PRE-EXISTING INFLAMMATION PRODUCES DEPRESSION FOLLOWING SPINAL CORD INJURY

A Dissertation

by

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ABSTRACT

Major depressive disorder (MDD) is a significant, but understudied, consequence of spinal cord injury (SCI). Approximately 11-24% of SCI patients experience MDD, compared to 8% in the general population, yet there are no therapies specific for SCI patients. Previously, we have shown that one-third of spinally injured rats exhibit behavioral, physiological, and immunological correlates of depression. This is concomitant with the human population, and, moreover, indicates that a biological mechanism is responsible for the rise in depression after SCI. As increased inflammation is strongly associated with depression in both animal and human studies, it is likely that the immune activation inherent to SCI fosters future depression. To address this hypothesis, the experiments presented here explored the impact of inflammation on depression in a rat model of SCI. The first experiment evaluated the protective effects of minocycline, an anti-inflammatory drug, on the development of depression. The results indicated that inflammation after injury may not influence depression, but that higher inflammatory profiles *before* injury predicted depressive outcomes. The next experiment explored the sufficiency of pre-existing inflammation for the development of depression after injury. Pre-treatment with the pro-inflammatory cytokine IL-6 produced a significant elevation in incidence of depression following SCI. Finally, I investigated the hypothesis that pre-existing inflammation may lead to glucocorticoid resistance, dysregulation of the immune response after injury, and subsequent depression. Refuting this hypothesis, I found that subjects with pre-existing inflammation did not have higher corticosterone

levels post-injury, although they did have elevated depression-like behaviors. Together, the findings here suggest that pre-existing inflammation predicts susceptibility to depression following a major stressor, such as SCI. These results underscore the importance of a deeper understanding of the relationship between stress and inflammation, and the impact of pre-existing differences on psychiatric health care.

DEDICATION

To Dr. Steiner, Dr. Van Zant, and Professor Pytel, who shaped the foundations of my journey in science.

And to Ellen, who convinced me to apply to Texas A&M. If it weren't for you, I wouldn't be writing this dissertation.

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The lesion analysis and neurogenesis data presented in Experiment 1 (Chapter III) was obtained with Aryana Garza and Grace Yoo, as independent undergraduate research projects under the supervision of Dr. Michelle Hook. The rtPCR data presented in Experiment 3 (Chapter V) was obtained with help from Nihal Salem, a graduate student of Dr. Rajesh Miranda of the Department of Neuroscience and Experimental Therapeutics. All other work for the dissertation was completed by the student independently.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW[1]

Depression following spinal cord injury

While paralysis or loss of limb function is the most recognizable consequence of a spinal cord injury (SCI), SCI also affects psychological wellbeing. Approximately 22.2% of patients with SCI suffer from major depressive disorder (MDD) (Williams and Murray, 2015), an incidence nearly three times greater than the 8.1% in the general US population (Brody et al., 2018). An additional 16-34% of SCI patients report significant clinical symptoms of depression but do not meet the criteria for MDD (Bombardier et al., 2012; Krause et al., 2000; Migliorini et al., 2009).

The impact of depression on both physical and psychological health after SCI is also substantial. Depression is associated with an increased risk of suicide (Cao et al., 2014; Craig et al., 2015; DeVivo et al., 1991; Soden et al., 2000), an increased incidence of urinary tract infections and pressure ulcers, longer hospitalization stays, less adherence to rehabilitation protocols, lower community involvement, and greater unemployment (Elliott and Frank, 1996; Fuhrer et al., 1993; Herrick et al., 1994). However, despite its prevalence and its significant impact on quality of life, there has been relatively little research conducted on the etiology of depression after SCI.

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Injury characteristics *per se* do not appear to affect mental health scores (Abrantes-Pais et al., 2007; Charlifue et al., 2011; Leduc and Lepage, 2002; Tramonti et al., 2014), but psychosocial changes are associated with the development of depression. As in the general population, individuals with poor social relationships are more likely to experience mental health problems than those with satisfactory social relationships (de la Vega et al., 2018; Dodd et al., 2015; Kraft and Dorstyn, 2015; Müller et al., 2012; Tough et al., 2017; Zürcher et al., 2019). Environmental barriers (i.e. lack of physical accessibility) and low access to assistance (e.g. health care and home caregiver services) are significant obstacles for social participation after SCI (Hammel et al., 2015; Tsai et al., 2017). Loss of employment, insurance, and income after SCI, together with the high costs of ongoing medical care and accommodations necessary to adapt with disability, can also lead to a decline in socioeconomic status (Fyffe et al., 2011; Paul et al., 2013). In the United States, even among people with SCI that do return to work, women, minorities, and those that have not completed a college degree earn less, further contributing to financial strain (Krause and Broderick, 2004; Krause and Pickelsimer, 2008; Krause and Saunders, 2009; Krause and Terza, 2006). Not surprisingly, individuals with financial strain are also more likely to report general mental health problems and depressive symptomatology after SCI (Fekete et al., 2014; Krause et al., 2000; Lim et al., 2017; Zürcher et al., 2019). Personal and environmental changes such as these are meaningful risk factors for development of depression following SCI.

