acceleration injuries, (Fromm et al., 2004; Pandey et al., 2009). Reduced exploratory behavior in the open field test has been reported following pneumatic cortical contusions and controlled cortical impact (Sonmez et al., 2007; Wagner et al., 2007). Following LFPI, significant decreases in exploration of the open field and elevated plus maze were reported (Jones et al., 2008).

More recent models have shown increases in both conditioned (Reger et al., 2012) and unconditioned (Rodgers et al., 2012) fear responses to both learned and novel stimuli following LFPI. Evidence of anxiety-like behavior following experimental TBI in rats suggests that animal models may be useful for exploring basic mechanisms of post-traumatic anxiety observed in humans. Yet in spite of reports that post traumatic anxiety is elevated in both humans and rats, little is known about the pathogenesis of anxiety following TBI. Increasing evidence now implicates excessive inflammatory actions of the neuroimmune system and a generalized inflammatory state in the development of anxiety disorders following TBI (Spivak et al., 1997; Gasque et al., 2000; Tucker et al., 2004; Shiozaki et al., 2005; von Kanel et al., 2007; Hoge et al., 2009).

NEUROINFLAMMATION FOLLOWING TBI

Neuroinflammation involves a complex integration of immediate local inflammatory responses by cells of the CNS (including neurons, macroglia, and microglia), followed by systemic recruitment of immune cells including macrophages, T cells and B cells (Medzhitov and Janeway, 1998; Lo et al., 1999; Carson et al., 2006). These inflammatory responses are initiated through intracellular signaling cascades immediately following cellular injury and damage to tissue. The primary injury is due to mechanical forces that occur at the time of the initial insult, resulting in focal (contusion, brain

laceration, intracerebral hemorrhage, subarachnoid or subdural hemorrhage) and/or diffuse (damage to blood vessels, ischemic infarct and axonal shearing) brain trauma (Rao and Lyketsos, 2000; Werner and Engelhard, 2007).

DANGER SIGNALS

Neuroimmune responses are initiated by both exogenous and endogenous danger signals, which are not present in healthy cells or those undergoing normal physiological deaths (Matzinger, 2001). Exogenous danger signals alert the organism to invading pathogens and involve pathogen-associated molecular patterns (PAMPs), which are a diverse set of microbial molecules that share various recognizable biochemical features (Bianchi, 2007; Namas et al., 2009). The inflammatory responses generated by PAMPs usually include inflammation and immunity; however, trauma is another cause of tissue and cell damage that involves another form of danger signaling (Bianchi, 2007).

Stressed, damaged, and injured cells release endogenous danger signals termed ‘alarmins’, which also trigger the local inflammatory response needed for tissue repair and regeneration (Gallucci and Matzinger, 2001; Oppenheim and Yang, 2005; Oppenheim et al., 2007; Hirsiger et al., 2012). Molecules in this class of inflammatory mediators include: High-mobility Group Box 1 (HMGB1), S100A and B, Uric acid, IL-1β, heat shock proteins,

and a growing list of additional molecules (Namas et al., 2009). Alarmins share similar structure and function with PAMPs and both share the same receptors, pattern recognition receptors (PRRs), which sense and respond to both exogenous and endogenous danger signals (Adib-Conquy and Cavaillon, 2007). Since alarmins are thought to be the endogenous equivalent of PAMPs, they are sometimes collectively classified as damage-associated molecular patterns (DAMPs), which play an important role in the propagation of the proinflammatory cascade of innate immunity through the production of cytokines and other inflammatory mediators (Bianchi, 2007; Namas et al., 2009).

Once activated, DAMPs initiate the innate immune response through the activation of antigen presenting cells (APCs), which then upregulate costimulatory and major histocompatibility complex (MHC) molecules (Gallucci and Matzinger, 2001; Matzinger, 2002a; Hirsiger et al., 2012). APCs respond to exogenous and endogenous signals through toll-like receptors (TLRs) and cytostolic nucleotide-binding oligomerization domain (NOD) receptors, which recognize a variety of PAMPs and act as PRRs for both exogenous and endogenous molecules. For example, TLR4 is a receptor for both LPS (exogenous bacterial product) and heat shock protein 70 (Hsp70, an endogenous proinflammatory protein), while TLR2 binds to exogenous bacterial lipoproteins and endogenous Hsp60 (Matzinger, 2002b). Microglia are the resident immunological cells and primary APCs of the CNS,

these cells remain quiescent until activated through TLRs engagement with DAMPs to perform effector inflammatory and APC functions (Olson and Miller, 2004).

Danger signaling from injured cells makes the neuroinflammatory response at the site of injury a potential source of activated microglia and astrocytes, which secrete multiple growth factors and cytokines that may lead to glial scar formation, preventing neuronal regeneration and functional recovery in damaged areas (Lotan and Schwartz, 1994). It has been well established that microglia and astrocytes are activated during the innate immune response to injury, leading to the expression of high levels of proinflammatory cytokines (Fan et al., 1996; Aloisi, 2001; Allan et al., 2005; Shiozaki et al., 2005; Riazi et al., 2008b), most notably interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). Given that these cytokines participate in autonomic, neuroendocrine and behavioral responses to brain injury, destabilize neurotransmitter release and re-uptake, negatively impact neuronal integrity and survival, and initiate neurotoxic processes, they may contribute to functional alterations of brain areas involved in post-traumatic anxiety-like behavior (Sternberg, 1997; Raison and Miller, 2003; Szelenyi and Vizi, 2007).

Glial activation is normally neuroprotective (Aloisi, 2001; Farina et al., 2007a); however, the chronic inflammatory responses and exaggerated proinflammatory cytokine levels observed following injury initiate neurotoxic

processes resulting in secondary tissue damage (Gasque et al., 2000; Simi et al., 2007; Hailer, 2008; Lehnardt, 2010), neuronal death (Sternberg, 1997; Brown and Bal-Price, 2003; Schmidt et al., 2005; Beattie et al., 2010), secondary injury cascades (Bains and Shaw, 1997; Cernak et al., 2001a, b; Ansari et al., 2008a, b) and neuronal hyperexcitability (Hailer, 2008; Riazi et al., 2008b; Rodgers et al., 2009; Beattie et al., 2010). In addition, human and animal studies have shown that neuroinflammation is an ongoing process that persists for months to years following the injury (Gentleman et al., 2004; Streit et al., 2004; Nagamoto-Combs et al., 2007; Ramlackhansingh et al., 2011), which could predispose TBI patients to develop anxiety disorders through the chronic release of proinflammatory cytokines.

INFLAMMATION AND ANXIETY

Evidence for chronic inflammation has been seen in a number of studies examining patients with anxiety disorders, reporting peripheral elevations of TNF-α, IFNγ, IL-1β and IL-6 in patients with PTSD (Spivak et al., 1997; Rohleder et al., 2004; Tucker et al., 2004; von Kanel et al., 2007) and elevations of TNF-α and IL-6 in patients with OCD (Konuk et al., 2007). A recent examination of cytokine levels in individuals with panic disorder (PD) or PTSD diagnosis, revealed significantly higher mean levels of 18 of 20 cytokines, as compared to healthy controls (Hoge et al., 2009). Another

study found elevated pro-inflammatory cytokine levels in patients with PTSD, and pro-inflammatory markers were found to correlate with PTSD symptoms indicating that inflammatory markers could possibly be utilized in diagnostic situations (Kanel et al., 2007).

Studies have shown that administration of proinflammatory cytokines in rats increases anxiety-like behavior in commonly used tests of anxiety. Central administration of TNF-α and IL-1β was found to increase anxiogenic effects in the elevated plus maze (Connor et al., 1998). Other reports indicate that systemic co-administration of TNF-α and IL-1β synergistically increases plasma corticosterone concentrations (Brebner et al., 2000). Postnatal administration of TNF-α has been found to induce anxiety-like behavior in elevated plus maze and open field exploration (Zubareva and Klimenko, 2009). Sub-pyrogenic doses of IL-1β resulted in increased anxiety-like behavior in the open field test within 5 days of treatment (Sokolova et al., 2007). Intracerebroventricular administration of IL-1β was found to induce anxiety-like behavior in the open field and elevated plus maze (Song et al., 2003). In addition, *in vivo* microdialysis studies in rats report elevated levels of IL-1β and TNF-α within hours of brain trauma (Woodroofe et al., 1991). Increased levels of activated microglia and astrocytes, IL-1β, TNF-α and IL-6 have been reported following controlled cortical impact and weight drop injury in rats (Chen et al., 2007; Homsi et al., 2009; Li et al., 2009; Homsi et al., 2010).

Overall, the findings of increased proinflammatory cytokine levels in anxiety disorders, increased anxiety-like symptoms following the administration of these cytokines in rats, and reports of elevated microglia, astrocytes, IL-1β, TNF-α and IL-6 in closed head injury models in rodents provide evidence of the role of the neuroimmune system in the pathophysiology of post-traumatic anxiety. The secondary outcomes of neuroinflammation following TBI may contribute to damage and dysfunction in brain regions associated with anxiety, as TBI has been found to induce both acute and chronic neurodegeneration that could be caused by delayed cellular death pathways initiated by complex signaling cascades in activated glial cells (Smith et al., 1997; Bendlin et al., 2008).

BRAIN STRUCTURES INVOLVED IN ANXIETY

As discussed above, TBI initiates many harmful biochemical cascades, these secondary injury pathways include inflammation, oxidative stress, increased vascular permeability, mitochondrial dysfunction, changes in neuronal excitability and neuroexcitotoxic damage (Aschner et al., 1999; Werner and Engelhard, 2007), which may be the key factor in hyperexcitability and damage to the brain regions associated with anxiety.

Extensive evidence has documented associations between PTSD and mild TBI, but due to high overlap in symptoms affecting brain function, many studies exclude TBI patients with histories of psychiatric or

neurological illness. In spite of substantial neuroimaging studies examining TBI only or PTSD only, there are no imaging studies that have compared the co-occurrence of PTSD and TBI (Dolan et al., 2012). The only study found that assessed any comorbid anxiety disorder was a comparison of ten patients with TBI and OCD, and the findings implicated dysfunction of frontal-subcortical circuits (Berthier et al., 2001). However, the patients had many comorbid conditions, including PTSD, depression, generalized anxiety, and aggressive personality change. In order to understand the complexities of post-traumatic anxiety there is a critical need for neuroimaging studies to compare patients with singular affective diagnoses and TBI, which may not be possible because the risk of multiple neuropsychiatric disorders are increased following TBI. Until these studies exist, neuroimaging data from studies assessing anxiety disorders may be the best indication of structural and functional alterations in post-traumatic anxiety.

Neuroimaging research in patients with obsessive/compulsive disorder, phobia and PTSD have revealed that aversive anticipation (a hallmark of anxiety) involves increased activation of the insula and amygdala (Simmons et al., 2006). The findings indicate that the amygdala may initiate the fear response after the detection of threat cues, while visceral changes within the insula contribute to an anxious state of aversive anticipation.

MRI studies have reported structural, neurochemical and functional abnormalities of medial prefrontal cortex in patients with PTSD, including

anterior cingulate cortex (Shin et al., 2006; Shin and Liberzon, 2010). Medial prefrontal cortex is involved in executive functions, decision-making, problem solving and mental flexibility, while the anterior cingulate modulates social comportment, motivation, reward and emotional behaviors (McAllister, 2011). Functional neuroimaging has shown diminished responses in medial prefrontal cortex, which is thought to play a role in personality changes and deficits in response inhibition and emotional modulation in a variety of anxiety disorders (Shin et al., 2006).

Neuroimaging studies of the hippocampus have found decreased functioning and volume in patients with PTSD, which is thought to be a risk factor for the development of pathological stress responses (Bremner et al., 1995; Sapolsky, 2000; Shin et al., 2006). Additionally, greater severity of PTSD symptoms has been associated with decreased hippocampal volumes (Shin et al., 2006).

NEUROTRANSMITTER DYSFUNCTION IN ANXIETY

Studies of the neurobiology of anxiety disorders implicate dysfunction of four primary neurotransmitters and receptors in the development and maintenance of anxiety disorders; these include serotonin (5-HT), noradrenaline (NE), dopamine (DA) and gamma aminobutyric acid (GABA). Dysregulation may have important consequences for behavioral homeostasis and the development of psychiatric sequelae, as these systems are involved

in mood regulation, arousal and cognition (McAllister, 2011). Hyperactive 5-HT and NE circuits between the cortex, thalamus, periaqueductal grey region, locus coeruleus, insula, amygdala and hypothalamus are implicated in generalized anxiety and panic disorders (Gorman et al., 2000; Stein, 2009).

Overactivity of DA neurons in the substantia nigra and excitatory glutamatergic projections from the cortex, thalamus and hippocampus are thought to produce excessive excitation in brain areas (cortex, thalamus, basal ganglia and striatum) implicated in obsessive compulsive disorder, while hypofunction of 5-HT neurons from the dorsal raphe nucleus may result in a lack of inhibition (Stein, 2000; Pittenger et al., 2011). The regions involved in memory processing in PTSD include the hippocampus, amygdala, prefrontal, orbitofrontal and anterior cingulate cortices, while the heightened stress response is thought to involve the NE, DA and 5-HT projections in thalamus, hypothalamus and locus coeruleus (Newport and Nemeroff, 2000; Nutt, 2000; McAllister, 2011). However, in spite of substantial neuroimaging and neurochemical reports, the cellular mechanisms potentially leading to TBI-induced neurochemical, structural, and functional abnormalities in these structures are poorly understood. However, we can gain some insight from treatments that are based on immune suppression, which are leading to better functional outcomes following TBI.

IMMUNE BASED TREATMENTS

Treatments targeting the neuroimmune system in cognitive dysfunction and motor impairments following TBI have shown decreases in activated microglia and astrocytes, IL-1β, TNF-α and IL-6 in controlled cortical impact and weight drop TBI models in rodents (Chen et al., 2007; Homsi et al., 2009; Li et al., 2009; Homsi et al., 2010). Immunosuppression has been found to be neuroprotective following TBI in rodents, resulting in increased structural preservation and improved functional outcomes (Hailer, 2008).

Minocycline, a tetracycline antibiotic with anti-inflammatory effects has been found to suppress microglial production of IL-1β, TNF-α and IL-6 in TBI models in rodents resulting in reduced cerebral edema and brain lesion volumes (Homsi et al., 2009; Homsi et al., 2010). The mechanism of action in minocycline involves inhibition of matrix metalloproteinases and p38 mitogen-activated protein kinase, resulting in inhibition of leukocyte recruitment (Hailer, 2008). Statins, a class of lipid-lowering drugs have been reported to suppress microglial production of IL-1β, TNF-α and IL-6 in TBI models in rodents, resulting in reductions of functional deficits and anatomical damage (Chen et al., 2007; Li et al., 2009). Statins show promise for treatment following TBI, mainly due to statins multifactorial effects, including the ability to serve as antioxidants, inhibitors of

inflammatory responses, immunomodulatory agents, and regulators of progenitor cells (Vink and Nimmo, 2009).

Cyclosporin A and FK506, are potent immunosuppressant drugs that have been found to suppress activation of microglia and astrocytes following lateral fluid percussion brain injury in rats, resulting in improved mitochondrial preservation, reductions in dendritic spine loss, and improvements cognitive performance and functional motor recovery (Alessandri et al., 2002; Campbell et al., 2011). The mechanisms of action in cyclosporine and FK506 treatments involve inhibition of calcium/calmodulin-dependent calcineurin activation and reduction in the expression of genes encoding various proinflammatory cytokines (Hailer, 2008; Vink and Nimmo, 2009). Steroid treatment with dexamethasone has been found to inhibit microglial proliferation and secretion of proinflammatory cytokines (TNF-α and IL-6) following experimental contusion injury in rats, resulting in reduced edema formation (Holmin and Mathiesen, 1996). Steroids mechanism of action is thought to involve nuclear modulation of gene expression (Hailer, 2008). The immunosuppressant drugs discussed above have direct inhibitory effects on microglia and astrocytes, leading to reductions in the secretion of proinflammatory cytokines following TBI. Immunosuppression of neuroinflammation may be a possible therapeutic target in the treatment of

anxiety disorders following TBI, therefore we designed 3 experiments to examine post-traumatic anxiety.

The introduction summarized the relevant data leading to the experiments conducted. Specific focus was directed towards the prevalence of anxiety disorders following TBI in humans, animal models of post-traumatic anxiety, the pathophysiology of post-traumatic anxiety, neuroinflammation, danger signals, reactive gliosis, proinflammatory cytokines, secondary injury cascades, brain regions involved, and possible preventative strategies. The development of post-traumatic anxiety involves a number of complex molecular, cellular, neurochemical and physiological changes, and future treatments will be needed that target multiple injury endpoints for successful pharmacological treatment in this population. Chronic inflammation plays an important role in the development of post-traumatic anxiety in humans and animals, and immunosuppression may be a key factor in attenuating dysregulation of the neuroimmune system and the subsequent development of post-traumatic anxiety. The goal of this thesis is to better understand the role of neuroinflammation in the development of anxiety disorders following TBI. The following chapters will demonstrate: 1) hyperexcitability in cortex following an immune challenge, 2) TBI resulting in anxiety-like behaviors in rats and peri-injury immunosuppressant treatment aimed at prevention, and 3) delayed, post-injury immunosuppressant treatment aimed at reversing established anxiety-like behaviors and reactive

gliosis in brain regions associated with human anxiety following TBI. The following study will illustrate hyperexcitability in the cortex following lipopolysaccharide (LPS) challenge to show that glial cell activation is sufficient for cortical excitability and disruption of normal cortical function.

CHAPTER II

. The cortical innate immune response increases local neuronal excitability leading to seizures

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ABSTRACT

Brain glial cells, five times more prevalent than neurons, have recently received attention for their potential involvement in epileptic seizures. Microglia and astrocytes, associated with inflammatory innate immune responses, are responsible for surveillance of brain damage that frequently results in seizures. Thus, an intriguing suggestion has been put forward that seizures may be facilitated and perhaps triggered by brain immune responses. Indeed, recent evidence strongly implicates innate immune responses in lowering seizure threshold in experimental models of epilepsy; yet, there is no proof that they can play an independent role in initiating seizures in vivo. Here, we show that cortical innate immune responses alone produce profound increases of brain excitability resulting in focal seizures. We found that cortical application of lipopolysaccharide (LPS) triples evoked field potential amplitudes and produces focal epileptiform discharges. These effects are prevented by pre-application of LPS-RS. Our results demonstrate how the innate immune response may participate in acute seizures, increasing neuronal excitability in response to TLR4 detection of the danger signals associated with infections of the central nervous system and with brain injury. These results suggest an important role of innate immunity in epileptogenesis and focus on glial inhibition, through pharmacological blockade of TLR4 and the pro-inflammatory mediators released by activated glia, in the study and treatment of seizure disorders in humans.

INTRODUCTION

Epileptic seizures affect 0.5-1.5% of the global population and remain the subject of concentrated neuroscientific investigation. Because seizures are characterized by pathologically synchronized interactions between neurons, epilepsy research remains justifiably "neurocentric" (Robert, 2008). Yet, glial cells far outnumber neurons in the forebrain and are perfectly situated to modulate neuronal function by encapsulating neuronal synapses and maintaining neurotransmitter balance (Araque et al., 1999; Halassa et al., 2007). With recent reports of dynamic neuro-glial interactions (Fields and Stevens-Graham, 2002), the possible modulating role of glial astrocytes in epileptogenicity has received intense interest (Zimmer et al., 1997; Wang and White, 1999; Plata-Salaman et al., 2000; Vezzani et al., 2000; Oprica et al., 2003; Rizzi et al., 2003; Vezzani, 2005; Halassa et al., 2007; Choi and Koh, 2008; Ravizza et al., 2008; Robert, 2008; Vezzani et al., 2008).