Resilience (the process of positive behavioral and psychological adaptation in the context of significant adversity) is vital for protection against depression after SCI, but it

can be weakened by a number of psychosocial and biological factors (Catalano et al., 2011; Dodd et al., 2015; Min et al., 2014; Todd and Worell, 2000). For example, personality and the ability to apply active coping strategies (problem solving, seeking support, exercising) have been shown to boost resilience, but work in animals models suggests that neurobiological processes, especially inflammation, reduce resilience and increase susceptibility to depression (Cairns et al., 2014; Gardner et al., 2009; Hodes et al., 2014; Maldonado-Bouchard et al., 2016; Menard et al., 2017; Oshio et al., 2018; Riolli et al., 2002; Wood et al., 2015). Because inflammation is a hallmark of SCI and has been strongly associated with susceptibility to stress, it may provide the link between SCI and subsequent depression.

SCI and inflammation

Inflammation resulting from SCI has been well described (Davies et al., 2007; Popovich et al., 1997). Immediately following injury, peripheral macrophages infiltrate into the spinal cord, and resident, resting microglia transform into activated microglia (Popovich et al., 1997). Inflammation persists as neutrophils, microglia, and macrophages are attracted to the injury site, where they attempt to remove dead cells and promote healing. While acute inflammation can have beneficial effects, the chronic inflammation that develops with SCI has serious negative outcomes, including neuropathic pain, metabolic disorders, and even, ironically, immune system impairment (Allison and Ditor, 2015a; Sun et al., 2016).

Importantly, inflammation is not confined to the primary site of injury, or even the spinal cord. After SCI, the brain also expresses pro-inflammatory cytokines associated

with activated microglia (Wu et al., 2014a). These microglia release cytotoxic and pro-inflammatory agents, producing neurotoxicity and causing cell death and related behavioral changes (Takeuchi, 2010; Wu et al., 2014a). Activated microglia have been identified in the thalamus, hippocampus, and frontal cortex in a rodent model of SCI; they activate as soon as 7 days after injury and remain so for at least 10 weeks post-SCI (Wu et al., 2014a; Wu et al., 2014b).

Further, the inflammatory response after SCI is not limited to the central nervous system (CNS). Emerging evidence indicates that activated, inflammatory microglia can migrate in and out of the spinal cord after a spinal nerve avulsion, carrying debris from the injury site to both the periphery and brain (Green et al., 2019). Additionally, SCI can lead to gut dysbiosis and bacterial translocation across the gut wall. Bacterial translocation, in turn, results in heightened immune activity in gut-associated lymphoid tissue and, subsequently, systemic immune activity and inflammation (Kigerl et al., 2016). Persistent peripheral inflammation does develop in a subset of patients after SCI. In fact, 1 year after injury, more than half of people living with SCI display elevated serum IL-2 and tumor necrosis factor-α (TNF-α) levels, while exhibiting no differences in anti-inflammatory cytokine levels (Hayes et al., 2002). Interestingly, even among SCI patients who are asymptomatic for other complications (pressure ulcers, pain, or urinary tract infections), there is a subset of individuals that have IL-6 levels about equal to those of patients with ongoing complications contributing to inflammation (Davies et al., 2007). These data indicate that there is a population of SCI patients who experience elevated inflammation, regardless their injury status. However, we do not know if these individuals

with high pro-inflammatory cytokine profiles also experience significantly different clinical outcomes. Given the association between depression and inflammation, as discussed below, it would be important to know whether high inflammatory profiles affect psychological wellbeing.

Inflammation and depression after SCI

As we learn more about depression, we have begun to realize that it is not a disease merely of the brain or the mind. In the past few decades, considerable evidence has suggested a connection between inflammation and depression. Pro-inflammatory cytokine upregulation has been described in both peripheral tissue and the central nervous systems of depressed individuals (Lindqvist et al., 2009; Liu et al., 2012; Loftis et al., 2010; Maes et al., 1997; Maes et al., 1995; Maes et al., 1993; Myint et al., 2005; Pandey et al., 2012). Chronic inflammation has been noted in many cases of major depression, and depression itself is often comorbid with diseases characterized by chronic inflammation or injury to the CNS. For example, there can be up to 50% lifetime prevalence of depression among patients with multiple sclerosis (Siegert and Abernethy, 2005), patients with psoriasis, inflammatory bowel disease, and arthritis also have increased likelihoods of developing depression (Jensen et al., 2016; Marrie et al., 2017; Olivier et al., 2010; Siegert and Abernethy, 2005). Further, interferon-α (IFN-α) immunotherapies for diseases such as cancer and hepatitis C cause a significant increase in depressive symptoms in patients, with up to 50% developing major depression (Capuron et al., 2009; Capuron et al., 2002). Similarly, in animal models, central or systemic administration of pro-inflammatory cytokines produces a "sickness" behavior that is characterized by behavioral and

physiological changes associated with depression (Anisman et al., 2005). These depressive-like symptoms can last for several weeks after administration (Anisman and Merali, 2003; Schmidt et al., 2003), long after the levels of exogenously applied cytokines have subsided. Symptoms of this inflammatory treatment are attenuated by the clinically-relevant antidepressant, fluoxetine (Merali et al., 2003). These data indicate a causative, rather than merely a correlative, relationship between pro-inflammatory cytokines and depression.

Animal studies have also found that depression after SCI is associated with increased inflammation. A clip compression injury resulted in elevated pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, and IL-6) in both blood plasma and the spinal cord of spinally injured female rats with depression-like symptoms (decreased sucrose preference and social interaction), compared to sham and injury-naïve rats, for up to 28 days post injury (do Espírito Santo et al., 2019a). Others have found behavioral deficits and signs of an activated immune response in the brain after SCI in mice. In a comprehensive evaluation of cerebral inflammation after SCI, Wu et al. (2014b) analyzed cognition, depressive-like behaviors (as measured by tail suspension test and sucrose preference test), cerebral microglial activation, and cell cycle activity in spinally injured mice. They found that spinally injured mice showed more depression-like behavior than their uninjured counterparts and that administration of CR8, a cyclin-dependent kinase inhibitor and cell-cycle inhibitor, decreased microglial activation, reduced neuronal cell death, and reversed the depression behaviors (Wu et al., 2014b).

Using a comprehensive behavioral ethogram and statistical analyses, our laboratory was one of the first to describe depression behavior in a subset of SCI rats (Luedtke et al., 2014). Using hierarchical clustering, we are able to identify specific subjects that show depression-like behavior after SCI, allowing us to more effectively explore individual differences between depression susceptible and resilient animals. Luedtke et al. (2014) found that 35% of spinally injured rats developed depression symptoms in the month following a T12 contusion injury, a percentage concomitant with the human population (Williams and Murray, 2015). Subsequently, we have found that "depressed" and "not-depressed" SCI rats differ in their molecular responses to injury (Maldonado-Bouchard et al., 2016). Inflammatory cytokines increased in both the serum and the hippocampi of depressed SCI rats, compared to both the intact controls and the not-depressed SCI rats. By 24 days post-injury, SCI rats exhibiting depression-like behaviors had higher IL-1β and IL-17A in their serum and higher IL-1α and TNFα in their hippocampi, indicating that depression after SCI is associated with increased inflammation, even relative to not-depressed SCI subjects. These studies show that even in the absence of external psychological stressors, inflammation is associated with depression after SCI.

One of the few studies examining the interaction between inflammation and depression after SCI in humans investigated the therapeutic impact of an anti-inflammatory diet (Allison and Ditor, 2015b). Participants eliminated inflammation-inducing foods, such as refined wheat and sugar, from their diets and introduced anti-inflammatory supplements, such as curcumin, omega-3 fatty acids, and antioxidants. One

month after the start of treatment, patients on the diet had lower IL-1β and lower scores on the Center for Epidemiological Studies Depression Scale than they did before the start of treatment. Though the treatment itself did not target one, specific, inflammatory mechanism of depression, this study measured kynurenine and tryptophan levels in patients' serum, which are products of an inflammation-driven alternative tryptophan pathway. The changes in patients' depression scores were positively correlated to the changes in kynurenine and the tryptophan/kynurenine ratios in these patients. These findings support both the inflammatory theory of depression and the tryptophan/kynurenine pathway as a mechanism of action, which will be discussed below.