Since glial cells also comprise the brain's immune system, it has been proposed that some seizures may result from brain immune responses (Vezzani, 2005; Ravizza et al., 2008; Riazi et al., 2008a). If so, this would suggest the involvement of astrocytes but, even more so, it would predict the involvement of microglia, a second major class of glial cells which has received less attention by epileptologists. This would be suggested because microglia are distinguished from astrocytes in their primary responsibility for immune surveillance of invading pathogens and danger signals produced by

brain damage (Aloisi, 2001), damage that frequently results in seizures. Relatively quiescent in their basal state (Davalos et al., 2005; Nimmerjahn et al., 2005), microglia are activated by central infection, trauma, and ischemia, releasing pro- and anti-inflammatory cytokines, chemokines, prostaglandins, and nitric oxide that comprise an early inflammatory response (innate immunity; (Nadeau and Rivest, 2000)).

Recent evidence indicates that chemically induced visceral inflammation is correlated with widespread activation of cortical microglia, by unknown mechanisms, and decreased thresholds for generalized pentylenetetrazole-induced seizures that can be reversed by cortical application of the anti-inflammatory microglial inhibitor minocycline (Riazi et al., 2008a). These results strongly implicate microglia and the accompanying innate immune response in the facilitation of generalized seizures when the cortex is challenged by convulsant compounds. While comorbity between peripheral inflammation and epilepsy has been decribed (Tellez-Zenteno et al., 2005), more importantly, these results raise the possibility that localized and direct activation of brain innate immunity, characteristic of more commonly observed cortical insult and focal epilepsy, could participate in epileptogenesis. It is possible that innate immune responses alone, in the absence of convulsive agents and prior seizures, could independantly increase cortical excitbility and serve as a source as well potentiator of epileptogenesis. To explore this possibility, we used field potential mapping

of sensory evoked potentials and spontaneous activity in rat somatosensory cortex to quantify changes in brain excitability associated with innate immune responses *in vivo*, induced by direct and focal cortical application of LPS.

MATERIALS AND METHODS

Evoked Potential Studies

Surgery

All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory animals in biological research. Seven adult male Sprague-Dawley rats (300-400 g) were anesthetized to surgical levels using an intraperitoneal injection of ketamine-xylazine-acepromazine (45-9-1.5 mg/kg body weight), placed on a regulated heating pad, and maintained with subsequent injections throughout the experiment so that the eye blink reflex could be barely elicited. While ketamine has been shown to attenuate LPS induced inflammatory responses of glial cultures (Shibakawa et al., 2005), it was required here to provide adequate anesthesia for the large bilateral craniotomy required for hemispheric mapping. The effects reported here may have been larger without ketamine. A bilateral craniotomy was performed over both hemispheres extending 2 mm rostral to bregma to 1 mm caudal of lambda and from the mid-sagittal suture to 2 mm below the

zygomatic arch, exposing a maximal area of the surgically accessible cortex. The dura was reflected and the exposed cortex regularly doused with Ringer Solution containing: NaCl 135 mM; KCl 3 mM; MgCl 2 mM; and CaCl 2 mM – pH 7.4 at 37º C.

Experimental procedures

Somatosensory responses were evoked by electrical stimulation of the forepaw that was shaved and coated with conductive jelly. A bipolar electrode (500 µm tips; 2 mm separation) attached to a constant current source delivered biphasic current pulses (1.0 ms; 0.5-1.0 mA) to the exposed skin. Auditory click stimuli (0.1 ms monophasic pulses) were delivered using a high frequency piezoelectric speaker placed approximately 15 cm lateral to the contralateral ear.

Epipial maps of auditory and somatosensory evoked potentials (AEP and SEP, respectively) were recorded using a flat multi-electrode array consisting of 256 stainless steel wires in a 16 × 16 grid (tip diameter: ~100 µm; inter-electrode spacing: 400 µm) covering a 6.0 × 6.0 mm area of the left hemisphere in a single placement. The array was pressed against the cortex with sufficient force to establish contact of all electrodes. The required pressure had no effect on evoked potential amplitude, post-stimulus latency, or morphology when compared to potentials recorded previously with more lightly placed small arrays. While both the surgical procedures (i.e.

craniotomy and dura removal) have the potential for producing inflammatory effects, we have not noted any progressive changes in SEP amplitudes, morphology, or spatial distribution for hours after this procedure in other studies, unlike the acute SEP changes induced by LPS application in this experiment. Recordings were referred to a stainless steel electrode secured over the frontal bone, and were simultaneously amplified (×2000; NerveAmp, Center for Neural Recording, Washington State University, Pullman, WA), analog filtered (band-pass cut-off = -6 db at 0.1 to 3000 Hz, roll-off = 5 db/octave) and digitized at 10 kHz. Evoked potentials were averaged over 32 stimulus presentations.

Regions of auditory and somatosensory cortex were estimated from interpolated (bicubic spline) maps of the root mean squared (RMS) power of the evoked potential (an example of the mapping procedure is shown in Fig. 1). Evoked potentials were recorded 1 min prior to, and 5, 10, 15, 20, 25 and 30 min subsequent to epicortical suffusion (0.02 µg in 10 µl saline) of the TLR4 agonist, LPS (Sigma, St. Louis, MO), alone (left hemisphere), or LPS after pretreatment with TLR4 antagonist, LPS-RS (LPS derived from the photosynthetic bacterium *Rhodobacter Sphaeroides*; InvivoGen, SanDiego, CA; right hemisphere). Manufacturer recommended concentrations of LPS for adequate stimulation of TLR4, and of LPS-RS to block these effects, are .0001 - .01 µg/µl. Other reports where LPS was injected into cortex have used concentrations ranging from .002 – 1.25 µg/µl and doses of .002 -5 µg

to effect inflammation (Andersson et al., 1992; Park et al., 2005; de Pablos et al., 2006; Park et al., 2007). Our low LPS concentration of .002 μg/μl and high concentration of .02 μg/μl (for epileptiform spikes and seizures; see below) were at the low end of previous reports. If all of the LPS penetrated the cortex, our low and high doses of LPS were .02 and .2 μg, which is also at the low end of the dose range from previous reports. Because the precise spread and penetration of epipially suffused LPS used here were not known, these doses should be considered approximate, but were chosen because they produced consistent functional results across animals.

LPS-RS antagonizes toxic LPS in both human and murine cells and also prevents LPS-induced shock in mice (Qureshi et al., 1999). The main mechanism it uses to block LPS-dependent activation of TLR4 consists of direct competition between under-acylated LPS and hexa-acylated LPS for the same binding site on MD-2, while the secondary mechanism involves the ability of under-acylated LPS:MD-2 complexes to inhibit hexa-acylated endotoxin:MD-2 complexes function at TLR4 (Saitoh et al., 2004; Coats et al., 2005; Teghanemt et al., 2005; Visintin et al., 2005). Suffusion of 10 μl, for both LPS and LPS-RS solutions, was chosen because this quantity completely saturated a 4x4 mm pledget of filter paper used to confine applications in our pilot studies. However, direct suffusion was used in the present experiment to permit immediate recording, presumably covering a wider cortical area but sufficiently confined to the approximate area of

somatosensory cortex as to produce no effects in auditory cortex, only 4 mm lateral to the application site (see Results). Here, and in all subsequent cortical applications, 10 µl of dimethyl sulphoxide (DMSO; Sigma, St. Louis, MO; 2.5% in saline) was first applied.

Epilepsy Studies

Surgery

An additional 12 adult male Sprague-Dawley rats (300-400 g) were anesthetized to surgical levels using an intraperitoneal injection of xylazine-acepromazine (X-A; 9-1.5 g/kg body weight) then supplemented with isoflurane (2%) for the duration of surgery (approximately 30 min). At the end of surgery, all surgical wounds were injected with lidocaine (2%) and isoflurane was discontinued. Frequent supplements of X-A were administered for the duration of the experiment (2-3 hr), resulting in a highly sedated but unanesthetized preparation. Both eye blink and toe pinch withdraw reflexes could be easily elicited, but no voluntary movements were observed, and animals were left unrestrained during recording. Recording commenced 1 hr following termination of isoflurane. The purpose of this anesthesia regime was to eliminate ketamine, which can influence epileptogenicity (Velisek et al., 1993; Borowicz and Czuczwar, 2003; Midzyanovskaya et al., 2004) and also attenuate LPS induced inflammatory responses of glial cultures

(Shibakawa et al., 2005). Isoflurane was also cleared because of its possible effects on epileptogenicity (Veronesi et al., 2008).

Surgery differed from the prior evoked potential preparation only in that the craniotomy was smaller (extending from bregma to lambda and from the mid-sagittal suture to 2 mm past the temporal ridge) to accommodate a smaller recoding array (8 × 8 electrodes; 500 μm spacing; 3.5 × 3.5 mm recording area).

Experimental procedures

In 6 animals, a unilateral craniotomy over the left hemisphere was performed and only spontaneous activity recorded (i.e. no evoked potentials). One min of spontaneous activity was recorded prior to, and at 10 min intervals for 30 min following, epipial application of LPS (0.2 μg in 10 μl saline). In an additional 6 animals, bilateral craniotomies were performed. One min of spontaneous activity was recorded prior to and immediately following epipial application of IL-1ra (Amgen, Thousand Oaks, CA; 100 μg in 10 μl saline). In two subsequent 15 min intervals, IL-1ra was reapplied along with LPS (0.2 μg in 10 μl saline) and 1 min of spontaneous activity recorded immediately before and after each application. In the right hemisphere, 1 min of spontaneous activity was recorded before, and at 15 min intervals for 30 min following LPS application (no IL-1ra treatment). If no epileptiform spikes were observed by 15 min following the first LPS

application, the same amount of LPS was applied a second time. This was required in 2 of the animals. Following recording, spontaneous activity in the right hemisphere was silenced by epipial application of lidocaine hydrochloride (2%). $GABA_A$ receptor antagonist bicuculline methiodide (BMI; Sigma, St. Louis, MO; 10 μM in 0.9% NaCl) was then applied (10 μl) to the left hemisphere to produce a simple model of cortical epilepsy independent of neuro-glial effects. One min epochs of spontaneous activity were recorded in 10 min intervals for approximately 20 min following application of BMI.

In 4 of the bi-hemisphere surgical preparations, SEPs were obtained for comparison to the previous study. Whisker stimulation was provided by 0.1 ms pulses delivered to a solenoid with attached 3 cm armature constructed from hypodermic tubing. Single whisker displacements were approximately 0.5 mm on the rostro-caudal axis. Whisker stimulation (contralateral to the recorded hemisphere) was used instead of electrical forepaw stimulation because it did not disturb the animals or elicit reflexive responses. Whisker evoked SEPs (32 trials) were obtained immediately following each recording of spontaneous activity.

Analysis

Changes in the SEP were quantified in three ways. First, the RMS power of SEP was computed at single electrode sites in the left and right hemisphere. Electrode sites were chosen based on those revealing the

largest amplitude response in the pre-treatment baseline. Second, to examine changes in the spatial distribution or area of the SEP independent of overall power, the responses across the array were normalized to the maximum power for a given condition (latency pre- or post-treatment) and then the number of electrode sites reaching at least 50% of this maximum were counted to determine active area in sq mm. Finally, total SEP power in each condition was summed across all electrodes of the array. This measure combined changes in power at each electrode with changes in the area of active cortex. Significant differences in SEP amplitude and spatial distribution are reported as the mean ± standard error and evaluated using a two-way repeated measures ANOVA followed by pair-wise post-hoc comparisons.

RESULTS

Innate immune responses increase cortical excitability

The SEP evoked by forepaw stimulation in untreated cortex (Fig. 1.1A; blue traces; averaged across 7 animals) consisted of a typical biphasic (positive/negative) slow wave of maximum amplitude at electrode sites in the dorsal and rostral region of a 16×16 epipial electrode array used to map the entire hemisphere, centered on the forepaw representation of primary somatosensory cortex. Click stimulation produced an AEP (Fig. 1.1A; red traces) of maximum amplitude at electrode sites covering primary and

secondary auditory cortex in the ventral and caudal area of the array. To better visualize the amplitude distribution of evoked responses, the RMS power of the waveform at each electrode site was computed, normalized to the electrode with the highest power, and displayed as an interpolated map for the SEP and AEP (Fig. 1.1B and C, respectively). In these and subsequent maps, a ratunculus has been superimposed as an anatomical reference, reflecting the complete body representation of primary and secondary somatosensory cortex (SI and SII, respectively). The ratunculus was adapted from a previous study (Benison et al., 2007) since comprehensive mapping was not performed in the present experiment. The SEP was of largest power over approximately 7 sq mm of the forepaw representation of SI, and the AEP covered a smaller region in auditory cortex (Fig. 1.1C; Aud).

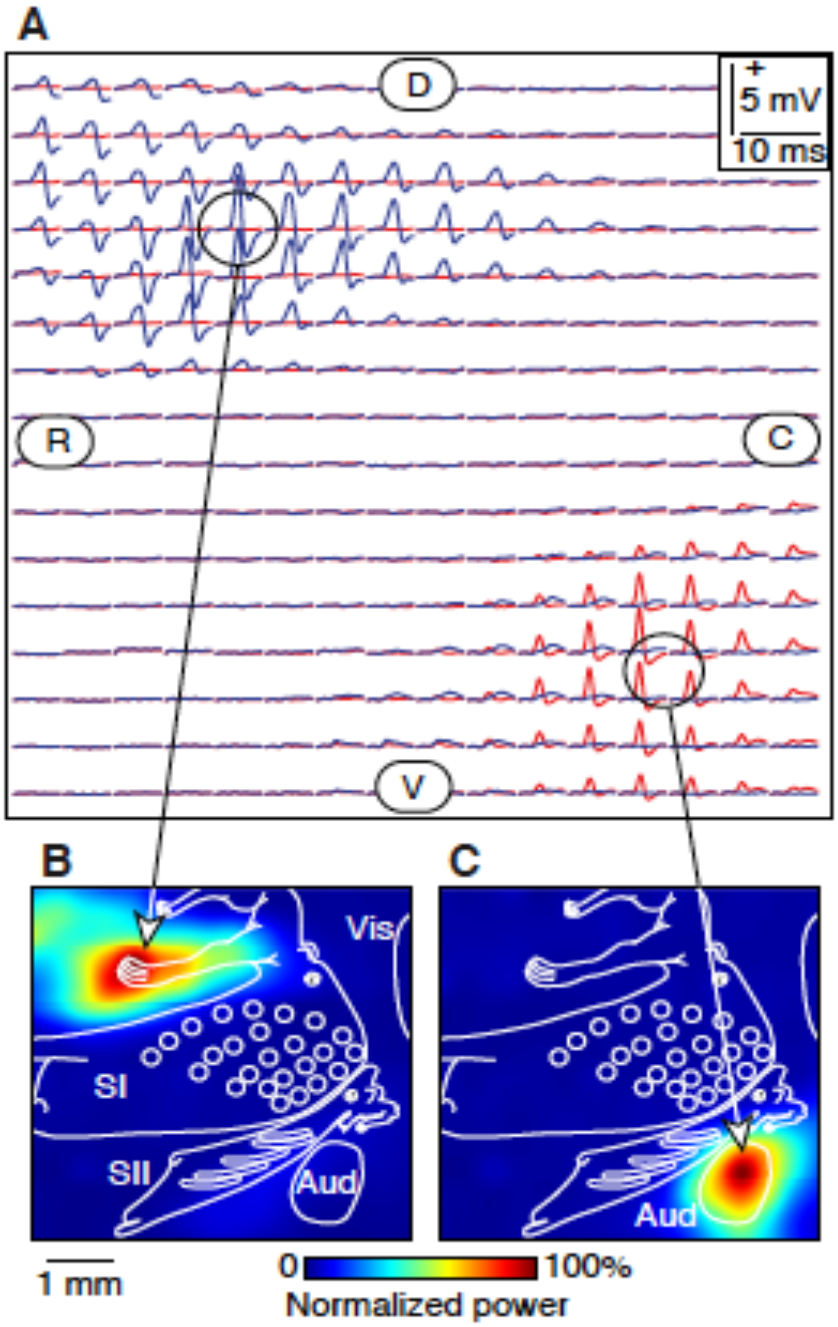

Figure 1.1 Hemispheric epipial mapping of averaged SEP and AEP recorded from the left hemisphere of seven animals. (A) The SEP produced by forepaw stimulation (blue traces) and AEP (red traces) produced by click stimulation. Rostral, caudal, dorsal and ventral are labeled R, C, D and V, respectively. (B, C) RMS power of the waveform at each electrode site displayed as an interpolated map for the SEP and AEP, respectively. Superimposed is a ratunculus, reflecting the complete body representation.

The same SEP map as shown in Figure 1.1B is depicted again in Figure 1.2A (left column, top), representing the baseline response prior to cortical application of LPS. Here, the amplitude of the response appears lower than in Figure 1.1 because the values were not normalized to the largest response within the map, but rather, to the largest response across experimental conditions and hemispheres. Local suffusion of LPS (0.02 µg in 10 µl saline) at the site of the forepaw representation in the left hemisphere markedly increased cortical excitability, reflected by increases of both the amplitude and spatial distribution of the SEP. By 20 min these parameters had nearly tripled (Fig. 1.2A; left column).

Excitability changes are specific to TLR4

Pre-application of LPS-RS prevented LPS induced excitability changes in the contralateral hemisphere (Fig. 1.2A; right column). However, both the spatial distribution and amplitude of the SEP remained unchanged from baseline. Thus, LPS-RS had only a blocking effect of LPS activation through antagonism of TLR4 and no direct effect on cortical excitability, demonstrating no detectable basal TLR4 activity or that which could potentially have been induced by the surgery. AEP in both hemispheres remained stable (Fig. 1.2B), suggesting that LPS spread remained largely confined to somatosensory cortex, although this was not histologically verified.

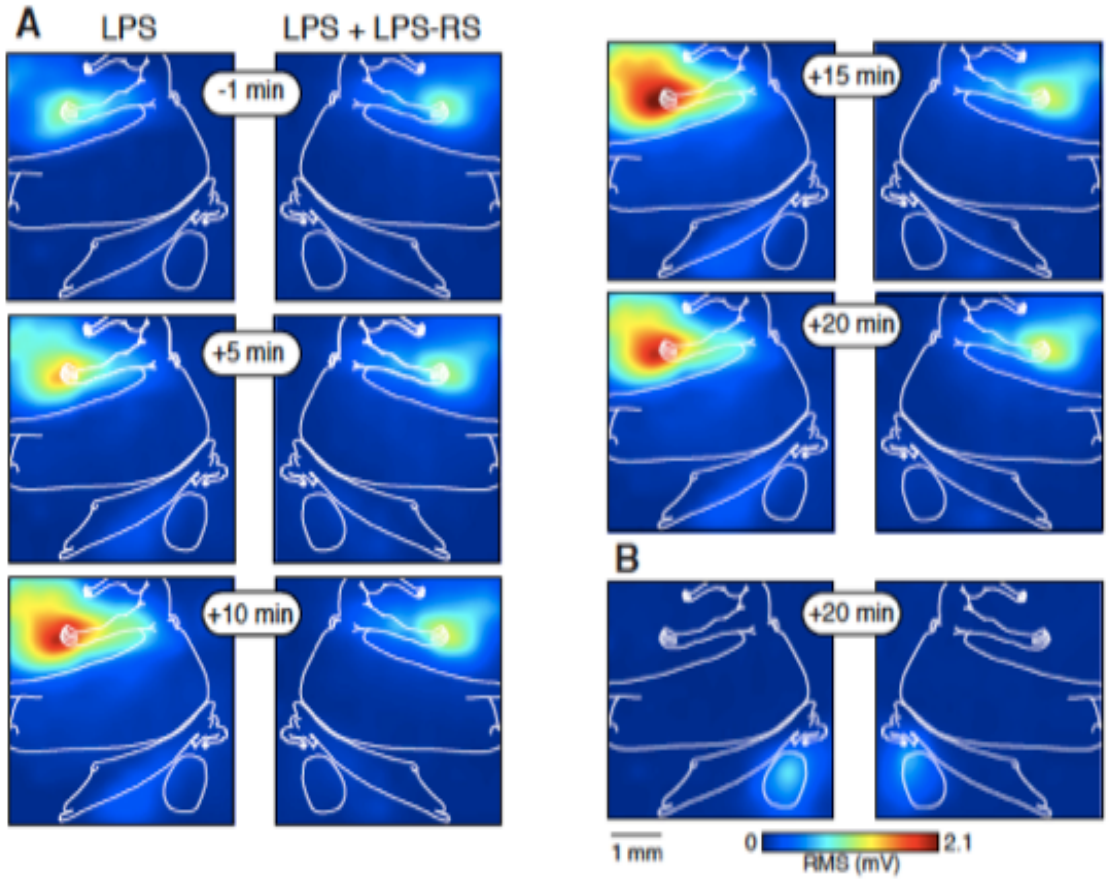

Figure 1.2 Increases in SEP power and area produced by LPS. (A) The left column of maps displays the grand average SEP evoked by forepaw stimulation at time points 1 min prior to, and 5, 10, 15 and 20 min subsequent to application of LPS. Right hemisphere was treated with an LPS-RS before application of LPS (right column). LPS-RS prevented the increased neuronal excitability produced by LPS without affecting the amplitude or area of normal SEPs. (B) Mapping of auditory AEPs in both hemispheres following LPS and LPS-RS application revealed no change from baseline.