Molecular mechanisms of inflammation-driven depression

Pharmacological studies have shown that interventions targeting inflammatory pathways significantly alter the expression of neurotransmitters implicated in depression. Peripheral inflammatory signals may impact brain function in several ways. As shown in Figure 1, peripheral inflammatory signals modulate serotonin (5-HT) and brain-derived neurotrophic factor (BDNF) pathways, and activate the hypothalamic-pituitary-adrenal (HPA) axis (Anisman, 2009; Audet and Anisman, 2013; Maes et al., 2011; Miller and Raison, 2016; Raison et al., 2010; Zhu et al., 2010). The link between decreased serotonin and depression is well-established, and this system is already targeted by standard antidepressants (Coppen and Doogan, 1988; Fakhoury, 2016; Kambeitz and Howes, 2015). More recent data, however, have shown that BDNF is also associated with depression, most likely through its ability to promote neurogenesis. BDNF expression and protein levels are lower in the prefrontal cortex and hippocampi of individuals who die

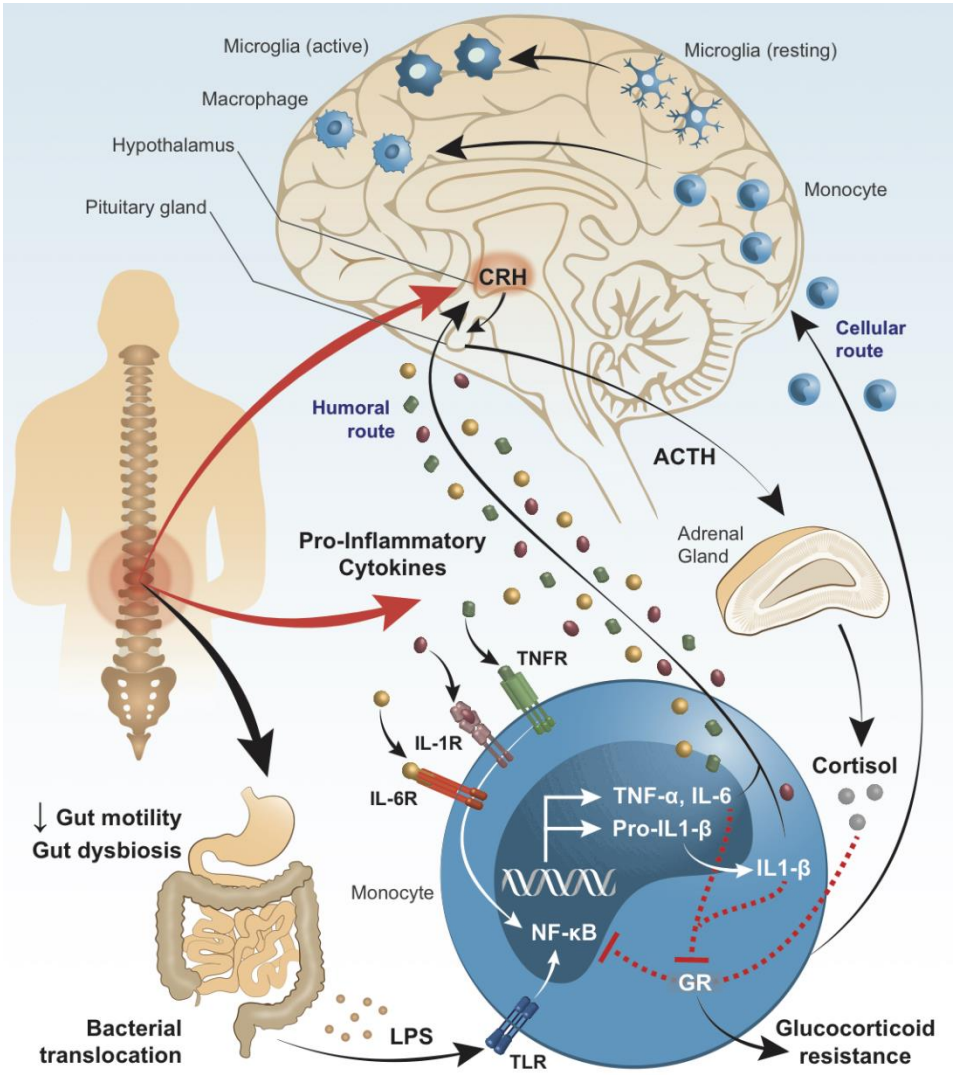

Figure 1. Effects of spinal cord injury on inflammation.
Spinal cord injury (SCI) has major effects throughout the body and influences inflammatory response in multiple systems. Under normal conditions, the hypothalamus creates corticosterone releasing hormone (CRH), which releases adrenocorticotropic hormone (ACTH) in the pituitary gland, which acts on the adrenal gland to release cortisol. In immune cells, cortisol binds to the glucocorticoid receptor (GR) to inhibit nuclear factor-κB (NF-κB) and promote negative feedback of the stress response. Inflammation or gut dysbiosis from SCI disrupts the HPA axis and initiates immune response in monocytes via cytokine receptors or toll-like receptors (TLR), increasing NF-κB production, which prevents transcription of anti-inflammatory factors and promotes transcription of pro-inflammatory cytokines. Pro-inflammatory cytokines can travel directly to the brain, where they continue to stimulate the HPA axis. Monocytes also travel to the brain via circulation and then release inflammatory factors that convert resting microglia into their active, inflammatory state.