Quantitative analysis of SEP changes at single electrode sites in the left hemisphere (treated with LPS alone) and right hemisphere (treated with LPS + LPS-RS), indicated significant effects of hemisphere group ($p<.005$), time after LPS application ($p<.001$), and an interaction between the two factors ($p<.002$). Pair-wise comparisons revealed no significant increases in SEP power from pre-treatment baseline when TLR4 activation was prevented with LPS-RS. There were also no significant decreases observed from the pre-treatment baseline (Fig. 1.3A; light grey bars). In contrast, SEP amplitude increased significantly and steadily with LPS alone (Fig. 1.3A; dark grey bars) compared to LPS+LPS-RS, reaching significance at 10 min. While there was a slight decrease in excitability at 20 min, this was not significant, and measurements conducted as late as 30 min revealed sustained power increase. To examine increases in the spatial distribution or area of the SEP, independent of overall power, the responses across the array were normalized to the maximum power for a given condition (latency pre- or post-treatment) and then the number of electrode sites reaching at least 50% of this maximum were counted to quantify the active area in sq mm. With measurements of spatial distribution, there were significant effects of hemisphere group ($p<.009$), time after LPS application ($p<.01$), and an interaction between the two factors ($p<.002$). Pair-wise comparisons revealed that increases in the area of active cortex with LPS compared to the LPS-RS control mirrored those of power increases with the exception that

significant changes were detected as early as 5 min post-treatment (Fig. 1.3B). Finally, SEP power summed across all 256 electrodes of the array, which combined changes in power at each electrode with changes in the area of active cortex (Fig. 1.3C), produced significant effects of hemisphere group ($p<.007$), time after LPS application ($p<.0001$), and an interaction between the two factors ($p<.0002$). Pair-wise comparisons mirrored increases of separate analyses of SEP amplitude and spatial distribution with LPS alone or LPS+LPS-RS.

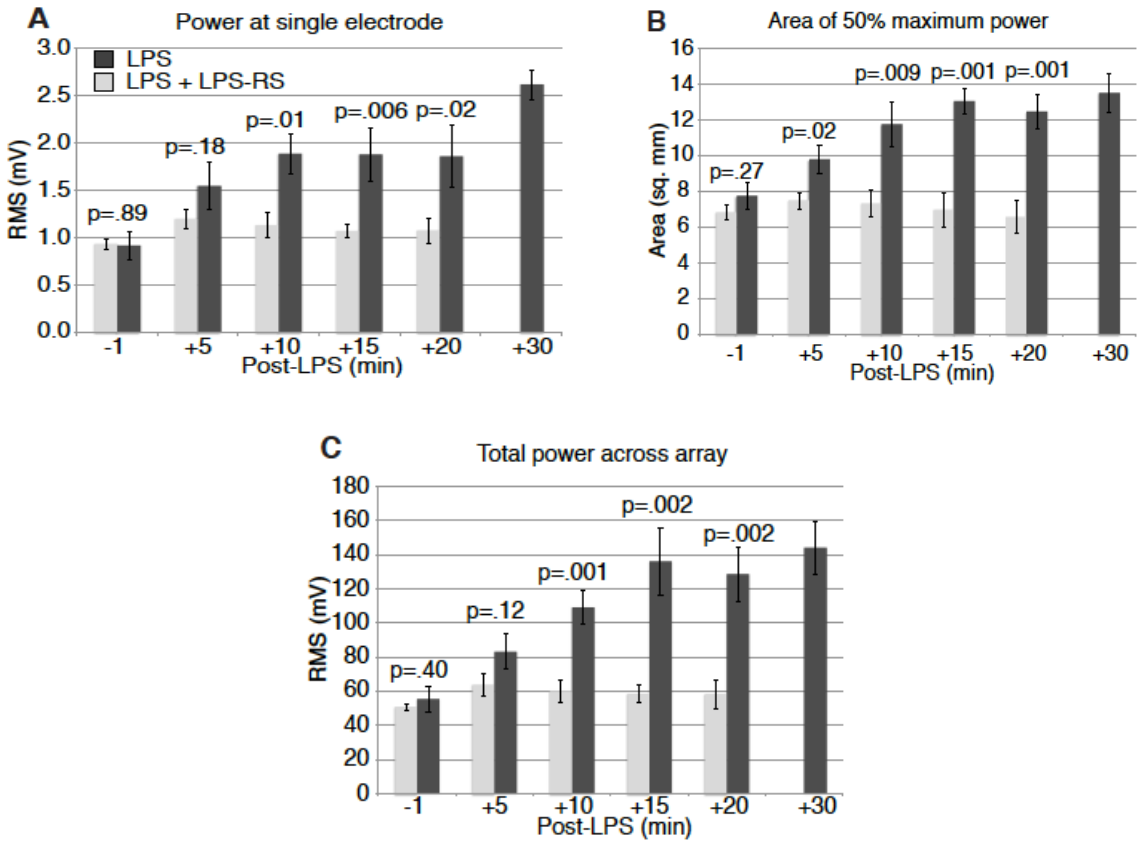

Figure 1.3 Quantitative analysis of SEP changes induced by LPS. (A) The RMS power of SEP at a single electrode site in the left hemisphere and right hemisphere. While no significant increases in SEP power were seen when TLR4 were blocked with LPS-RS (light grey bars), neither were there any significant decreases from the pre-treatment baseline. In contrast, SEP amplitude increased significantly and steadily with LPS alone (dark grey bars). (B) To examine increases in the spatial distribution or area of the SEP, independent of overall power, the responses across the array were normalized to the maximum power for a given condition (latency pre- or post-treatment) and then the number of electrode sites reaching 550% of this maximum were counted to determine active area in sq mm. Increases in the area of active cortex with LPS compared with the LPS-RS control mirrored those of power increases (A) with the exception that significant changes were detected as early as 5 min post-treatment. (C) Similar to (A), but reflecting SEP power summed across all 256 electrodes of the array. This measure combines changes in power at each electrode (A) with changes in the area of active cortex (B).

Innate immune responses produce spontaneous epileptiform discharge and seizures

Epicortical application of more concentrated LPS (0.2 µg in 10 µl saline) in an additional 12 animals produced spontaneous epileptiform discharge (Fig. 1.4A a-l) within 10-20 min Three animals exhibited motor characteristics of focal seizures, with extension and superimposed twitching of the tail, contralateral hindpaw and/or forepaw. Ipsilateral motor involvement was not observed. Electrographic records were characterized by periodic episodes of high frequency spikes (Fig. 1.4A a-c; 164±3.2 spikes per min; 3.5±.25 mV peak to peak) followed by suppression of all spontaneous activity for 10-30 sec, but no distinct tonic/clonic phases typical of more generalized seizures. Seizures recurred at approximately 1-2 min intervals for the 30 min recording period. The remaining 9 animals (Fig. 1.4A d-l) only exhibited continuous lower frequency epileptiform spikes (38.8±.25 spikes per min; 3.3±.41 mV) during this period with no accompanying motor signs.

IL-1 is involved in innate immune control of cortical excitability

Epicortical application of IL-1ra prior to LPS prevented both seizures and epileptiform spikes in all animals in which it was tested (Fig. 1.4B) for the entire recording period, indicating IL-1 as an important mediator of LPS induced epileptiform activity in these animals. In 4 bilateral preparations

(surgically exposed right and left hemispheres; Fig. 1.4B; lower 4 traces) prevention of epileptiform spiking was achieved with IL-1ra in the left hemisphere while subsequent LPS application to the untreated contralateral hemisphere resulted in substantial epileptiform discharges (corresponding to Fig. 1.4A a, d, e and h, respectively). Yet, effects of IL-1ra were selective to LPS induced hyper-excitability. Reduced inhibition effected by application of $GABA_A$ receptor antagonist BMI to the IL-1ra treated cortex resulted in epileptiform spikes (42.1±2.75 spikes per min; 4.5±.16 mV peak to peak), demonstrating that, despite previous findings showing that IL-1ra over-expressing mice are less susceptible to BMI induced seizures (Vezzani et al., 2000), neural circuits remained capable of paroxysmal synchronization when sufficiently challenged (Fig. 1.4B; right traces). Indeed, SEP amplitudes in the bilateral animals did not differ from baseline after application of either IL-1ra or subsequent application of IL-1ra + LPS in the left hemisphere (Fig. 1.4C; light grey bars), suggesting normal cortical excitability was maintained despite the IL-1ra treatment. In contrast, LPS application to the untreated contralateral hemisphere resulted in significantly increased SEP amplitude (Fig. 1.4C; dark grey bars).

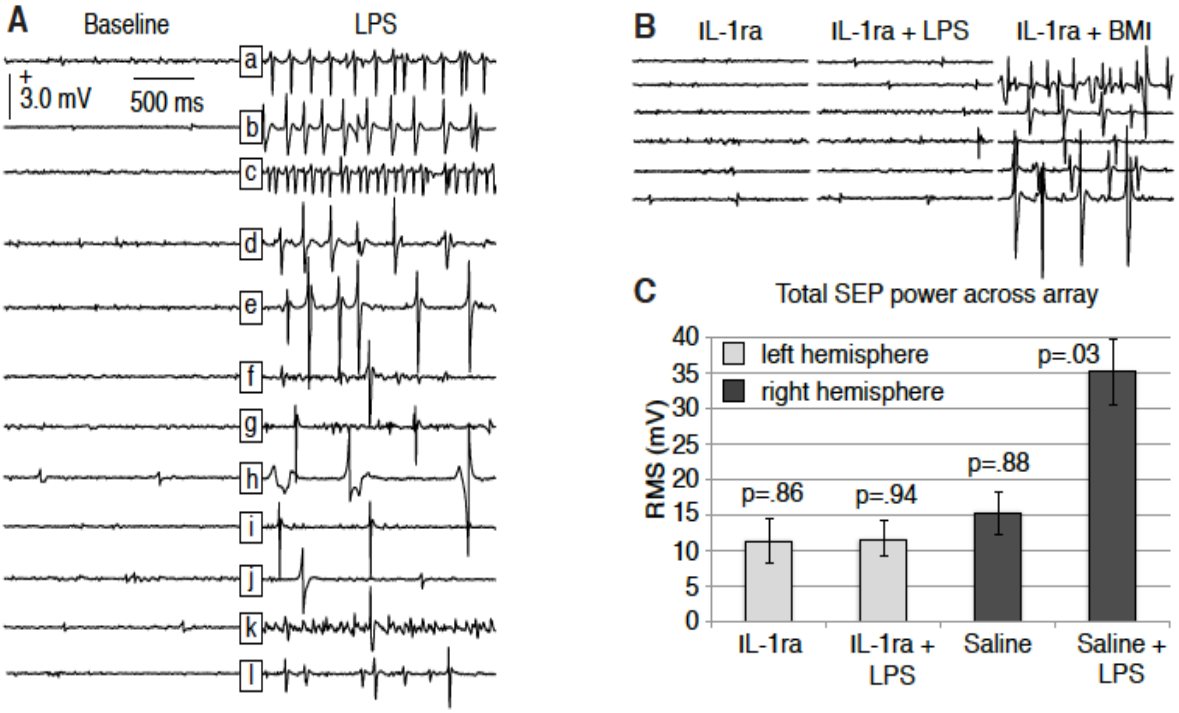

Figure 1.4 Increased excitability and epileptiform spikes produced by higher LPS concentrations are due to glial release of IL-1. (A) Spontaneous activity recorded from an electrode in the center of somatosensory cortex before (left) and 30–40 min after application of higher concentrations of LPS. The top three animals (a–c) exhibited seizures, the others (d–l) produced only lower frequency epileptiform discharges. (B) Application IL-1ra prevented LPS-induced hyper-excitability. Spontaneous activity, recorded up to an hour following LPS administration (middle traces), was indistinguishable from baseline (left traces). However, BMI remained effective in evoking epileptiform discharges (right traces). (C) In four animals with bi-hemispheric surgeries, LPS approximately doubled the SEP amplitude in the untreated right hemisphere (dark grey bars), whereas pre-application of IL-1ra to the left hemisphere maintained normally responsive SEPs in the presence of LPS (light grey bars).

DISCUSSION

Glial cells have long been thought to provide only metabolic/structural support in the cerebral cortex and, until recently, have been largely ignored with respect to their potential influence on normal or abnormal electrical activity of neural circuits. With insults to the brain, such as percussive or penetrating wounds, tumor, infarct and infection, glial cells accumulate in the regions of damage (reactive gliosis) for the purpose of repair and reconstruction (Sofroniew, 2005). However, as early as 1970 (Pollen and Trachtenberg, 1970), it was suspected that astrocytes in regions of cortical damage may also function abnormally and alter the excitability of neurons sufficiently to trigger acute or chronically occurring epileptic seizures. This glial hypothesis of epilepsy was based on the theory that damaged astrocytes in an epileptic focus may fail to buffer excess extracellular K+ accumulated near active neurons, resulting in hyper-excitability and epileptic seizures.

While the glial/K+ buffering theory of epilepsy fell out of favor for several decades for lack of strong supporting evidence (however, see (Frohlich et al., 2008) the potential role of glia in epileptogenesis has received a resurgence of interest with discovery of neuro-glial interactions. The continued focus of this work has been on astrocytes since these are known to surround the synaptic junctions between nerve cells, forming a tripartite synapse (Araque et al., 1999; Halassa et al., 2007) that not only

can buffer K+, but can respond to, release, and remove chemical transmitters in these critical zones. It is possible that dysfunction of astrocytes may disrupt regulatory control of neuronal circuits, resulting in runaway excitation (i.e. seizures (Parpura et al., 1994). In the same light, a repertoire of recently discovered glio-transmitters and proinflammatory cytokines (IL-1 and tumor necrosis factor-α [TNFα]), that can be released by microglia, astrocytes and endothelial cells influencing neuronal excitability, are under intense investigation for their possible contributions to epileptogenesis (Merrill, 1992; Vitkovic et al., 2000).

The present data indicate that the innate immune response to TLR4 ligands, most likely involving activated microglia, results in increased neuronal excitability sufficient to trigger seizures *in vivo*. While microglia are regarded to be the major cell type in the CNS capable of transducing LPS signals (Lehnardt et al., 2002), TLR4 have also been described in astrocyte cultures (Bowman et al., 2003; Carpentier et al., 2005; Borysiewicz et al., 2008; Li et al., 2008) and LPS stimulation of cultured astrocytes results in production of IL-1 and TNFα (Lieberman et al., 1989; Chung and Benveniste, 1990; Bowman et al., 2003; Borysiewicz et al., 2008; Konat et al., 2008). While TLR4 on astrocytes have not yet been demonstrated *in vivo* (Farina et al., 2007b), their contribution to the LPS induced seizures reported here is a distinct possibility. It has also been shown that, although fluorescently tagged LPS binds to microglia but not to neurons (Lehnardt et al., 2003),

neurons can express and up-regulate TLR4 in response to ischemia (Tang et al., 2007). Yet, unlike microglia and astrocytes, neuronal responses to LPS have not been demonstrated (Tang et al., 2007). This does not rule out the potential contribution of neuronal TLR4 to pathological excitability in situations of brain trauma where ischemia is a distinct possibility. Similarly, while it is not clear that TLR4 expression on endothelial cells in their basal state would be sufficient to contribute to LPS effects observed in the present study (however, see (Zeuke et al., 2002), TLR4 is markedly up-regulated in endothelial cells of brain capillaries following subarachnoid hemorrhage (Zhou et al., 2007). Given the capacity of micro-vascular endothelial cells to secrete proinflammatory cytokines IL-1 and TNFα (Behling-Kelly et al., 2007), they may also contribute to neuronal hyper-excitability following traumatic injury.

The remarkably rapid onset of both LPS induced SEP increases and epileptiform spikes demonstrated here (<10.0 min) may, at least in part, have been facilitated by the requisite tissue trauma and elapsed time of surgical preparation, priming the cells for upregulation of cytokines. However, in this study, significant changes in excitability were not detectable until administration of LPS and associated cytokine release. In astrocytic cultures, LPS exposure times as short as 15 min result in a sharp upregulation of IL-1ß, however the time course of the response is slow, requiring 24 hr to reach maximum (Krasowska-Zoladek et al., 2007). Yet,

studies concerned with the time-course of cytokine expression evoked by LPS focus on changes with a temporal resolution of hours and not minutes. Our data suggest functional responses to LPS reach significance at latencies as short as 5 min, and are sustained for at least an hr (the longest time point evaluated here) without abatement. This rapid LPS response mirrors *in vitro* results from LPS application to slices of adult rat parietal cortex, resulting in increased glutamate and noradrenaline within 10 min, potentially resulting in rapid increases of neuronal excitability (Wang and White, 1999).

Glia are clearly activated following seizures in experimentally induced epileptic foci, producing elevated IL-1 and TNFα (Minami et al., 1991; Zimmer et al., 1997; Plata-Salaman et al., 2000; Oprica et al., 2003; Rizzi et al., 2003). Our results support the possibility that IL-1 is an important glia mediated proinflammatory cytokine participating in these excitability changes since IL-1ra prevented the effects of LPS, a finding in close agreement with reports of anticonvulsant actions of IL-1ra in experimental models of epilepsy (Vezzani et al., 2000). However, IL-1 is only one of several proinflammatory cytokines capable of increasing neuronal excitability and lowering seizure threshold. For example, CNS inflammation triggered by peripheral chemically-induced visceral inflammation is correlated with widespread activation of cortical glial cells, decreased thresholds for generalized PTZ-induced seizures, increased levels of TNFα, and a marked increase in seizure threshold with central antagonism of TNFα (Riazi et al.,

2008a). IL-1 released by microglia may increase neuronal excitability through its activating effect on astrocytes, imbalancing their control of glutamate homeostasis at pyramidal synaptic junctions (Choi and Koh, 2008). However, IL-1 released by microglia and/or astrocytes may also have direct effects on neuronal channels and excitability. It is notable that studies of seizure induced IL-1 expression reveal rapid dynamics in agreement with the LPS induced excitability effects shown here. Expression of IL-1 has been detected within 30 min of PTZ injection and rapid onset (5-10 min post-injection) seizures (Minami et al., 1990). While the molecular mechanisms of the convulsive actions of IL-1 are not known, such a rapid time-course would suggest a non-transcriptional glia to neuron pathway such as recently reported by Balosso and coworkers (Balosso et al., 2008).

Ravizza and colleagues (Ravizza et al., 2008) have hypothesized that persistent inflammation may be a fundamental mechanism of chronic epilepsy. Our data indicate that early inflammation associated with innate immunity produces large but short-term increases in neuronal excitability. Early inflammatory responses to traumatic brain injury (D'Ambrosio et al., 2004) and to infection (Vezzani and Granata, 2005), modeled in this study by LPS activated innate immunity via TLR4, result in the glial release of a variety of proinflammatory cytokines. The rapid modulation of brain excitability demonstrated here would be expected to result in inflammation-induced decreases in seizure threshold, as observed by others in a variety of

experimental epilepsy models (Vezzani et al., 2008). However, this rapid modulation may also serve as an acute source of epileptiform neuro-excitability with sufficient activation of the innate immune response. In this light, the innate immune response may be viewed, not just as a consequence and possible facilitator of seizures, but also as a potential precursor to seizures. The results suggest an important role of innate immunity and focus on glial inhibition, through pharmacological blockade of TLR4 and the proinflammatory mediators released by activated glia, in the study and possible prevention of epilepsy in humans.

This work established that glial cells are sufficient to produce excitability and disruption of normal cortical function, and that glial inhibition was able to block the development of cortical excitability. We used these results as an indication that immunosuppression may be able to attenuate injury-induced cortical excitability in brain regions associated with anxiety, thus reducing the subsequent development of post-traumatic anxiety behaviors following lateral fluid percussion injury (LFPI) in rats. The following chapter details LFPI-induced increases in anxiety-like behavior and reactive gliosis in brain regions associated with anxiety in humans, in a rodent model of post-traumatic anxiety. Immunosuppressant treatment resulted in significant attenuation of behavioral and immunohistological impairments.

CHAPTER III

Acute neuroimmune modulation attenuates the development of anxiety-like freezing behavior in an animal model of traumatic brain injury

Rodgers, K.M., Bercum, F.M., McCallum, D.L., Rudy, J.W., Frey, L.C., Johnson, K.W., Watkins, L.R. and Barth, D.S. (2012). J. Neurotrauma, Jul 1;29(10):1886-97.

ABSTRACT

Chronic anxiety is a common and debilitating result of traumatic brain injury in humans. While little is known about the neural mechanisms of this disorder, inflammation resulting from activation of the brain's immune response to insult has been implicated in both human TBI-induced anxiety and in recently developed animal models. In this study, we used a lateral fluid percussion injury (LFPI) model of traumatic brain injury in the rat and examined freezing behavior as a measure of TBI-induced anxiety. We found that LFPI produced anxiety-like freezing behavior accompanied by increased reactive gliosis (reflecting neuroimmune inflammatory responses) in key brain structures associated with anxiety: the amygdala, insula and hippocampus. Acute peri-injury administration of Ibudilast (MN166), a glial cell activation inhibitor, suppressed both reactive gliosis and freezing behavior, and continued neuroprotective effects were evidenced several months post-injury. These results support the conclusion that inflammation produced by neuroimmune responses to traumatic brain injury play a role in TBI-induced anxiety, and that acute suppression of injury-induced glial cell activation may have eventual promise for prevention of TBI-induced anxiety in humans.

INTRODUCTION

The long-term consequences of traumatic brain injury (TBI) include heightened risk of neuropsychiatric disorders, of which anxiety disorders are the most prevalent (Rao and Lyketsos, 2000; Moore et al., 2006; Vaishnavi et al., 2009). Studies of the etiology of anxiety disorders implicate exaggerated responses of the amygdala and insula (Rauch et al., 1997; Simmons et al., 2006; Stein et al., 2007; Shin and Liberzon, 2010; Carlson et al., 2011), impaired inhibition of medial prefrontal cortex and anterior cingulate (Davidson, 2002; Shin et al., 2006; Milad et al., 2009; Shin and Liberzon, 2010) and decreased hippocampal volume (Bremner et al., 1995; Sapolsky, 2000; Shin et al., 2006). Yet, whether and how TBI induces neurochemical, structural, and functional abnormality in these structures is poorly understood.

There is increasing evidence that excessive inflammatory actions of the neuroimmune system may contribute to the development of anxiety disorders following TBI (Spivak et al., 1997; Gasque et al., 2000; Tucker et al., 2004; Shiozaki et al., 2005; von Känel et al., 2007; Hoge et al., 2009). Microglial cells are the first line of defense and primary immune effector cells in the CNS and respond immediately to even small pathological changes from damaged cells, producing proinflammatory cytokines and toxic molecules that compromise neuronal survival (Gehrmann, 1996; Gonzalez-Scarano and Baltuch, 1999; Aloisi, 2001; Town et al., 2005). This rapid

microglial response often precedes the more delayed, yet prolonged activation of astrocytes and is thought to be involved with the onset and maintenance of astrogliosis (Graeber and Kreutzberg, 1988; McCann et al., 1996; Hanisch, 2002; Iravani et al., 2005; Herber et al., 2006; Zhang et al., 2010). It has been well established that microglia and astrocytes are activated during the innate immune response to brain injury, leading to the expression of high levels of proinflammatory cytokines, most notably interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). While glial activation is typically neuroprotective (Aloisi, 2001; Farina et al., 2007a), the chronic inflammatory responses and exaggerated proinflammatory cytokine levels observed following injury initiate neurotoxic processes resulting in secondary tissue damage (Gasque et al., 2000; Simi et al., 2007; Hailer, 2008; Lehnardt, 2010), neuronal death (Sternberg, 1997; Brown and Bal-Price, 2003; Schmidt et al., 2005; Beattie et al., 2010), secondary injury cascades (Bains and Shaw, 1997; Cernak et al., 2001a, b; Ansari et al., 2008a, b) and neuronal hyperexcitability (Hailer, 2008; Riazi et al., 2008b; Rodgers et al., 2009; Beattie et al., 2010), all of which may contribute to the dysfunction of brain regions associated with anxiety.

Recently developed animal models of post-traumatic anxiety (O'Connor et al., 2003; Vink et al., 2003; Fromm et al., 2004; Sönmez et al., 2007; Wagner et al., 2007; Jones et al., 2008; Baratz et al., 2010; Liu

et al., 2010) permit examination of the possible contributions of brain inflammation. Tests of post-traumatic anxiety in these models have typically included standard measurements of exploratory preference in mildly stressful environments, such as an open-field or elevated-plus testing apparatus. However, it has been frequently noted that measures of exploratory preference may be confounded by a marked overall decrease in exploration in brain-injured animals (O'Connor et al., 2003; Vink et al., 2003; Fromm et al., 2004). Decreased exploration cannot be attributed to TBI-induced motor deficits since numerous studies report only transient (~ 1 week) deficits following trauma (Yan et al., 1992; Taupin et al., 1993; Dixon et al., 1996; Fassbender et al., 2000; Goss et al., 2003; Cutler et al., 2005; Cutler et al., 2006b; Cutler et al., 2006a; Kline et al., 2007; Wagner et al., 2007; Bouilleret et al., 2009; Frey et al., 2009; Baratz et al., 2010; Liu et al., 2010). Rather, TBI-induced decreases in exploration have been attributed to the indirect effects of freezing (a primary component of the rodent's natural defensive behavior repertoire; Blanchard and Blanchard, 1988), suggesting an abnormally heightened response to stress in brain-injured rats (O'Connor et al., 2003; Vink et al., 2003; Fromm et al., 2004).

Based on these results, we tested the hypothesis that trauma-induced innate immune responses contribute to the development of anxiety-like behaviors in rats by directly examining freezing responses to a minor (novel environment) and major (foot-shock) stressor following Lateral Fluid

Percussion Injury (LFPI; a clinically relevant animal model of human closed head injury). We also tested the effectiveness of a glial cell activation inhibitor, Ibudilast (MN166), in attenuating post-injury freezing behavior and reducing reactive gliosis in brain regions associated with hyperexcitability in anxiety disorders.

MATERIALS AND METHODS

Sixty adult viral-free male Sprague-Dawley rats (275-325g; Harlan Laboratories, Madison, WI) were housed in pairs in temperature (23 ± 3 °C) and light (12:12 light: dark) controlled rooms with *ad libitum* access to food and water. All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory rats in biological research. Rats were randomly assigned to 1 of 10 groups (n = 6/group). Six groups (surgically naïve, sham operated, sham operated+vehicle, sham operated+MN166, LFPI+vehicle and LFPI+MN166) were shocked immediately after behavioral testing at 1 month post-surgery (sham operation or LFPI in the experimental rats). Surgically naïve rats received no injections or surgery, whereas sham operated rats received surgery but were not injected, the final 4 groups received sham or LFPI surgery and either vehicle injections or MN166 treatment. Another 4 groups (sham operated+vehicle, sham operated+MN166, LFPI+vehicle and LFPI+MN166) were run separately in a sucrose preference test to assess

anhedonia (the inability to experience pleasure, a core symptom of human depression) without exposure to stressors (anxiety tests and foot shock).

Lateral Fluid Percussion Injury

LFPI rats were anesthetized with halothane (4% induction, 2.0-2.5% maintenance) and mounted in a stereotaxic frame. The lateral fluid percussion injury used in this study has been described previously (McIntosh et al., 1989; Thompson et al., 2005; Frey et al., 2009) utilizing a PV820 Pneumatic PicoPump (World Precision Instruments, Inc., Sarasota, FL) to deliver standardized pressure pulses of air to a standing column of fluid. A 3.0 mm diameter craniotomy was centered at 3 mm caudal to bregma and 4.0 mm lateral of the sagittal suture, with the exposed dura remaining intact. A female Luer-Loc hub (inside diameter of 3.5 mm) was secured over the craniotomy with cyanoacrylate adhesive. Following hub implantation, the animal was removed from the stereotaxic frame and connected to the LFPI apparatus. The LFPI apparatus delivered a moderate impact force (2.0 atmospheres; 10 ms). The injury cap was then removed, scalp sutured and the rats returned to their home cages for recovery. Sham operated rats underwent identical surgical preparation, but did not receive the brain injury.

Ibudilast (MN166) administration

MN166 (MediciNova, San Diego, CA) is a relatively non-selective phosphodiesterase inhibitor with anti-inflammatory actions via glial cell attenuation, which has been found to reduce glia-induced neuronal death through the suppression of nitric oxide, reactive oxygen species, and proinflammatory mediators (Mizuno et al., 2004; Rolan et al., 2009). Treated rats received a 5-day dosing regimen of once-daily MN166 injections (10 mg/kg, 1 ml/kg subcutaneously in corn oil) 24 hr prior to LFPI, the day of surgery and LFPI, and 3 days following LFPI. Weight was recorded prior to each dosing and treatment administered at the same time each day to maintain constant levels across a 24 hr period. Dose selection was based on prior animal pharmacology results (Ellis AL, SFN, 2008b) showing MN166 to be safe and well tolerated, yielding plasma concentration-time profiles commensurate with high dose regimens in clinical development. MN166 administered via this regimen yields plasma and CNS concentrations that are linked to molecular target actions including, most potently, macrophage migration inhibitory factor (MIF) inhibition (Cho et al., 2010) and, secondarily, PDE's -4 and -10 inhibition (Gibson et al., 2006). The relevance of MIF inhibition in disorders of neuroimmune function such as neuropathic pain has recently been well demonstrated (Wang et al., 2011). Such dosing regimens have clearly been linked to glial attenuation in other animal models (Ledeboer et al., 2007), and the anti-inflammatory actions of MN166 have

recently been shown to suppress cerebral aneurysms in a dose-dependent manner (Yagi et al., 2010).

Tests of motor, vestibular and locomotive performance

Baseline testing of motor, vestibular and locomotive performance in all groups was conducted immediately prior to surgery and again, following a 1-week recovery period. These tests included ipsilateral and contralateral assessment of forelimb and hindlimb use to assess motor function, locomotion, limb use and limb preference (Bland et al., 2000; Bland et al., 2001), toe spread to assess gross motor response (Nitz et al., 1986), placing to assess visual and vestibular function (Schallert et al., 2000; Woodlee et al., 2005), catalepsy rod test to assess postural support and mobility (Sanberg et al., 1988), bracing to assess postural stability and catalepsy (Schallert et al., 1979; Morrissey et al., 1989) and air righting to assess dynamic vestibular function (Pellis et al., 1991a; Pellis et al., 1991b). Scoring ranged from 0 (severely impaired) to 5 (normal strength and function). The individual test scores were summed and a composite neuromotor score (0–45) was then generated for each animal. In addition to the composite neuromotor score, limb-use asymmetry was assessed during spontaneous exploration in the cylinder task, a common measure of motor forelimb function following central nervous system injury in rats (Schallert et al., 2000; Schallert, 2006) and post-injury locomotor activity was assessed

through distance traveled on a running wheel, both tasks were scored for 5 minutes under red light (~90 lux).

Behavioral measures

A novel environment was used to assess freezing behavior in response to a minor stressor (Dellu et al., 1996). The environment consisted of a standard rat cage with one vertically and one horizontally striped wall. No aversive stimuli were introduced in this context and no conditioning occurred. Rats were tested (5 minutes) and the percent of freezing behavior was assessed. Freezing was defined as the absence of movement except for heart beat/respiration, and was recorded in 10 sec intervals.

Freezing behavior in the novel environment was measured before and after administration of a foot shock in a separate shock apparatus. The shock apparatus consisted of two chambers placed inside sound-attenuating chests. The floor of each chamber consisted of 18 stainless steel rods (4 mm diameter), spaced 1.5 cm center-to-center and wired to a shock generator and scrambler (Colbourn Instruments, Allentown, PA). An automated program delivered a 2-sec/1.5 mA electric shock. Rats were transported in black buckets and shocked immediately upon entry to chambers. Following shock, rats were returned to their home cages.

A sucrose preference test was also performed in separate groups of rats that did not receive foot-shock or testing in the novel environment. This

task is commonly used to measure anhedonia in rodent models of depression (Monleon et al., 1995; Willner, 1997). The sucrose preference task was included because anxiety and depression share high rates of co-morbidity in humans (Moore et al., 2006) and was assessed as a possible confound to freezing behavior, due to possible co-occurrence of depression-like behavior. Rats were first habituated to sucrose solution, and were tested during the dark phase of the light/dark cycle to avoid the food and water deprivation necessary when testing during the light phase. Day 1 and day 2 consisted of habituation, day 3 and day 4 were baseline (averaged) and day 5 was the first test day. The rats were presented with two pre-weighted bottles containing 2% sucrose solution or tap water for a period of 4 hours. Thirty minutes into the task the bottles were swapped to force preference and counter for placement effects. Total sucrose intake and sucrose preference (sucrose intake/(sucrose intake + water intake * 100) were measured.

Timeline for behavioral testing

Following a 2-week recovery period from sham operation or LFPI in experimental animals, all groups except those to be evaluated for sucrose preference were tested in the novel context. Testing was performed at 2 weeks, 1, 2 and 3 months post-surgery. Shock was delivered after behavioral testing was completed at the 1 month time-point. Tests for

sucrose preference were performed at 2 weeks, 1 month and 3 months post-surgery with no intervening foot-shock.

Immunohistochemistry

Immunoreactivity for OX-42 (targets CD11b/c, a marker of microglial activation) and glial fibrillary acidic protein (GFAP; a marker of astrocyte activation) was measured using an avidin-biotin-horseradish peroxidase (ABC) reaction Loram et al., 2009. Brain sections (12 μm) were cut on a cryostat and mounted onto poly-L-lysine-coated slides and stored at -80 °C. Sections were post-fixed with 4% PFA for 15 min at room temperature, then treated with 0.03% H_2O_2 for 30 min at room temperature. The sections were incubated at 4 °C overnight in either mouse anti-rat OX-42 (1:100; BD Biosciences Pharmingen, San Jose, CA) or mouse anti-pig GFAP (1:100; MP Biomedicals, Aurora, OH). The next day, sections were incubated at room temperature for 2 h with biotinylated goat anti-mouse IgG antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Sections were washed and incubated for 2 h at room temperature in ABC (1:400 Vector Laboratories, Burlingame, CA) and reacted with 3', 3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO). Glucose oxidase and β-D-glucose were used to generate hydrogen peroxide. Nickelous ammonium sulfate was added to the DAB solution to optimize the reaction product. Sections were air-dried over night and then dehydrated with graded alcohols, cleared in Histoclear and

coverslipped with Permount (Fisher Scientific, Fairlawn, NJ). Densitometric analysis was performed using Scion Image software.

Image Analysis

Slides were viewed with an Olympus BX-61 microscope, using Olympus Microsuite software (Olympus America, Melville, NY) with bright-field illumination at 10X magnification. Images were opened in ImageJ, converted into gray scale and rescaled from inches to pixels. Background areas were chosen in the white matter or in cell-poor areas close to the region of interest (ROI). The number of pixels and the average pixel values above the set background were then computed and multiplied, giving an integrated densitometric measure (integrated gray level). Four measurements were made for each ROI; the measurements were then averaged to obtain a single integrated density value per rat, per region. All measurements were taken while blind to treatment group.

Statistical Analyses

Results are expressed as mean ± SEM. Analyses for all behavioral variables used analysis of variance (ANOVA) with repeated measures (time after injury), and treatment as the independent variable. The integrated density from the histology was only conducted at one time point and utilized one-way ANOVAs to compare regions between groups. Data were analyzed

using SPSS® Statistics software and, in all cases, statistical significance was set at $p < 0.05$.

RESULTS

Neuromotor composite scores of the brain-injured groups (LFPI+MN166, LFPI+vehicle) did not significantly differ from controls ($F(3,20) = 0.803$, $p = 0.508$). Rats in all groups consistently received normal scores on forelimb and hindlimb use, toe spread, placing, catalepsy rod, bracing, and air righting tests, indicating no impairments in motor, vestibular or locomotive functioning due to TBI. There were also no significant between group differences in limb-use asymmetry observed for contralateral ($F(5,29) = 0.544$, $p = 0.741$) and ipsilateral ($F(5,29) = 0.428$, $p = 0.826$) forelimb use during vertical exploratory behavior in the cylinder task, indicating no limb-use bias due to injury (Fig. 2.1A). No significant between group differences were found in locomotor performance evidenced by distance traveled during the running wheel activity ($F(5,29) = 0.069$, $p = 0.996$), revealing no post-injury impairments in locomotion (Fig. 2.1B). Nor were there significant between group differences in the sucrose preference task ($F(3,21) = 0.338$, $p = 0.798$), indicating no impairments in hedonic states.