by suicide than in age- and sex-matched controls (Dwivedi et al., 2003). BDNF levels also inversely correlate with the degree of clinical impairment, as well as with reductions of hippocampal volume, and they normalize with successful antidepressant treatment (Huang et al., 2008; Shimizu et al., 2003). Importantly, there is evidence that SCI itself reduces BDNF in both the spinal cord and hippocampus (Fumagalli et al., 2009; Garraway et al., 2011; Hajebrahimi et al., 2008; King et al., 2000; Liebl et al., 2001). While dysregulation of other neurotransmitters, such as dopamine, have also been associated with depression in other models, the literature reviewed here will focus on serotonin and BDNF, as both have been heavily implicated in depression and SCI. The following sections review the molecular mechanisms that may underlie the development of depression in conditions characterized by inflammation, such as SCI.

Cytokine mediated effects on depression via the kynurenine cycle

The kynurenine pathway of tryptophan metabolism has been heavily implicated as a causal factor in the development of inflammation-induced depression. As shown in Figure 2, cytokine-induced activation of the enzyme indoleamine 2,3-dioxygenase (IDO) decreases serotonin and BDNF levels, in part by diverting the metabolism of tryptophan (the primary precursor of serotonin) into kynurenine, decreasing serotonergic availability (Maes et al., 2011; Müller, 2016; Raison et al., 2010). Cytokines like IL-1β and IFN-γ can also increase kynurenine 3-monooxygenase (KMO) levels in microglia and neurons (Connor et al., 2008; Corona et al., 2010; Gonzalez-Pena et al., 2016; Guillemin et al., 2003; Laumet et al., 2017). KMO is a key enzyme for the metabolism of kynurenine into 3-hydroxy-kynurenine (3-HK). 3-HK is then transformed into quinolinic acid (QUIN) by