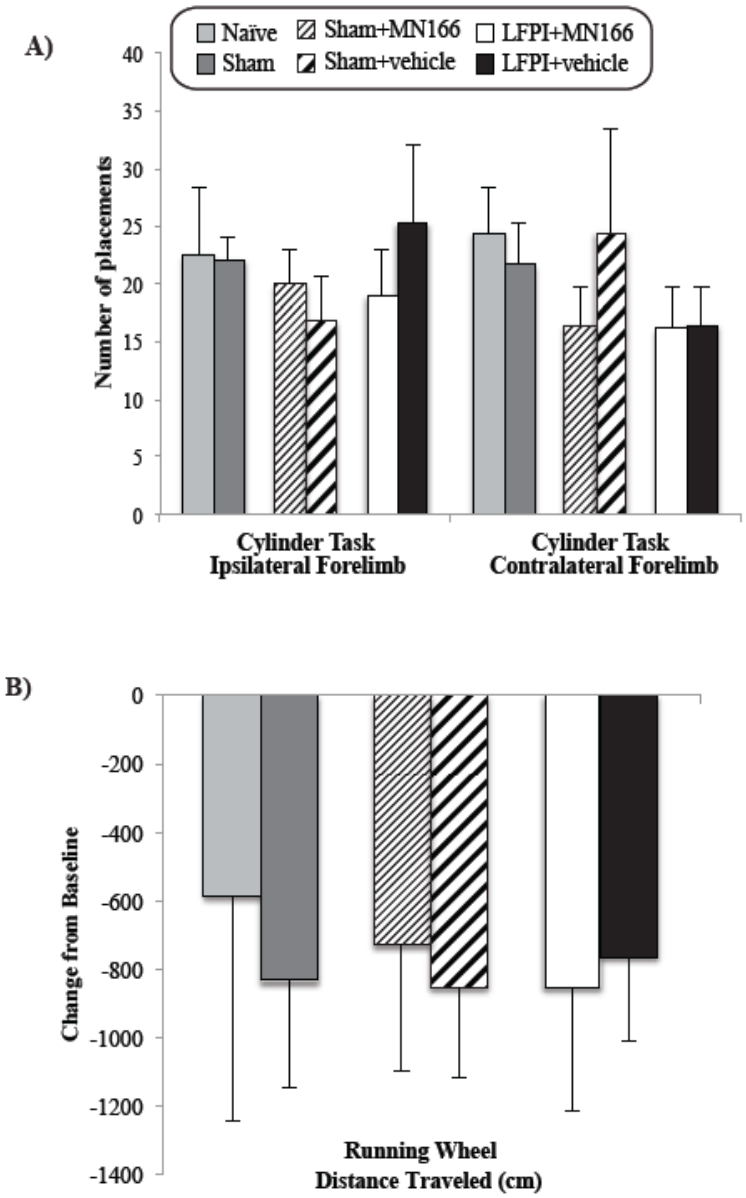

Figure 2.1 Cylinder task and running wheel activity at 1 week post-injury. (A) LFPI rats mean number of spontaneous forelimb placements (ipsilateral and contralateral) during exploratory activity in the cylinder test did not differ from controls at 1 week post-injury. A reduction was seen in contralateral limb-use in injured rats, but this reduction did not reach significance (p=0.741). (B) LFPI rats mean change in distance traveled in the running wheel activity did not significantly differ from controls at 1 week post-injury. Data represent mean ± SEM.

Despite normal motor, vestibular and locomotive function, LFPI produced large increases in freezing behavior when rats were placed in a novel context (Fig. 2; $F(5,30) = 9.539$, $p < 0.0001$). Exposed only to this minor stressor (i.e. at 2 week and 1 month post-injury measurements conducted prior to shock), LPFI rats injected with either MN166 or vehicle (Fig. 2.2; white and black bars, respectively) froze approximately twice as long as naïve or sham operated rats (Fig. 2.2; light and dark grey bars, respectively; $p < 0.01$). At 2 and 3-month measurement times, following the additional major stressor of shock (Fig. 2.2; arrows), freezing in both naïve and sham operated rats remained constant at approximately 10%. Freezing in LFPI rats treated with MN166 remained consistently higher than these controls ($p < 0.001$), but, while appearing higher compared to earlier post-injury measurements in the same animals, this increased freezing compared to naïve and sham operated rats before (1 month) and following (2 month) shock did not reach significance ($p=0.316$). By contrast, LFPI+vehicle rats nearly doubled their freezing time to approximately 50% (Fig. 2.2; black bars) compared to pre-shock values ($p < 0.001$), freezing approximately twice as long as LFPI+MN166 rats ($p < 0.001$) and 5 times as long as naïve and sham operated controls ($p < 0.001$) at the 2 and 3 month post-injury measurement times.

The behavioral effects of injections alone, independent of LFPI, are reflected in sham surgery groups with injections of either MN166 or vehicle

(Fig. 2.2; narrow and broad diagonal lines, respectively). Sham operated rats tended to freeze more than un-injected naïve and sham operated controls, reaching significance for both groups at the 2 and 3-month measurement points ($p < 0.01$) and suggesting that injections alone are aversive and can contribute to subsequent freezing. However, even at pre-shock measurement points, LFPI animals that received the same injections of MN166 or vehicle froze significantly more than injected controls ($p < 0.01$), indicating substantial enhancement of freezing produced by LFPI. This effect became more apparent following shock, where LFPI+vehicle rats froze twice as long as the injected controls ($p < 0.001$). By contrast, LFPI+MN166 rats were not distinguishable from either injected control group following shock, suggesting that their elevated freezing compared to naïve and sham operated animals was the result of injections alone and that MN166 eliminated the exaggerated freezing response to shock characterizing LFPI+vehicle rats.

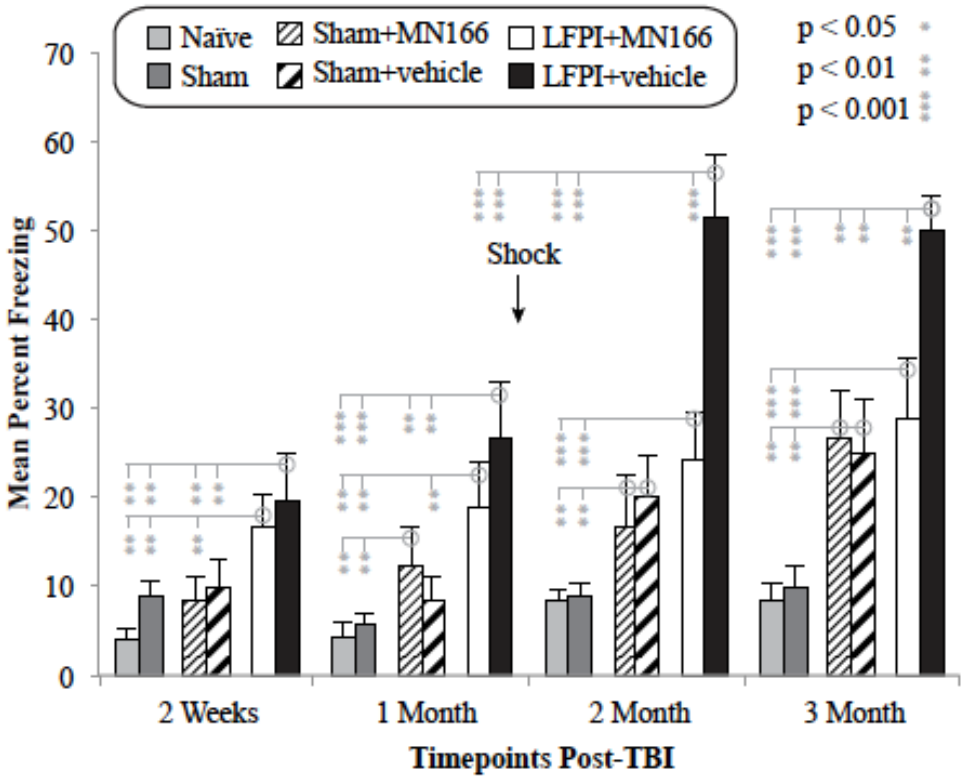

Figure 2.2 Freezing behavior in a novel context. Both surgically naïve and sham operated rats froze approximately 5-10% at post-surgical measurement points before (2 weeks and 1 month) after (2 and 3 month) foot-shock (arrow). In contrast, LFPI rats froze significantly longer (~20%) than these controls before shock. After shock, untreated LFPI rats (LFPI-vehicle) nearly doubled in time freezing (~50%) whereas treated LFPI rats (LFPI+MN166) showed only a slight increase (~25%) that did not reach significance (p=0.316). The effect of injections alone (Sham+Mn166 and Sham+vehicle) were to increase freezing behavior compared to un-injected naïve and sham operated rats, particularly at the 2 and 3 month post-shock measurement points where freezing in these rats could not be distinguished from LFPI rats treated with MN166. Data represent mean ± SEM.

OX-42 and GFAP immunoreactivity (reflecting microglia and astrocytic activation) was assessed in the insula, amygdala and hippocampus in brain-injured rats for comparison to sham operated and surgically naïve rats. Representative images (40X), showing GFAP immunoreactivity in several of these regions, are shown in Figure 2.3, revealing normal astrocyte morphology in surgically naïve and sham operated rats. LFPI+vehicle rats showed clear signs of reactive astrocytes (Fig. 2.3; bottom row). LFPI rats treated with MN166 (Fig. 2.3; third row) were difficult to differentiate from sham operated or surgically naïve control groups.

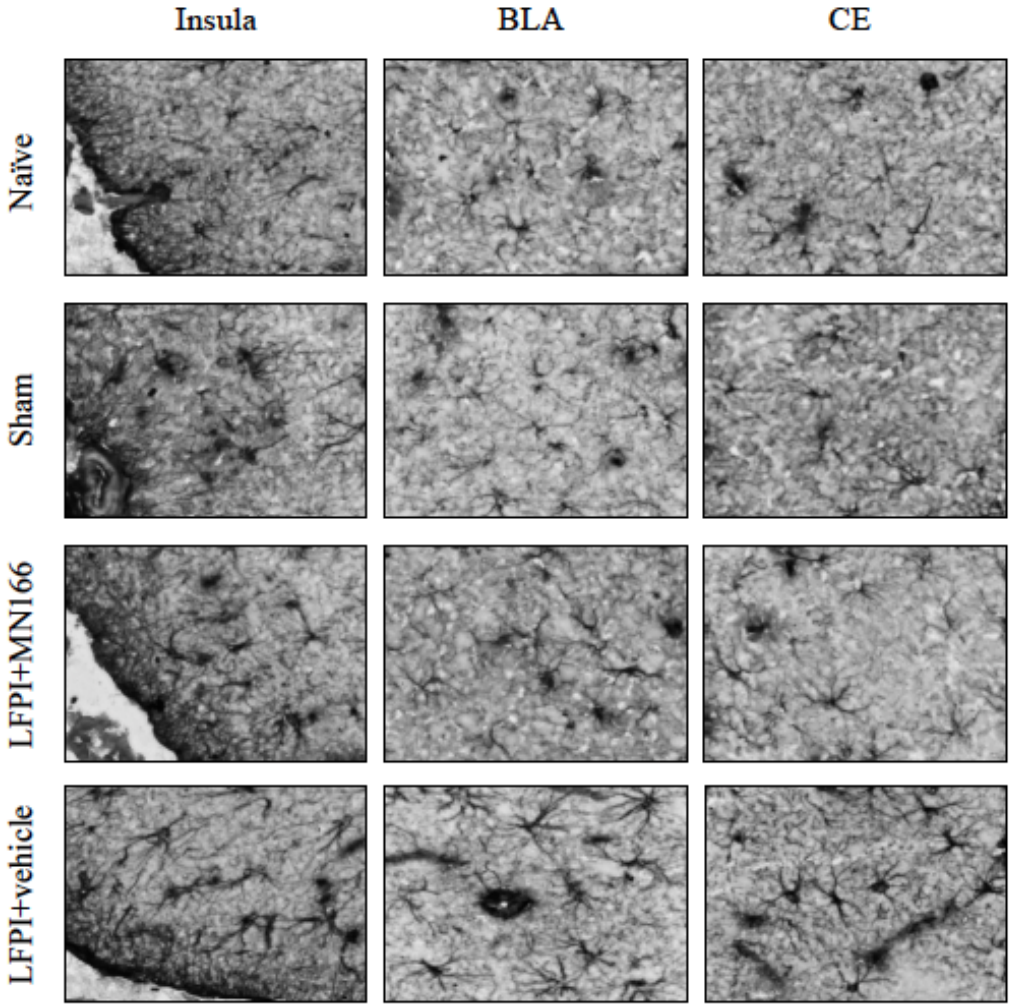

Figure 2.3 Representative images depicting GFAP immunoreactivity (reflecting astrocytic activation) assessed in the hippocampus, amygdala and insula at 3 months post-injury. LFPI rats injected with vehicle showed clear signs of reactive astrocytes (bottom row), while naïve and sham operated rats appeared to have normal astrocyte morphology. LFPI rats treated with MN166 (third row) were difficult to differentiate from surgically naïve and sham operated groups.

Densitometry of GFAP labeling in all areas examined confirmed that activation of astrocytes was significantly greater in LFPI compared to all other groups in insula (Fig. 2.4A; left bars; $F(3,19) = 13.17$, $p < 0.0001$), amygdala (Fig. 2.4B; left bars; $F(3,18) = 7.54$, $p < 0.002$) and hippocampus (Fig. 2.4C; left bars; $F(3,15) = 8.47$, $p < 0.002$). In contrast, no differences in GFAP labeling were observed between surgically naïve, sham operated and LFPI+MN166 groups in any of the regions examined. While MN166 treated LFPI rats were not distinguishable from surgically naïve or sham operated controls, post-hoc analyses revealed that LFPI+vehicle rats had significantly greater astrocyte activation in all 3 brain regions as compared to controls (Fig. 2.4A-C): insula ($p < 0.002$ vs. surgically naive, sham operated and LFPI+MN166), amygdala ($p < 0.02$ vs. surgically naive, sham operated and LFPI+MN166) and hippocampus ($p < 0.03$ vs. surgically naive, sham operated and LFPI+MN166).

Analysis of GFAP immunoreactivity in sub-regions of the insula (Fig. 2.4A; right bars), amygdala (Fig. 2.4B; right bars), and hippocampus (Fig. 2.4C; right bars), also revealed no differences between surgically naïve, sham operated and LFPI+MN166 groups. As in the regional analysis, LFPI+vehicle rats showed increased astrocyte activation over controls in most sub-regions examined. In the insula, LFPI+vehicle rats showed significantly increased GFAP labeling in agranular ($F(3,19) = 16.778$, $p <$

0.0001), dysgranular ($F(3,19) = 6.042$, $p < 0.005$) and granular ($F(3,19) = 5.277$, $p < 0.008$) regions, as compared to control groups.

In the amygdala, GFAP labeling in LFPI+vehicle rats was significantly increased in the BLA ($F(3,18) = 4.050$, $p < 0.023$) and CE ($F(3,18) = 5.012$, $p < 0.011$) nuclei, as compared to controls. LFPI+vehicle rats also showed increased GFAP expression in the hippocampus, but this was only significant in CA3 ($F(3,18) = 3.810$, $p < 0.03$) and approached significance in CA1 ($F(3,17) = 3.234$, $p = 0.055$).

LFPI+vehicle rats also showed significantly increased microglia activation compared to control groups, as measured by OX-42 labeling, but this was restricted to the insula (Fig. 2.4D; $F(3,19) = 5.59$, $p < 0.007$). Analysis of sub-regions of the insula also revealed increases in microglial activation for LFPI+vehicle rats, and post-hoc comparisons showed that LFPI alone significantly increased OX-42 labeling in agranular ($F(3,19) = 11.186$, $p < 0.0001$), granular ($F(3,18) = 3.740$, $p < 0.03$), and approaching significance ($F(3,19) = 2.742$, $p < 0.072$) in dysgranular areas. No differences in OX-42 labeling were observed between surgically naïve, sham operated and LFPI+MN166 groups in any insular regions examined. No significant between group differences were found in OX-42 expression for the amygdala or hippocampus.

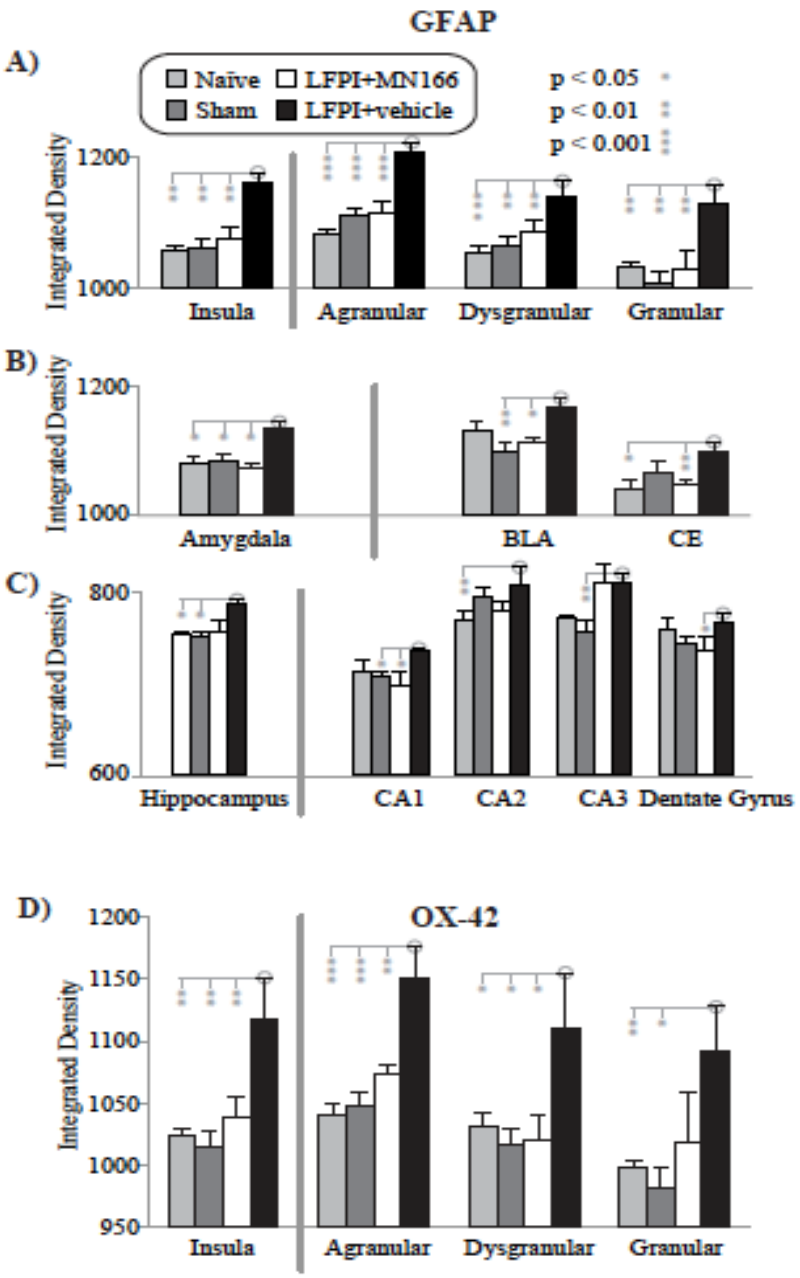

Figure 2.4 Regional and sub-regional analyses of microglial and astroglial activation in hippocampus, amygdala and insula at 3 months post-injury. (A-C) LFPI vehicle injections induced a significant increase in GFAP labeling in all three regions, compared to surgically naïve, sham operated and LFPI+MN166 treated rats. (D) In the insula, OX-42 activation was greater in LFPI rats compared to surgically naïve, sham operated and LFPI+MN166 treated rats. There were no significant differences found between surgically naïve, sham operated and LFPI+MN166 treated rats in either analysis. Data represent mean± SEM integrated densities of immunoreactivity.

DISCUSSION

These data suggest a link between injury-induced brain inflammation and post-traumatic anxiety. Rats with LFPI display freezing responses to the minor stress of a novel environment that is 2-3 times normal and which, unlike controls, is nearly doubled by the delivery of a major foot-shock stressor. LFPI also results in marked reactive gliosis in brain regions associated with anxiety. The possibility that post-traumatic brain inflammation and gliosis may contribute to anxiety-like behavior observed here, is supported by the effects of glial-cell activation inhibitor MN166. MN166 reduces reactive gliosis and TBI-induced freezing behavior, rendering these animals histologically and behaviorally indistinguishable from naïve and sham operated controls. To our knowledge, this is the first study to report pharmacological immunosuppression resulting in the reduction of anxiety-like behaviors following TBI.

A possible mechanism for neuroimmune induced post-traumatic anxiety

Our finding of prolonged reactive gliosis in brain structures including, but likely not confined to, the hippocampus, amygdala and insular cortex, suggests that these structures may contribute to the persistent enhanced freezing of our brain-injured animals in reaction to a novel environment. All three structures have been implicated in rodent research investigating the pathogenesis of anxiety (Davis, 1992; Davis et al., 1994; Davidson, 2002; Vyas et al., 2004; Paulus and Stein, 2006; Rauch et al., 2006; Canteras et

al., 2010) and fear behavior in the rat (Sullivan, 2004; Rosen and Donley, 2006; Milad et al., 2009; Liu et al., 2010).