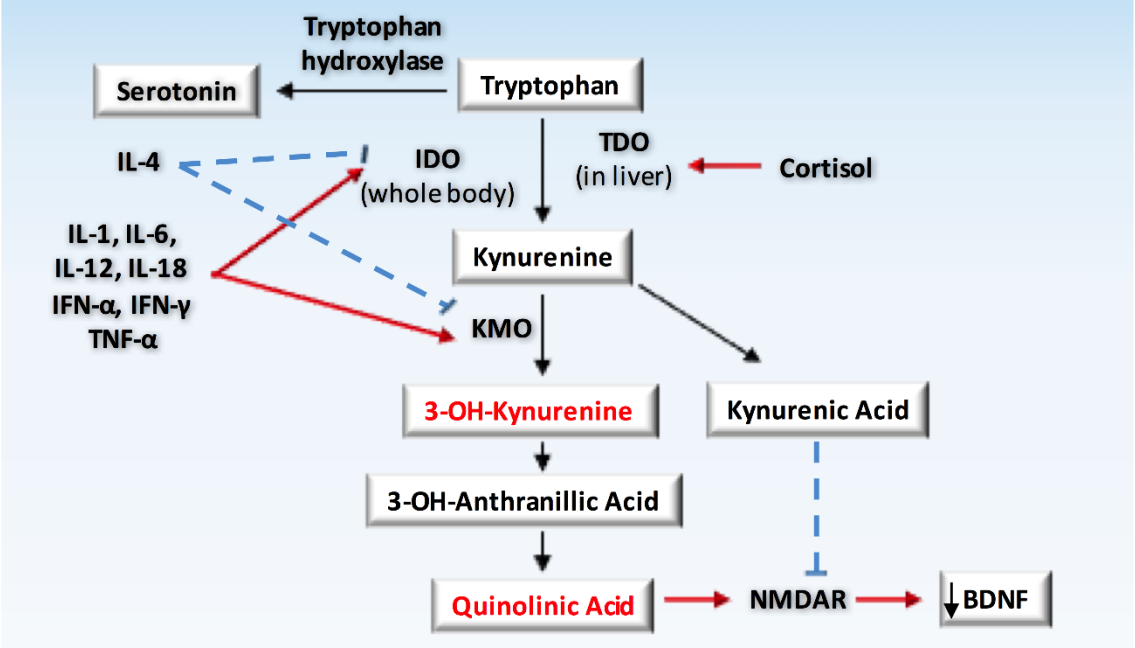

Figure 2. Kynurenine pathway.
The amino acid tryptophan is an essential precursor to serotonin. However, it can also be converted to kynurenine, most commonly in the liver, through tryptophan 2,3-dioxygenase (TDO), or, throughout the rest of the body and especially the central nervous system, through indolamine 2,3-dioxygenase (IDO). Kynurenine is further metabolized into kynurenic acid, an N-methyl-D-aspartate receptor (NMDAR) antagonist or into quinolinic acid, an NMDAR agonist, which can produce neurotoxicity and decrease brain derived neurotrophic factor (BDNF) in the brain. Cortisol can activate TDO, while proinflammatory cytokines activate both IDO and kynurenine monooxygenase (KMO). Anti-inflammatory cytokines, such as IL-4, can inhibit IDO. *Red text: neurotoxic, red arrows: activation, blue dashes: inhibition.*

kynureninase and 3-hydroxyanthralinic acid dioxygenase (Schwarcz and Stone, 2017). QUIN, which is excitotoxic, over-activates the N-methyl-D-aspartate (NMDA) receptor (Heyes et al., 1992; Heyes et al., 1991; Schwarcz et al., 1983), and, together with cytokine-induced reductions in astrocytic glutamate reuptake and stimulation of astrocytic glutamate release, leads to excessive glutamate both within and outside the synapse (Bryleva and Brundin, 2016). Excessive glutamate binding to extrasynaptic NMDA

receptors can, in turn, lead to increased excitotoxicity and decreased BDNF (Santana-Martínez et al., 2018).

Dysregulation of kynurenine metabolism is linked to depression at multiple levels. In rodents, lipopolysaccharide (LPS) administration induces expression of IDO, IFN-γ, TNF-α, and IL-1β, while also decreasing sucrose preference and increasing immobility in the forced swim and tail suspension tests (O'connor et al., 2009). IDO is the rate-limiting step of kynurenine production, and its upregulation leads to decreased serotonin and increased neurotoxic factors. O'Connor and colleagues found that administration of 1-methyltryptophan, a competitive IDO inhibitor, blocked the development of depression after LPS administration (O'connor et al., 2009; Salazar et al., 2012). Similarly, the attenuated *Mycobacterium bovis*, bacille Calmette-Guerin (BCG) increased expression of IDO, IFN-γ, and TNF-α in the brain and produced depression-like behavior (Moreau et al., 2008). O'Connor et al. (2009) showed that the depressive phenotype and kynurenine dysregulation produced by BCG is absent in IDO and IFN-γ knockout mice.

KMO activity is also significantly increased following a peripheral immune challenge with LPS (Parrott et al., 2016b). Targeted deletion of the KMO gene prevented the expression of depression-like behaviors on the tail suspension test and increased spontaneous alterations on the Y-maze (a measure of working memory) after LPS administration, suggesting that hippocampus-dependent behaviors may be particularly vulnerable to neurotoxic dysregulation of kynurenine metabolism (Parrott et al., 2016a). Similarly, Laumet et al. (2017) showed that KMO activity is necessary for the development of depression after a spared nerve injury. They found that inhibition of KMO

activity reverses depression-like behavior in the forced swim test after injury (Laumet et al., 2017). Interestingly, Wang et al. (2017) also found an association between polymorphisms of the *KMO* gene and the incidence of postpartum depression symptoms in women. Heightened KMO activity, arising from KMO rs1053230 G/A genetic variations, were associated with a higher serum 3-HK/KYN ratio and increased susceptibility to develop depression (Wang et al., 2017). KMO appears to be a pivotal mediator of depression-like behaviors.

Increased KMO activity leads to elevations in neurotoxic metabolites including QUIN. This metabolite is produced by microglia and macrophages, and it exerts neurotoxic effects through multiple different mechanisms, including activation of the NMDA receptor (Guillemin, 2012). Suicide victims have increased levels of QUIN in their brains, and patients with major depression have increased microglial production of QUIN in the subgenual anterior cingulate cortex, a brain region implicated in the neurobiology of depression and often targeted for deep brain stimulation therapy (Harrison et al., 2009; Steiner et al., 2011). Additionally, QUIN can decrease BDNF in the brain by activating the inflammatory Jun N-terminal kinase (JNK) pathway (Santana-Martínez et al., 2018). Depression after SCI may depend on cytokine-mediated decreases in BDNF expression. Notably, current anti-depressants do not target BDNF. It has been proposed, however, that the rapid action of ketamine, an NMDAR antagonist which acts as an antidepressant in treatment-resistant patients may be mediated by the release of BDNF in the prefrontal cortex (Lepack et al., 2014; Murrough, 2012). Together, these results

illustrate the impact inflammatory cytokines can have on vital mechanisms involved in brain function and homeostasis.