The mechanisms by which immune responses may contribute to dysfunction of these structures remain to be determined. It is well established that LFPI in the rat results in activation of microglia and astrocytes as part of the innate immune response to insult. A number of studies indicate that LFPI-induced reactive gliosis follows a distinct time-course, beginning with predominant microglia activation that peaks within a week (Hill et al., 1996; Nonaka et al., 1999; Grady et al., 2003; Gueorguieva et al., 2008; Clausen et al., 2009; Yu et al., 2010) but continues for several weeks and overlaps later with persistent astrocytic activation (D'Ambrosio et al., 2004; Yu et al., 2010). Microglia are resident macrophages and first responders to pathogens and neuronal insults in the CNS. They react rapidly, leading to activation of astrocytes and prolonged disruption of neuronal (Iravani et al., 2005; Herber et al., 2006; Zhang et al., 2009; Zhang et al., 2010). Several lesion paradigms have also shown rapid microglial response followed by delayed astrocyte (Gehrmann et al., 1991; Dusart and Schwab, 1994; Frank and Wolburg, 1996; McCann et al., 1996; Liberatore et al., 1999).

Our results support this well-documented temporal relationship suggesting that microglial activation precedes astrocytic activation and plays a role in the onset and maintenance of astrogliosis (Graeber and Kreutzberg,

1988; McCann et al., 1996; Hanisch, 2002; Iravani et al., 2005; Herber et al., 2006; Zhang et al., 2010). This time-course is consistent with behavioral freezing responses in the present study, appearing rapidly within 2 weeks but persisting unabated for the 3-month post-injury measurement period. It is also consistent with our immunohistochemistry results, indicating injury-induced astrocytic activation in all 3 regions of interest, insula, amygdala and hippocampus at 3 months post-injury, but less activation of microglia, only significant in the insula. The lower levels of microglia expression are likely due to assessment at 3 months post-injury.

Trauma-related reactive gliosis is well known to result in the release of high levels of pro-inflammatory cytokines, specifically tumor necrosis factor-aalpha (Taupin et al., 1993; Fan et al., 1996; Lloyd et al., 2008), interleukin-1 beta (Taupin et al., 1993; Fan et al., 1995; Fassbender et al., 2000; Yan et al., 2002; Lloyd et al., 2008), and interleukin-6 (Taupin et al., 1993; Yan et al., 2002; Lloyd et al., 2008), which are central mediators of neuroinflammation following head injury (Fan et al., 1995; Rothwell and Hopkins, 1995; Rothwell and Strijbos, 1995; Fan et al., 1996; Simi et al., 2007). Release of these pro-inflammatory cytokines, particularly IL-1β and TNF-α, pathologically increases neuronal excitability in all brain regions where it has been measured (Riazi et al., 2008b; Schafers and Sorkin, 2008; Rodgers et al., 2009; Beattie et al., 2010; Maroso et al., 2010). While neuronal excitability and proinflammatory cytokine levels were not measured

in the present study, neuroinflammation has been implicated in neuronal excitability of amygdala and insular cortex and anxiety-like behavior by others using c-Fos labeling (Abrous et al., 1999; Ikeda et al., 2003; Kung et al., 2010). These same regions have also consistently been reported to be hyperexcitable in human imaging data across a variety of anxiety disorders (Rauch et al., 1997; Shin et al., 2006; Simmons et al., 2006; Stein et al., 2007; Shin and Liberzon, 2010; Carlson et al., 2011).

Attenuation of post-traumatic anxiety with MN166

Meta-analysis of the impact of pharmacological treatments on behavioral, cognitive, and motor outcomes after traumatic brain injury in rodent models (Wheaton et al., 2011) indicates that of 16 treatment strategies evaluated to date, improved cognition and motor function have been reported, but almost no treatments have improved behaviors related to psychiatric dysfunction in general and anxiety in specific. Exceptions to this are recent promising reports of treatments such as magnesium sulphate to limit excitotoxic damage (Vink et al., 2003; Fromm et al., 2004; O'Connor, 2003, 533-41) and resevatrol to limit excitotoxicity, ischemia, hypoxia (Sönmez et al., 2007), both increasing open field exploration (resulting from decreased freezing) and therefore presumably decreasing post-injury anxiety.

Glial targeted immunosuppression has also been found to be neuroprotective following TBI in rodents, resulting in increased structural

preservation and improved functional outcomes (Hailer, 2008); including recent reports that MN166 significantly attenuated brain edema formation, cerebral atrophy and apoptosis in neuronal cells following ischemic brain injury in rats, increasing neuronal survival rates (Lee et al., 2011). MN166 may reduce neuronal damage in regions involved in anxiety, mitigating the role of glial activation, neurotoxicity and hyperexcitability in the subsequent development of anxiety-like behaviors. While not focused on post-traumatic anxiety, MN166 has been found to reduce intracellular calcium accumulation (Yanase et al., 1996), apoptosis, functional damage and passive avoidance behaviors following a transient ischemia model in rats (Yoshioka et al., 2002). Increasing evidence supports neuroinflammation, chronic inflammatory responses, proinflammatory cytokines, neuronal hyperexcitability, and secondary injury cascades in the pathophysiology of post-traumatic anxiety. The mechanisms of the effect of MN166 on TBI-induced anxiety-like behavior are not fully known. However, the results of this study provide evidence of a neuroprotective role for MN166 in attenuating and perhaps preventing development of post-traumatic anxiety.

Further establishing a relationship between TBI, neuroimmune responses, neurocircuitry and anxiety disorders, is important to further understand the sequelae of TBI and to the development of effective treatment strategies. The development of anxiety disorders following TBI is a complex and multifaceted problem, and finding treatments that work will

require multifaceted approaches. The injury itself initiates many complex biological events including glial activation, breakdown of the blood brain barrier, excitotoxicity and chronic neuroinflammation. While primary injury often cannot be prevented, it may be possible to reduce secondary injury, leading to better functional and behavioral recovery following TBI. The present results, using peri-injury treatment with MN166 to prevent post-traumatic freezing behavior, not only suggest a role for neuroimmune inflammation in anxiety physiology, but similarly successful results with post-injury treatment could introduce a promising and clinically realistic translational possibility for prevention of post-traumatic anxiety in humans.

This research validated the use of LFPI in investigating post-traumatic anxiety in rats, and established the efficacy of MN166 in altering behavioral and immunohistological outcomes following LFPI. Treatment with MN166 significantly reduced anxiety-like behaviors and reactive gliosis, providing the first evidence that immunosuppression may be a potential therapeutic target for the treatment of post-traumatic anxiety. However, peri-injury treatment has limited clinical potential and narrows the critical window for intervention following injury. The following study aimed to replicate the current findings at long-term post-injury timepoints, treatment administration was delayed until 1 month post-injury and measurements of anxiety-like behavior and alterations in glial reactivity were extended through 6 months post-injury.

CHAPTER IV

Delayed, post-injury neuroimmune suppression reduces anxiety-like behavior following lateral fluid percussion injury in rats

ABSTRACT

Traumatic brain injury (TBI) increases the risk for developing neuropsychiatric disorders, particularly anxiety disorders. Research now implicates chronic neuroinflammation in the development of human post-traumatic anxiety and in recently developed animal models. In a previous study, we used a lateral fluid percussion injury (LFPI) model of traumatic brain injury in the rat and examined freezing behavior as a measure of post-traumatic anxiety (Rodgers et al., 2012). We found that LFPI produced anxiety-like freezing behavior accompanied by increased reactive gliosis in brain structures associated with anxiety. Acute peri-injury administration of Ibudilast (MN166), a glial cell activation inhibitor, suppressed both reactive gliosis and freezing behavior. In the current study, we examined the efficacy of MN166 in reducing anxiety-like freezing behavior at a more clinically relevant time point, one month following injury. Remarkably, treatment with MN166 reduced established anxiety-like freezing behavior, as compared to vehicle-injected controls, and continued neuroprotective effects were evidenced several months post-injury. These results support the conclusion that chronic neuroinflammation is involved in the development of anxiety-like behaviors following TBI and that delayed treatment is effective in reducing TBI-induced behavioral and immunological impairments, substantially expanding the therapeutic window for treatment of post-traumatic anxiety.

INTRODUCTION

More than 5.3 million people in the United States are living with traumatic brain injury (TBI)-related permanent disabilities. TBI has been described as a silent epidemic because many of the post-injury disabilities are neuropsychiatric in nature, and are not always apparent following primary injury (Rao and Lyketsos, 2000; Moore et al., 2006; Vaishnavi et al., 2009). Following TBI, a broad range of neurobehavioral disorders are common, which typically manifest as a wide spectrum of psychiatric conditions. Some of the more commonly reported syndromes are anxiety disorders, psychosis, depression and mood disorders (van Reekum et al., 1996; van Reekum et al., 2000; Fann et al., 2004; Hoge et al., 2008; Schneiderman et al., 2008; Whelan-Goodinson et al., 2009). Anxiety disorders are among the most prevalent, with rates of 10-70% reported across studies (Rao and Lyketsos, 2000; Hiott and Labbate, 2002; Vaishnavi et al., 2009).

However, in spite of the high rates of co-occurrence many researchers have focused on physical injuries (cognitive/memory impairments and motor deficits) and very few studies have examined psychiatric aftermaths, which are not generally thought to have a neurobiological basis. Increasing evidence implicates both structural and neurobiological alterations in the pathophysiology of anxiety disorders following TBI, including dysregulation of the neuroimmune system.

In response to tissue injury, cells of the innate immune system rapidly produce endogenous danger signals or alarmins (danger-associated molecular patterns, DAMPs), which function as potent effectors of innate defense. DAMPs play a role in generating tissue inflammation following injury, but may also work synergistically with microbial antigens to increase the inflammatory reaction (Adib-Conquy and Cavaillon, 2007; Pugin, 2012). These signals act to alarm the immune system by promoting the recruitment and activation of host leukocytes, which relay and amplify the inflammatory response through the release of cytokines and other inflammatory mediators (Pugin, 2012). DAMPs activate antigen presenting cells (APCs) for recruitment of leukocytes, and microglia are the resident immunological cells and primary APCs of the CNS (Matzinger, 1998; Hirsiger et al., 2012). Microglia then release proinflammatory cytokines, chemokines and other proinflammatory mediators in response to activating signals (Gehrmann et al., 1993; Gehrmann et al., 1995).

Activation of brain glial cells during the innate immune response leads to the expression of high levels of pro-inflammatory cytokines, most notably interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α). Several new lines of evidence support this hypothesis, including reports of peripheral elevations of these cytokines in patients with PTSD and panic disorder (Spivak et al., 1997; Tucker et al., 2004; von Kanel et al., 2007; Hoge et al., 2009), innate immune responses that are clearly

triggered by TBI (Gasque et al., 2000; Hailer, 2008; Lehnardt, 2010) and the resultant release of pro-inflammatory cytokines by activated glial cells (Sternberg, 1997; Simi et al., 2007; Loane and Byrnes, 2010; Zhang et al., 2010). When these cytokines are administered centrally or systemically in rats, they also induce anxiety-like behavior (Connor et al., 1998; Cragnolini et al., 2006; Sokolova et al., 2007; Zubareva and Klimenko, 2009) and increase plasma corticosterone concentrations (Brebner et al., 2000). Finally, our laboratory and others have found IL-1β, IL-6 and TNF-α to be powerfully excitatory to neurons (Hailer, 2008; Riazi et al., 2008b; Rodgers et al., 2009; Beattie et al., 2010), presenting a potential for inducing dysfunction based on hyperexcitability in neural circuits of affected structures. These secondary outcomes may contribute to the dysfunction of brain regions and neurotransmitter systems associated with anxiety.

The goals of the present study were to examine anxiety-like behavior and reactive gliosis in brain regions associated with anxiety in rats following TBI to support our earlier results showing these behavioral and immunological responses could be attenuated with acute immunosuppression (Rodgers et al., 2012). Those results set up the present study, where treatment with a glial cell activation inhibitor (Ibudilast) was delivered at one month post-injury to assess its efficacy in reversing established anxiety-like behaviors and reducing TBI-induced immunological damage.

MATERIALS AND METHODS

Twenty-four adult viral-free male Sprague-Dawley rats (275-325g; Harlan Laboratories, Madison, WI) were housed in pairs in temperature (23 ± 3 °C) and light (12:12 light: dark) controlled rooms with *ad libitum* access to food and water. All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory rats in biological research. Rats were randomly assigned to the following groups (n = 6/group): sham operated+vehicle, sham operated+MN166, LFPI+vehicle and LFPI+MN166.

Lateral Fluid Percussion Injury

LFPI rats were anesthetized with halothane (4% induction, 2.0-2.5% maintenance) and mounted in a stereotaxic frame. The lateral fluid percussion injury used in this study has been described previously (McIntosh et al., 1989; Thompson et al., 2005; Frey et al., 2009). Briefly, a 3.0 mm diameter craniotomy was centered at 3 mm caudal to bregma and 4.0 mm lateral of the sagittal suture, with the exposed dura remaining intact. A female Luer-Loc hub (inside diameter of 3.5 mm) was secured over the craniotomy with cyanoacrylate adhesive. Following hub implantation, the animal was removed from the stereotaxic frame and connected to the LFPI apparatus. The LFPI apparatus delivered a moderate impact force (2.0 atmospheres; 10 ms). The injury cap was then removed, scalp sutured and

the rats returned to their home cages for recovery. Sham operated rats underwent identical surgical preparation, but did not receive the brain injury.

Ibudilast (MN166) administration

MN166 (MediciNova, San Diego, CA) is a relatively non-selective phosphodiesterase inhibitor with anti-inflammatory actions via glial cell attenuation (Mizuno et al., 2004; Rolan et al., 2009). Treated rats received a 5-day dosing regimen of once-daily MN166 injections (10 mg/kg), beginning at 30 days following LFPI. Weight was recorded prior to each dosing and treatment administered at the same time each day to maintain constant levels across a 24 hr period. Dose selection was based on prior animal pharmacology results (Ellis AL, SFN, 2008a) showing MN166 to be safe and well tolerated, yielding plasma concentration-time profiles commensurate with high dose regimens in clinical development. MN166 administered via this regimen yields plasma and CNS concentrations that are linked to molecular target actions including, most potently, macrophage migration inhibitory factor (MIF) inhibition (Cho et al., 2010) and, secondarily, PDE's -4 and -10 inhibition (Gibson et al., 2006). The relevance of MIF inhibition in disorders of neuroimmune function such as neuropathic pain has recently been well demonstrated (Wang et al., 2011).

Neuromotor Tests

Baseline testing of motor, vestibular and locomotive performance in all groups was conducted immediately prior to surgery and again, at 1 month following injury. These tests included ipsilateral and contralateral assessment of forelimb and hindlimb use to assess motor function, locomotion, limb use and limb preference (Bland et al., 2000; Bland et al., 2001), toe spread to assess gross motor response (Nitz et al., 1986), placing to assess visual and vestibular function (Schallert et al., 2000; Woodlee et al., 2005), catalepsy rod test to assess postural support and mobility (Sanberg et al., 1988), bracing to assess postural stability and catalepsy (Schallert et al., 1979; Morrissey et al., 1989) and air righting to assess dynamic vestibular function (Pellis et al., 1991a; Pellis et al., 1991b). Scoring ranged from 0 (severely impaired) to 5 (normal strength and function). The individual test scores were summed and a composite neuromotor score (0–45) was then generated for each animal. In addition to the composite neuromotor score, limb-use asymmetry was assessed during spontaneous exploration in the cylinder task, a common measure of motor forelimb function following central nervous system injury in rats (Schallert et al., 2000; Schallert, 2006) and post-injury locomotor activity was assessed through distance traveled on a running wheel, both tasks were scored for 5 minutes under red light (~90 lux).

Behavioral measures

A novel environment was used to assess freezing behavior in response to a minor stressor (Dellu et al., 1996). The environment consisted of a standard rat cage with one vertically and one horizontally striped wall. No aversive stimuli were introduced in this context and no conditioning occurred. Rats were tested (5 minutes) and the percent of freezing behavior was assessed. Freezing was defined as the absence of movement except for heart beat/respiration, and was recorded in 10 sec intervals.

Freezing behavior in the novel environment was measured after administration of a foot shock in a separate shock apparatus. The shock apparatus consisted of two chambers placed inside sound-attenuating chests. The floor of each chamber consisted of 18 stainless steel rods (4 mm diameter), spaced 1.5 cm center-to-center and wired to a shock generator and scrambler (Colbourn Instruments, Allentown, PA). An automated program delivered a 2-sec/1.5 mA electric shock. Rats were transported in black buckets and shocked immediately upon entry to chambers. Following shock, rats were returned to their home cages.

Timeline for behavioral testing

Testing was performed at months 1 through 6 post-injury. The single shock was delivered after neuromotor testing was completed at the 1 month timepoint.

Immunohistochemistry

Rats were intracardially perfused with 0.9% saline and tissue was collected, then fixed with 4% paraformaldehyde overnight at 4°C. Tissue was transferred to a 30% sucrose PBS solution for 1-2 days, then stored at -80 °C. Brain sections (20 μm) were post-fixed with 4% PFA for 15 min at room temperature, then treated with 0.03% H_2O_2 for 30 min at room temperature. Immunoreactivity in brain regions associated with anxiety (insula and amygdala) was assessed for markers of microglia (CD11b/c; OX42 labeling) and astrocytes (glial fibrillary acidic protein; GFAP), using an avidin-biotin-horseradish peroxidase (ABC) reaction (Loram et al., 2009). The sections were incubated at 4 °C overnight in either mouse anti-rat OX-42 (1:100; BD Biosciences Pharmingen, San Jose, CA) or mouse anti-pig GFAP (1:100; MP Biomedicals, Aurora, OH). The next day, sections were incubated at room temperature for 2 h with biotinylated goat anti-mouse IgG antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Sections were washed and incubated for 2 h at room temperature in ABC (1:400 Vector Laboratories, Burlingame, CA) and reacted with 3', 3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO). Sections were air-dried over night and then dehydrated with graded alcohols, cleared in Histoclear and coverslipped with Permount (Fisher Scientific, Fairlawn, NJ). Densitometric analysis was performed using Scion Image software.

Image Analysis

Slides were viewed with an Olympus BX-61 microscope, using Olympus Microsuite software (Olympus America, Melville, NY) with bright-field illumination at 10X magnification. Densitometric analysis was performed using Scion Image software. Images were opened in ImageJ, converted into gray scale and rescaled from inches to pixels. Background areas were chosen in the white matter or in cell-poor areas close to the region of interest (ROI). The number of pixels and the average pixel values above the set background were then computed and multiplied, giving an integrated densitometric measure (integrated gray level). Six measurements were made for each ROI; the measurements were then averaged to obtain a single integrated density value per rat, per region.

Statistical Analyses

Results are expressed as mean ± SEM. Analyses for behavioral measures used analysis of variance (ANOVA) with repeated measures (time after injury), and treatment as the independent variable. The integrated density from the histology was only conducted at one time point and utilized one-way ANOVAs to compare regions between groups. Data were analyzed using SPSS® Statistics software and, in all cases, statistical significance was set at $p < 0.05$.

RESULTS

Neuromotor composite scores of the brain-injured groups (LFPI+MN166, LFPI+vehicle) did not significantly differ from controls ($F(3,20) = 0.383$, $p = 0.766$). Rats in all groups consistently received normal scores on forelimb and hindlimb use, toe spread, placing, catalepsy rod, bracing, and air righting tests, indicating no impairments in motor, vestibular or locomotive functioning due to TBI. There were also no significant between group differences in limb-use asymmetry observed for contralateral ($F(3,20) = 0.058$, $p = 0.981$) and ipsilateral ($F(3,20) = 0.285$, $p = 0.836$) forelimb use during vertical exploratory behavior in the cylinder task, indicating no limb-use bias due to injury (Fig. 3.1A). No significant between group differences were found in locomotor performance evidenced by distance traveled during the running wheel activity ($F(3,20) = 0.152$, $p = 0.464$), revealing no post-injury impairments in locomotion (Fig. 3.1B).