Hypothalamic pituitary adrenal (HPA) axis hyperreactivity

Acute spinal cord injury not only increases inflammation, it also increases the glucocorticoid expression. In humans, urinary cortisol remains elevated for months after SCI (Campagnolo et al., 1999; Cruse et al., 2000). Similarly, after thoracic contusion injuries, rats and mice have elevated serum corticosterone levels (Gaudet et al., 2018; Gezici et al., 2009; Lucin et al., 2007; Popovich et al., 2001). Circulating corticosterone is elevated for 24 h after SCI and remains above control levels for up to 1 month postinjury in a rodent T8 contusion model (Popovich et al., 2001).

Typically, corticosteroids act as part of a homeostatic mechanism designed to control inflammation and regulate cell stress responses (Fig. 1). Glucocorticoids bind to glucocorticoid receptors (GR) in the cell cytosol, causing morphological change, phosphorylation of the receptor, and translocation into the cell nucleus, where the receptor regulates the expression of target genes (Fig. 3). GR translocation is necessary for the inhibition of nuclear factor-kappaB (NF-κB) and the transcriptional repression of inflammatory genes controlled by NF-κB (Bekhbat et al. 2017). Transactivation of anti-inflammatory genes by the GR, such as the IκB proteins, also suppresses the nuclear translocation of NF-κB and inflammation (Newton et al., 2007). Activation of the GR plays a significant role in modulating the effects of stress on the immune system.

Depression, however, has been associated with glucocorticoid resistance (the inability of glucocorticoids to exert their effects on target tissues), which undermines the