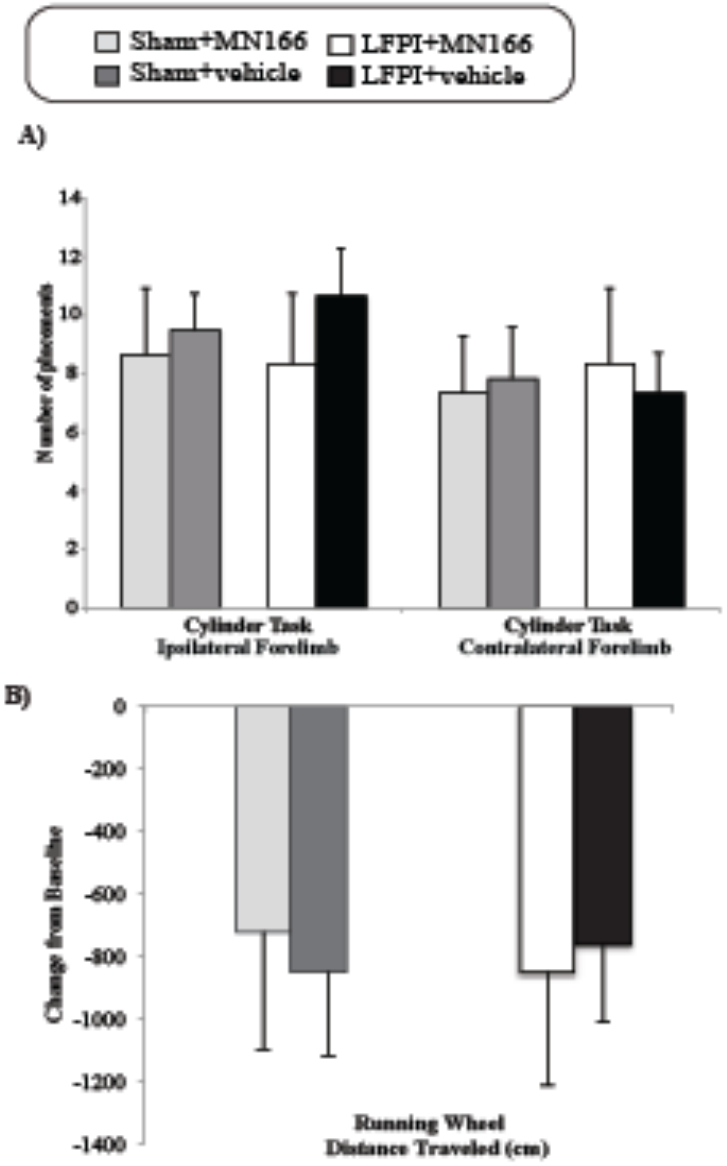

Figure 3.1 Cylinder task and running wheel activity at 1 month post-injury. (A) LFPI rats mean number of spontaneous forelimb placements (ipsilateral and contralateral) during exploratory activity in the cylinder test did not differ from controls at 1 month post-injury. (B) LFPI rats mean change in distance traveled in the running wheel activity did not significantly differ from controls at 1 month post-injury. Data represent mean ± SEM.

LFPI-induced increases in freezing behavior were observed when rats were placed in a novel context (Fig. 3.2; $F(3,20) = 9.029$, $p < 0.001$). Exposed only to this minor stressor, LPFI rats injected with either MN166 or vehicle (Fig. 3.2; white and black bars, respectively) froze approximately twice as long as treated or vehicle injected sham operated rats (Fig. 3.2; dark and light striped bars, respectively; $p < 0.03$) at the 1 month timepoint.

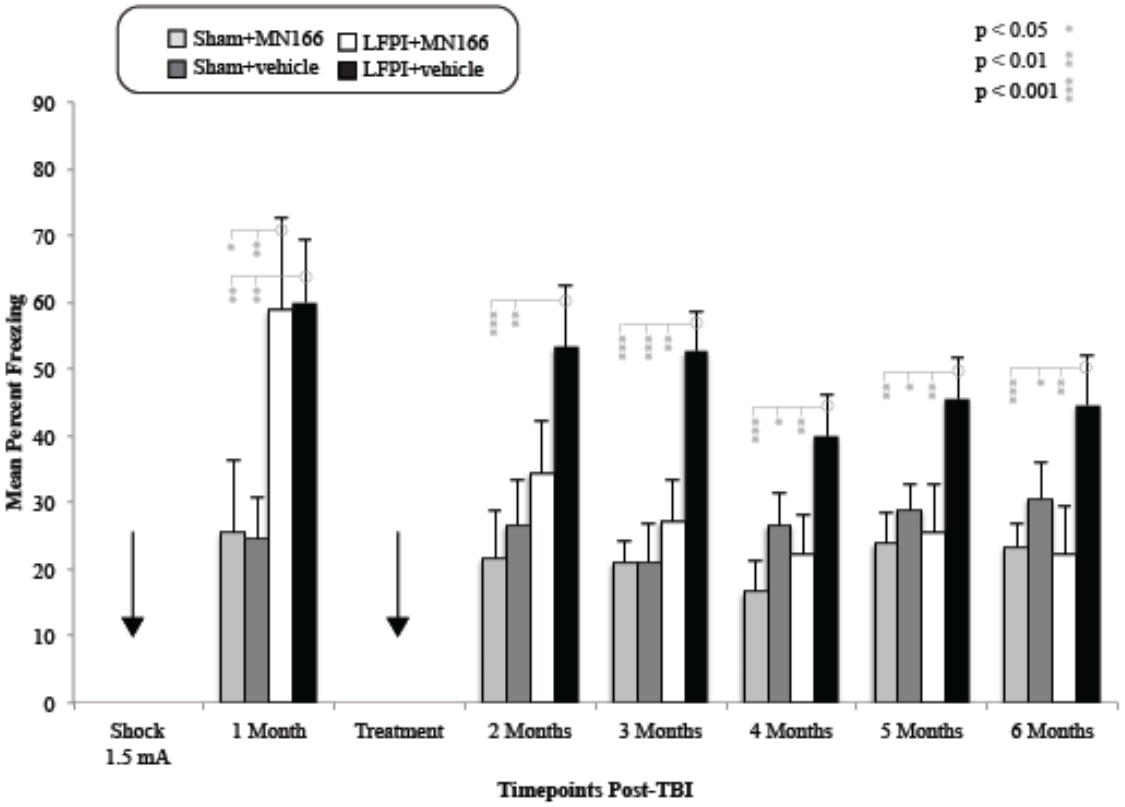

Figure 3.2 Freezing behavior in novel context. Sham operated rats froze approximately 25% before treatment with MN166 or vehicle, while LFPI rats froze at significantly higher rates (~60%). Following treatment, LFPI+MN166 rats freezing behavior was reduced to (~25%) compared to LFPI+vehicle rats (~50%). This effect was significant at 3 months, and remained through 6 months following injury ($p < 0.03$). Freezing in Sham+MN166 and Sham+vehicle rats could not be distinguished from LFPI+MN166 treated rats at all timepoints following treatment ($p > 0.257$), while LFPI+vehicle injected rats froze significantly more than both sham groups at all post-treatment timpoints ($p < 0.05$) with the exception of LFPI+vehicle and Sham+vehicle, which did not differ at the 4 month and 6 month timepoints ($p = 0.102$ and 0.133, respectively). Data represent mean ± SEM.

At 2 months post-injury, following treatment with MN166 or vehicle (Fig. 3.2; arrows), freezing in both sham operated groups remained constant at approximately 25%. Freezing behavior in vehicle injected LFPI rats remained consistently higher than these controls ($p < 0.03$), while freezing differences between both sham control groups and LFPI+MN166 rats did not reach significance ($p > 0.257$). LFPI+vehicle rats froze approximately 20% (Fig. 3.2; black bars) more than LFPI+MN166 rats; although this did not reach significance at the 2 month timepoint ($p = 0.100$) and 30% more than sham operated controls ($p < 0.03$) at the 2 month post-injury measurement.

At 3 through 6 months post-injury, freezing averages for Sham+MN166 and Sham+vehicle groups again remained constant (20% and 25%, respectively). Freezing behavior in vehicle injected LFPI rats remained consistently higher than these controls ($p < 0.05$) at 3 month through 6 month post-treatment timepoints, with the exception of LFPI+vehicle and Sham+vehicle, which did not differ at the 4 month and 6 month timepoints ($p = 0.102$ and 0.133, respectively). Freezing differences between sham treated and sham vehicle injected groups and LFPI+MN166 treated rats did not reach significance ($p > 0.343$) at any of the 3 through 6 month post-treatment timepoints. LFPI+vehicle injected rats froze approximately 20% (Fig. 3.2; black bars) more than LFPI+MN166 treated rats ($p < 0.034$) across the 3 month through 6 month post-treatment timepoints.

OX-42 and GFAP immunoreactivity (reflecting microglia and astrocytic

activation) was assessed in the insula and amygdala in treated and vehicle injected LFPI rats for comparison to sham operated controls. Representative images (40X), showing GFAP immunoreactivity in several of these regions, are shown in Figure 3.3, revealing normal astrocyte morphology in both MN166 treated and vehicle injected sham controls. LFPI+vehicle rats showed clear signs of reactive astrocytes (Fig. 3.3; bottom row), while LFPI rats treated with MN166 (Fig. 3.3; third row) were difficult to differentiate from sham operated control groups.

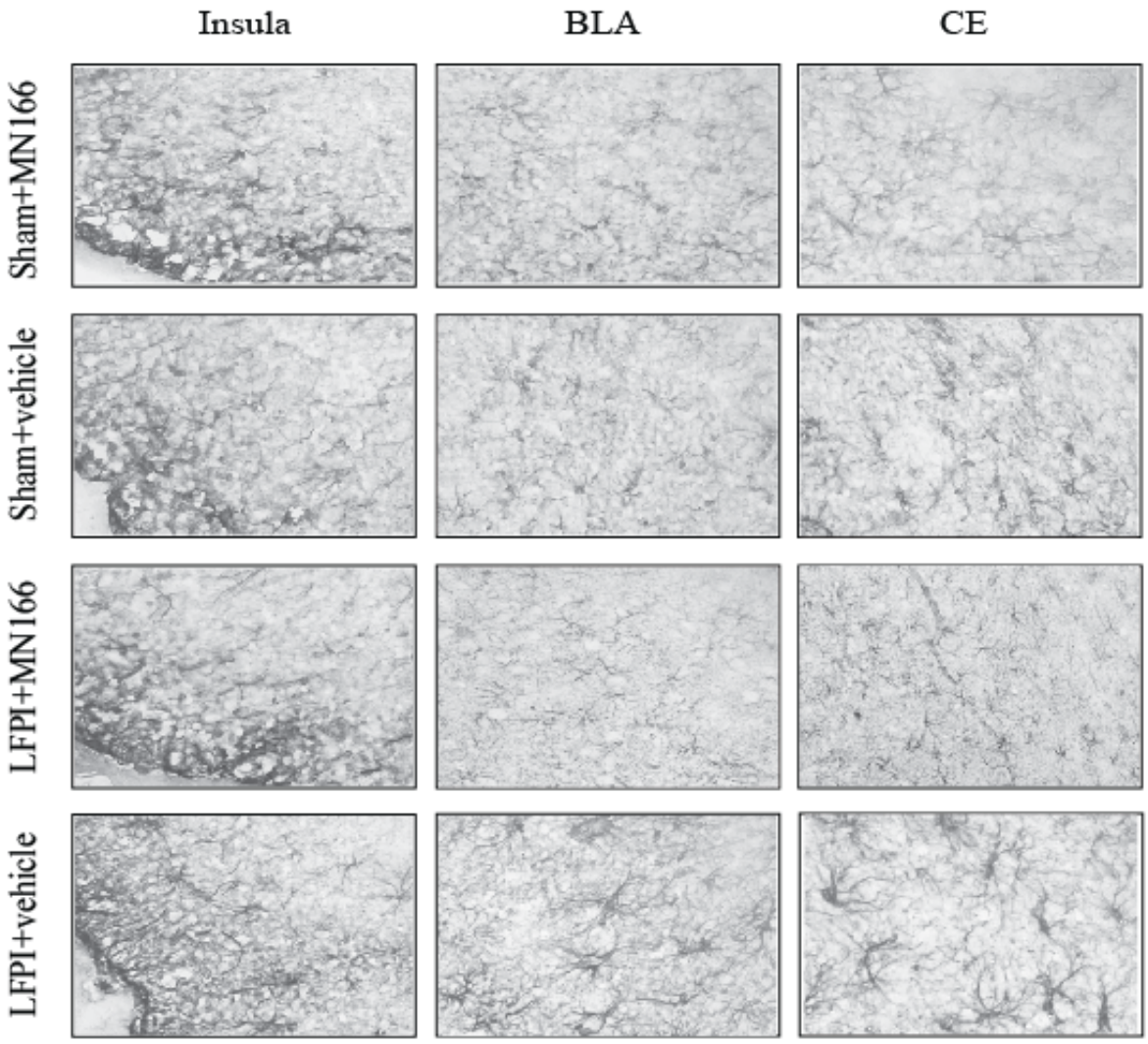

Figure 3.3 Representative images depicting GFAP immunoreactivity (reflecting astrocytic activation) assessed in the amygdala and insula at 6 months post-injury. LFPI rats injected with vehicle showed clear signs of reactive astrocytes (bottom row), while sham operated rats appeared to have normal astrocyte morphology (top rows). LFPI rats treated with MN166 (third row) were difficult to differentiate from sham operated groups.

Immunohistochemistry revealed increased GFAP labeling in both brain regions examined, confirming that astroglial activation was significantly greater in LFPI compared to control groups in insula (Fig. 3.4A; left graph; $F(3,140) = 3.761$, $p = 0.012$) and amygdala (Fig. 3.4B; left graph; $F(3,140) = 6.025$, $p < 0.001$). In contrast, no differences in GFAP labeling were observed between sham operated and LFPI+MN166 groups in overall insula or amygdala ($p > 0.419$). While MN166 treated LFPI rats were not distinguishable from sham operated controls, post-hoc analyses revealed that LFPI+vehicle rats had significantly greater astrocytic activation in both brain regions as compared to controls (Fig. 3.4A-B, left graphs): insula ($p < 0.02$ vs. sham operated groups and LFPI+MN166) and amygdala ($p < 0.005$ vs. sham operated groups and LFPI+MN166).

Analysis of GFAP immunoreactivity in sub-regions of the insula (Fig. 3.4A; right graph), also revealed that LFPI+vehicle rats had increased GFAP labeling in agranular ($F(3,140) = 2.493$, $p = 0.063$), dysgranular ($F(3,140) = 7.388$, $p < 0.0001$) and granular ($F(3,140) = 2.998$, $p < 0.033$) insular regions. No significant differences between sham operated and LFPI+MN166 groups were found in the sub-regions of the insula ($p > 0.153$). While MN166 treated LFPI rats were not distinguishable from sham operated controls, post-hoc analyses revealed that LFPI+vehicle rats had significantly greater astrocytic

activation in all three sub-regions as compared to controls (Fig. 3.4A, right graph): granular ($p < 0.003$ vs. Sham+vehicle), dysgranular ($p < 0.002$ vs. sham operated groups and LFPI+MN166) and agranular ($p < 0.03$ vs. Sham+vehicle and LFPI+MN166 groups).

In the sub-regions of the amygdala (Fig. 3.4B; right graph), GFAP labeling in LFPI+vehicle rats was significantly increased in BLA ($F(3,140) = 39.154$, $p < 0.001$) and CE ($F(3,140) = 12.073$, $p < 0.001$) nuclei. Post-hoc analyses revealed that LFPI+vehicle rats had significantly greater astrocytic activation in both sub-regions as compared to controls: CE ($p < 0.001$ vs. sham operated groups and LFPI+MN166) and BLA ($p < 0.001$ vs. sham operated groups and LFPI+MN166). MN166 treated LFPI rats had significantly less GFAP expression than Sham+vehicle controls in CE ($p = 0.021$), and significantly greater GFAP expression than Sham+MN166 controls in BLA ($p = 0.031$) nuclei.

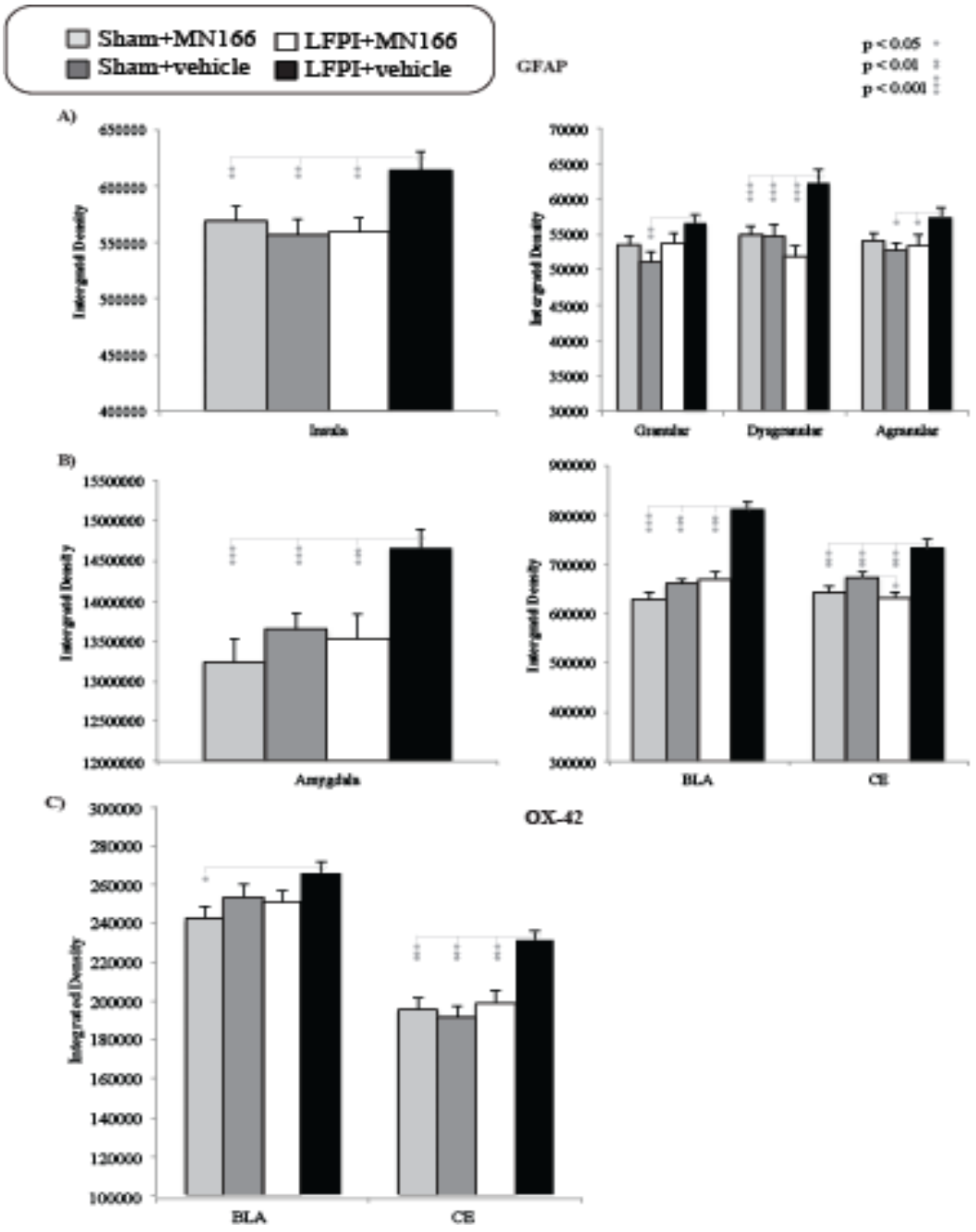

Figure 3.4 Astroglial and microglial activation in insula and amygdala at 6 months post-injury. (A-B) LFPI+vehicle rats had significantly increased in GFAP labeling in both regions, indicating higher astroglial activation compared to sham operated and LFPI+MN166 treated rats. (C) In the central amygdala (CE), microglial activation was greater in LFPI+vehicle injected rats compared to both sham operated groups and LFPI+MN166 treated rats ($p < 0.001$), and was approaching significance ($p = 0.071$) for basolateral amygdala (BLA). Data represent mean± SEM.

LFPI+vehicle rats also showed significantly increased microglial activation compared to control groups (Fig. 3.4C), as measured by OX-42 labeling, but this was restricted to sub-regions of the amygdala: CE $F(3,140) = 9.290$, $p < 0.001$), and also approached significance in BLA $F(3,140) = 2.399$, $p < 0.071$) nuclei. Post-hoc analysis revealed increases in microglial activation for LFPI+vehicle rats in CE ($p < 0.001$ vs. sham operated groups and LFPI+MN166). No differences in OX-42 labeling were observed between sham operated and LFPI+MN166 groups in amygdala, nor were any significant between group differences found in OX-42 expression for the insula.

DISCUSSION

LFPI-induced anxiety-like behaviors were found at long-term post-injury timepoints in untreated LFPI animals, as compared to MN166 treated, implicating chronic neuroinflammation in the development of anxiety-like behaviors following TBI. The remarkable finding was that pharmocological suppression of immune responses at one month post-injury reduced behavioral and immunological impairments through six months following TBI and restored MN166 treated rats to similar levels of sham operated controls, thus showing that immunosuppression is capable of reversing established post-traumatic anxiety behaviors. To our knowledge, this is the first study to report immunosuppression at long-term, post-injury timepoints, as other immunosuppressive treatments targeting anxiety-like behaviors have been administered prior to or within hours of injury (Kovesdi et al., 2012; Rodgers et al., 2012; Siopi et al., 2012). These results have important clinical implications for existing anxiety disorders following TBI, that MN166 treatment was successful in attenuating behavioral and immunological consequences when delivered at one month post-injury is a very important finding that substantially expands the therapeutic window for treatment of post-traumatic anxiety.

Chronic neuroinflammation must be due to a self-perpetuating positive feedback loop that, if interrupted, can improve functional

outcome following TBI. There is substantial support for chronic inflammation following TBI, both human and animal studies have shown that neuroinflammation is an ongoing process that persists for months to years following the injury (Gentleman et al., 2004; Streit et al., 2004; Nagamoto-Combs et al., 2007; Ramlackhansingh et al., 2011). The ongoing inflammatory response to tissue injury may contribute to damage and dysfunction in brain regions associated with anxiety, as TBI has been found to induce both acute and chronic neurodegeneration that could be caused by delayed cellular death pathways initiated by complex signaling cascades in activated glial cells (Smith et al., 1997; Bendlin et al., 2008).

Evidence for chronic inflammation has been seen in a number of studies examining patients with anxiety disorders, which report peripheral elevations of TNF-α, IFNγ, IL-1β and IL-6 in patients with PTSD (Spivak et al., 1997; Rohleder et al., 2004; Tucker et al., 2004; von Kanel et al., 2007) and elevations of TNF-α and IL-6 in patients with OCD (Konuk et al., 2007). A recent examination of cytokine and chemokine levels in well-characterized individuals with a primary DSM-IV panic disorder (PD) or PTSD diagnosis, revealed significantly higher mean levels of 18 of 20 cytokines measured, and elevations in six or more detectable levels of nine well-described proinflammatory cytokines and chemokines (MCP-1, MIP-1α. IL-1α, IL-1β, IL-6, IL-8,

Eotaxin, GM-CSF and IFNγ) as compared to healthy controls, suggesting the presence of a more generalized inflammatory cytokine signature (Hoge et al., 2009). The current results provide evidence of chronic neuroinflammation in the development of post-traumatic anxiety, as evidenced by elevated astroglial and microglial immunoreactivity in the amygdala and insula at 6 months post-injury, these brain regions are associated with hyperexcitability in anxiety disorders in humans.

Possible mechanisms of neuroinflammation

A possible mechanism of chronic neuroinflammation is endogenous and exogenous danger signals released from stressed or injured tissue, which signal various cells to produce cytokines, chemokines, and DAMPs (Gallucci and Matzinger, 2001; Oppenheim and Yang, 2005; Oppenheim et al., 2007;(Bianchi, 2007). DAMPs reactivate and further promote the production of inflammatory mediators, initiating a positive feedback loop of inflammation-damage-inflammation (Namas et al., 2009). Once activated, DAMPs initiate the innate immune response through the activation of antigen presenting cells (APCs), and microglia are the primary APCs of the of the CNS (Olson and Miller, 2004).

Resident microglia and invading macrophages have been observed many years following injury and are also key cells mediating

inflammatory processes in long-term survivors of TBI (Gentleman et al., 2004; Streit et al., 2004; Nagamoto-Combs et al., 2007; Ramlackhansingh et al., 2011). They may contribute to neuroinflammation through the secretion of proinflammatory cytokines such as IL-1 and TNF-α, which amplify the inflammatory response by initiating the production of other cytokines, promoting microglial proliferation and activation of astrocytes (Namas et al., 2009). The so called 'cytokine cycle' may initiate an inflammatory response in the brain that is normally neuroprotective, but can become self-perpetuating and cause neurodegenerative changes (Griffin et al., 1998; Gentleman et al., 2004). Sustained glial responses have been shown to result in secondary tissue damage (Gasque et al., 2000; Simi et al., 2007; Hailer, 2008; Lehnardt, 2010) and neuronal death (Sternberg, 1997; Brown and Bal-Price, 2003; Schmidt et al., 2005; Beattie et al., 2010). Evidence of these mechanisms is supported in our findings of reactive gliosis present in the insula and amygdala at long-term post-injury timepoints.

Treatment of post-traumatic anxiety with MN166

While the exact role of the immune system in the pathogenesis of anxiety disorders following TBI remains unknown, neuroinflammation is emerging as a potential target, as inflammation, reactive gliosis, and proinflammatory cytokine release are elevated in

many patients with anxiety disorders following TBI (Spivak et al., 1997; Rohleder et al., 2004; Tucker et al., 2004; von Kanel et al., 2007). The present findings of reductions in anxiety-like behaviors and reactive gliosis in brain regions associated with anxiety in humans supports the use of immunosuppression to improve functional outcome following TBI, other studies have shown neuroprotective effects through attenuation of immune responses. Many successful treatments for cognitive dysfunction and motor impairments following TBI have targeted the neuroimmune system, reporting decreases in activated microglia and astrocytes, IL-1β, TNF-α and IL-6 in controlled cortical impact and weight drop TBI models in rodents (Chen et al., 2007; Homsi et al., 2009; Li et al., 2009; Homsi et al., 2010). Immunosuppression has been found to be neuroprotective following TBI in rodents, resulting in increased structural preservation and improved functional outcomes (Hailer, 2008).

Minocycline, a tetracycline antibiotic with anti-inflammatory effects has been found to suppress microglial production of IL-1β, TNF-α and IL-6 in TBI models in rodents resulting in reduced cerebral edema and brain lesion volumes (Homsi et al., 2009; Homsi et al., 2010; Siopi et al., 2012). Statins, a class of lipid-lowering drugs have been reported to suppress microglial production of IL-1β, TNF-α and IL-6 in TBI models in rodents, resulting in reductions of functional

deficits and anatomical damage (Chen et al., 2007; Li et al., 2009). Cyclosporin A and FK506, are potent immunosuppressant drugs that have been found to suppress activation of microglia and astrocytes following lateral fluid percussion brain injury in rats, resulting in improved mitochondrial preservation, reductions in dendritic spine loss, and improvements cognitive performance and functional motor recovery (Alessandri et al., 2002; Campbell et al., 2011). The immunosuppressant drugs discussed above have direct inhibitory effects on microglia and astrocytes, leading to reductions in the secretion of proinflammatory cytokines following TBI.

Delayed treatment administration

The present study finding that delayed (one month) immunosuppression is capable of reversing established post-traumatic anxiety behaviors and immunological impairments through six months following TBI, restoring MN166 treated rats to similar levels of sham operated controls, contributes to evidence that the critical window for treatment following TBI could be expanded to include those suffering from long-term TBI-related disabilities. Studies have shown that delayed treatment (24 hr) with erythropoietin (EPO), a novel neuroprotective cytokine found to improve neuronal survival through the attenuation of cytokine production and inflammation, improved sensorimotor functional recovery, reduced hippocampal cell loss,

enhanced neurogenesis, and improved neurological outcomes following controlled cortical impact and weight drop TBI models in rodents (Yatsiv et al., 2005; Xiong et al., 2010).

A recent study reported reduced chronic inflammation and neurodegeneration after activation of metabotropic glutamate receptor 5 (mGluR5) with the specific agonist (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), which has been shown to decrease microglial activation and release of associated proinflammatory mediators (Byrnes et al., 2012). This study delayed treatment until one month following controlled cortical impact in mice, delivering a single intracerebroventricular (icv) injection of CHPG, and the results revealed reductions in reactive gliosis, hippocampal cell loss, reduced lesion progression, and improved motor and cognitive recovery compared to untreated controls. The findings of these studies provide support that delayed treatment can result in reduction of neuroinflammation and lead to better functional outcomes following TBI.

Immunosuppression of neuroinflammation may be a possible therapeutic target in the treatment of anxiety disorders following TBI. The above mentioned and current results add further support to the conclusion that inflammation produced by neuroimmune responses play a role in TBI-induced anxiety, and suggest that delayed post-

injury suppression of glial cell activation could expand the clinical window for treatment of TBI-induced anxiety disorders in humans.

CHAPTER V

DISCUSSION

DISCUSSION

TBI remains one of the leading causes of mortality and morbidity in the U.S. and worldwide, yet no successful neuroprotective therapies have been found in human clinical trials to date (Kumar and Loane, 2012). TBI is usually thought to involve a single pathological insult; however, new research is showing that neuroinflammation is an ongoing process that may contribute to new clinical disorders emerging years following primary injury. Studies have found that chronic inflammation is still present many years following TBI (Gentleman et al., 2004; Streit et al., 2004; Nagamoto-Combs et al., 2007; Ramlackhansingh et al., 2011) and convincing evidence for chronic inflammation in the pathophysiology of anxiety has also been shown (Spivak et al., 1997; Rohleder et al., 2004; Tucker et al., 2004; von Kanel et al., 2007).

The results presented with this thesis illustrate that acute and chronic neuroinflammation can cause hyperexcitability in the brain and that peri-injury and delayed post-injury immunosuppression can result in better functional outcomes following TBI. While the precise mechanisms involved in the development of neurodegenerative pathology and subsequent neuropsychiatric disorders are unclear, neuroinflammation is emerging as a potential treatment target for both the prevention of development and reversal of post-traumatic anxiety.

The present experiments confirm that LPFI produces trauma-induced, long lasting anxiety-like behavior and immunological changes in rats. The results also provide evidence of the efficacy of Ibudilast in attenuating the development of anxiety and reactive gliosis in key brain regions found to be associated with anxiety in both humans and rodents.

In experiment 1, we show that glial activation is sufficient to produce hyperexcitability in neurons and may be a mechanism by which neuroinflammation produces changes in brain regions associated with anxiety following TBI. The findings showed that LPS administration results in increased neuronal excitability capable of triggering seizures *in vivo*. The findings that pre-application of LPS-RS (the receptor antagonist for LPS) prevented the LPS-induced excitability suggested that LPS was not exerting direct effects on cortical excitability. Our results also supported the role of proinflammatory cytokines in hyperactivity following an LPS immune challenge and IL-1β has been found to induce anxiety in many rodent models. Specifically, the results showed that IL-1 is an important glia-mediated pro-inflammatory cytokine participating in these excitability changes since IL-1ra prevented the effects of LPS, a finding in close agreement with reports of anti-convulsant actions of IL-1ra in experimental models of epilepsy (Vezzani et al., 2000). However, IL-1

is only one of several proinflammatory cytokines capable of increasing neuronal excitability and lowering seizure threshold.

It has been well established that trauma related reactive gliosis results in the acute release of high levels of proinflammatory cytokines. The most prominent cytokines expressed are IL-1β and TNF-α, which are central mediators of neuroinflammation following head injury. While we have shown evidence here of IL-1β in neuronal excitability, other researchers have found that the release of TNF-α also pathologically increases neuronal excitability in all brain regions where it has been measured (Riazi et al., 2008b; Schafers and Sorkin, 2008; Rodgers et al., 2009; Beattie et al., 2010; Maroso et al., 2010). Overall, the findings of increased proinflammatory cytokine levels in anxiety disorders, increased anxiety-like symptoms following the administration of these cytokines in rats, and reports of elevated microglia, astrocytes, IL-1β, TNF-α and IL-6 in closed head injury models in rodents provide evidence of the role of the neuroimmune system in the pathophysiology of post-traumatic anxiety.

In experiment 2, we extended to findings from experiment 1 by demonstrating that LFPI could induce anxiety-like behaviors in an animal model of post-traumatic anxiety. Further, we showed the involvement of neuroinflammation by attenuating the behaviors and trauma-induced changes in reactive gliosis through

immunosuppression with a non-selective phosphodiesteraise inhibitor, Ibudilast. In this experiment we found that peri-injury administration resulted in a marked reduction in anxiety behaviors, which may have been due to attenuation of the inflammatory response post injury, as we found that astroglial and microglial activation was significantly reduced in the amygdala and insula, brain regions consistently implicated in anxiety in both rats and humans. This experiment had important clinical underpinnings and we were the first to report a reduction in anxiety-like behavior following immunosuppression.

Lastly, this experiment validated the use of LFPI to induce anxiety-like behavior following experimental TBI in rats and suggests that animal models may be useful for exploring basic mechanisms of post-traumatic anxiety observed in humans. In spite of overwhelming evidence that TBI is a risk for the development of anxiety disorders, this topic has been largely overlooked in animal research, likely because it is not thought to have a biological basis. However, our work provides support to a growing body of literature that suggests otherwise. Indeed, rat behavior typically associated with anxiety has been reported across a range of injury models including: impact-acceleration, pneumatic cortical contusions, controlled cortical impact and LFPI. Following impact-acceleration injuries, researchers have reported elevations in marble-burying behavior and decreases in social

interaction and exploratory behavior in the open field test (Fromm et al., 2004; Pandey et al., 2009). Rats that received pneumatic cortical contusions and controlled cortical impact have also showed reduced exploratory behavior in the open field test (Sonmez et al., 2007; Wagner et al., 2007). Significant decreases in exploration of the open field and elevated plus maze were also reported following LFPI (Jones et al., 2008).

In experiment 3, we replicated the finding of LFPI-induced increases in anxiety-like behavior and showed that acute treatment with Ibudilast resulted in a reduction of anxiety-like behaviors and reactive gliosis in brain regions important to the development of anxiety. However, this study expanded the clinical relevance of our previous findings because treatment was not administered until one month post-injury and the reductions in anxiety-like behavior persisted out to six months. The findings of elevated astroglial and microglial levels at timepoints this long post-injury supports the hypothesis of chronic neuroinflammation in anxiety disorders following TBI. The results also broaden the critical treatment window for those with post-traumatic anxiety disorders and suggest neuroinflammation as a possible treatment target long after injury and behavior have been established.

This was an important finding because studies indicate that prevalence rates continue to increase years after injury (Morton and Wehman, 1995; Deb et al., 1999; Koponen et al., 2002). Evidence for long-term risk in the development of post-traumatic anxiety was documented in a study of TBI patients that were assessed 30 years following injury, the results indicated that almost half (48.3%) of TBI participants developed a psychiatric disorder following injury, and almost half (23.3%) of the reported disorders were anxiety disorders, thus showing the importance of psychiatric follow-up and chronic treatment management after injury (Koponen et al., 2002).

Very few animal studies have reported success in treating post-traumatic anxiety. A recent meta-analysis (1989-2009, including 91 treatments and over 200 pre-clinical studies) assessed the impact of pharmacological agents on cognitive, motor and behavioral outcomes in rats following TBI, and the results revealed that no treatment improved behavioral outcomes, including anxiety, depression and aggression (Wheaton et al., 2011).

Three studies that have been found to be effective in reducing anxiety-like aftermaths following TBI, as evidenced by increased exploratory behavior in open field and elevated plus tests in treated animals, utilized magnesium, resveratrol and progesterone (Fromm et al., 2004; Cutler et al., 2006b; Sonmez et al., 2007). Although the

mechanism of action for magnesium treatment is unknown, the neuroprotective effect is likely due to reductions in glutamate excitoxicity, mitochondrial damage, and apoptosis (Vink and Nimmo, 2009). Resveratrol is a potent polyphenol with many antioxidant properties, which have been shown to decrease oxidative stress following TBI, resveratrol has also been found to be neuroprotective against excitotoxicity, ischemia, and hypoxia (Sonmez et al., 2007). Progesterone is thought to protect against glutamate excitotoxicity by interacting with inhibitory $GABA_A$ receptors and through modulation of excitatory neurotransmitter (kainite, glycine, serotonin, and acetylcholine) receptors (Vink and Nimmo, 2009). Recent studies have found that progesterone is a potent neuroactive steroid that reduces pro-apoptotic NGF precursor (proNGF) and pro-apoptotic BDNF precursor (proBDNF), while simultaneously increasing pro-survival neurotrophin signaling, leading to reduced apoptosis and improved behavioral recovery following TBI (Cekic et al., 2012). Following many years of successful clinical trials, progesterone is now in Stage III clinical trials for the treatment of moderate-to-severe TBI and Stage III international trials to treat pediatric brain injury (Stein and Wright, 2010; Stein, 2011a, b).

While the above treatments did show reductions in anxiety-like behavior following TBI, all treatments required rapid administration

reducing the therapeutic window to the day of injury. Pre-clinical studies utilizing animals have failed to translate a successful pharmacological intervention to date, likely because highly-controlled investigations may not be reflective of clinical trial designs, pre-clinical studies tend to only include one injury severity, exclude animals with secondary insults, only include young/healthy animals and typically only investigate a single sex (Vink and Nimmo, 2009). Also, the above mentioned weakness in pre-clinical trials includes the use of pre-treatment or very early interventions (30 min – 6 hours), in spite of evidence that many molecular, biochemical and immunological changes occur for many months to years following injury, and that clinical intervention may not be possible at such early stages of TBI. To better understand the pathophysiology of post-traumatic anxiety, pre-clinical treatments will need to target multiple injury factors in a wide range of injury severities, across both sexes and multiple age groups. Future research in our lab will focus on delayed MN166 treatment across injury severities; targeting mild, moderate and severe injuries to extend the findings of neuroprotective immunosuppression on functional and immunological outcomes following TBI across a range of injury severities.

CONCLUSION

The development of anxiety disorders following TBI is a complex and multifaceted problem, finding treatments that work will require multifaceted approaches. The injury itself initiates many complex biological events including glial activation and breakdown of the blood brain barrier, excitotoxicity and chronic neuroinflammation. Although studies are beginning to shed light on the role of neuroimmunity in anxiety, the lack of neuroimaging studies and neurochemical alterations in patients with TBI and anxiety disorders remains a barrier in finding a successful treatment. Emerging immunosuppressive treatments have shown success in treating cognitive and motor impairments following TBI, and have also been found to reduce mitochondrial dysfunction, lesion volumes and edema. Treatments with direct inhibitory effects on microglial proliferation and secretion of proinflammatory cytokines may be effective in the treatment of anxiety disorders following TBI, as the pathophysiology underlying the development of these disorders indicates chronic inflammatory states. Although we lack understanding of the brain regions and cellular origin of dysfunction in post-traumatic anxiety, targeting neuroinflammation is emerging as a promising approach to treatment.

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