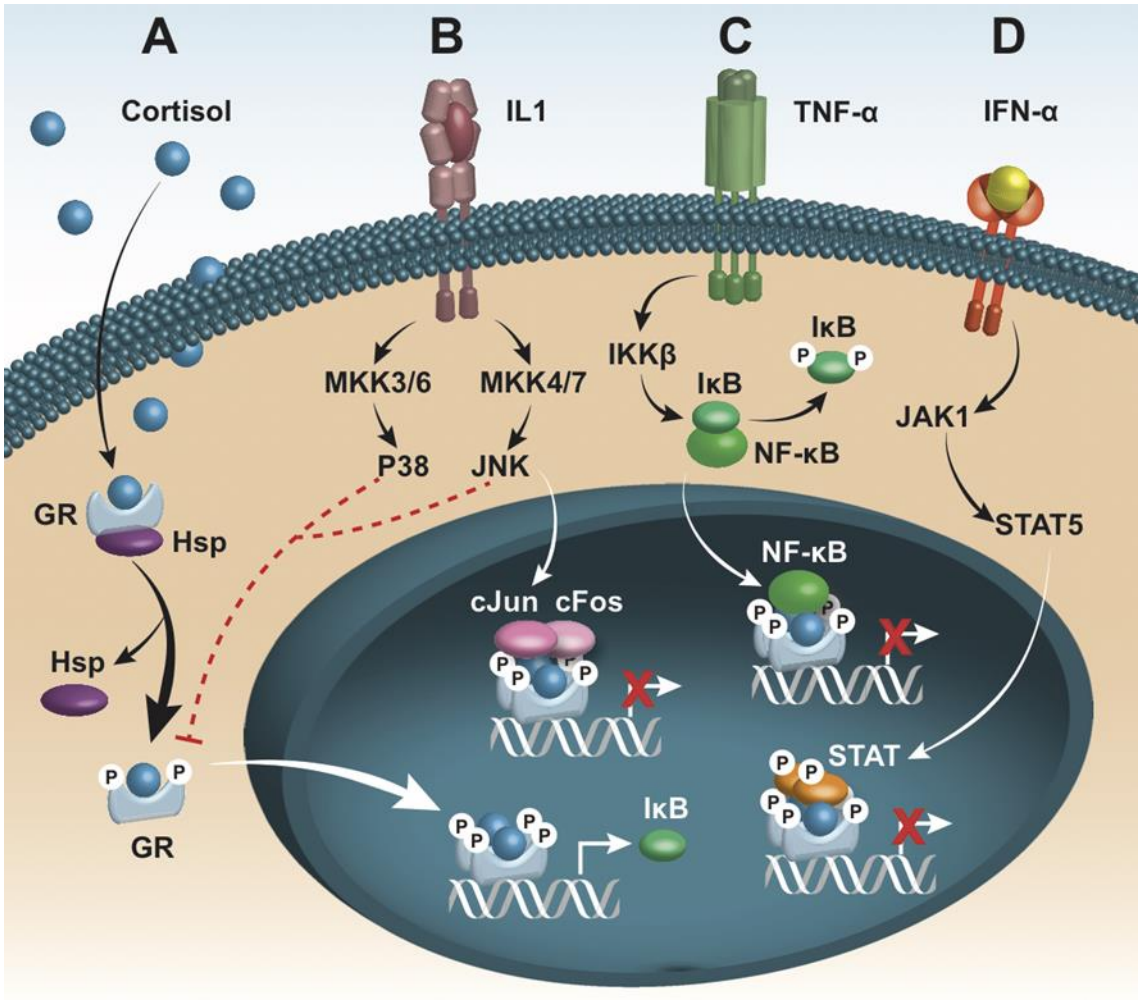

Figure 3. Glucocorticoid receptor and inhibition.
A) Cortisol binds to the glucocorticoid receptor (GR), which causes dissociation of the heat shock protein and phosphorylation of GR. GR then translocates into the nucleus, where it dimerizes and acts as a transcription factor for a number of genes, such as inhibitor κ-β (Iκβ), and directly binding to and inactivating NF-κB. B) IL-1 binds to its receptor and activates mitogen activated protein kinase (MAPK) kinases (MKK), which results in the activation of Jun amino-terminal kinase (JNK) and p38 kinase, both of which can phosphorylate GR further and inactivate it. JNK can also phosphorylate cJun, allowing it to create a heterodimer with cFos and interact with the GR. C) TNF-α binds to its receptor and activates IκB kinase β, which phosphorylates IκB and allows NF-κB to enter the nucleus and bind to GCR, inactivating both molecules. D) IFN-α binds to its receptor and causes Janus kinase (JAK) phosphorylation, which then phosphorylates signal transducers and activators of transcription (STAT) proteins, which inhibit GR activity in the nucleus.

glucocorticoid-mediated inhibition of inflammatory processes. Under stressful conditions, the hypothalamus releases corticotropin releasing hormone (CRH), which induces the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH subsequently travels to the adrenal glands and causes them to release glucocorticoids (cortisol in humans or corticosterone in rodents). Binding to their receptors in the HPA axis, endogenous glucocorticoids serve as potent negative regulators of HPA axis activity (De Kloet et al., 1998). When the HPA axis fails to induce negative feedback, however, it continues producing cortisol. In patients with major depression, numerous studies have shown loss of glucocorticoid-mediated negative feedback (Gold et al., 1988; Heuser et al., 1994; Holsboer and Barden, 1996). For example, Heuser et al. (1994) found that administering dexamethasone, an exogenous glucocorticoid, lowered levels of circulating cortisol and ACTH in healthy controls but had no effect in depressed patients. A significant percentage of depressed individuals also have higher levels of cortisol throughout their bodies, in urine, cerebrospinal fluid, plasma, and serum. They also have larger, hyperactive pituitary and adrenal glands, indicating that they are producing excess glucocorticoids and that they may not be able to effectively metabolize these glucocorticoids (Nemeroff and Vale, 2005; Pariante and Miller, 2001).

Several mechanisms have been identified as contributors to the development of glucocorticoid resistance. Depressed patients and people subject to early childhood stress (a predictor for future depression) tend to have mutations in their GR genes, and they have decreased levels of the active GR isoform (GRα) compared to non-depressed controls (Alt et al., 2010; Bet et al., 2009; Carvalho et al., 2014). Inflammatory cytokines also have a

direct impact on GR function. A number of studies have demonstrated that treatment with pro-inflammatory cytokines, like IL-1 and IL-6, decreases GR function, which reduces sensitivity to the functional effects of glucocorticoids and lowers GR affinity for its ligand (Maddock and Pariante, 2001; Miller et al., 1999; Pariante et al., 1999). Additionally, stimulating IL-2, IL-4, IL-6, or IL-10, as well as IFN-α/β or IFN-γ, activates the Jak-STAT pathway, and IL-1 or TNF-α activates the mitogen-activated protein kinase (MAPK) pathway. Both pathways ultimately produce more inflammatory products and phosphorylate the GR at key residues to prevent its action in the nucleus (Fig. 3) (Pace et al., 2007). The large body of work indicating GR inhibition upon the addition of inflammatory molecules link cytokine activity with glucocorticoid resistance.

Interestingly, it has recently been proposed that dysfunctional glucocorticoids not only signal less effectively, but that they may also produce a pro-inflammatory response (Frank et al., 2011; Horowitz and Zunszain, 2015). Glucocorticoid signaling has been demonstrated to enhance inflammation under both stress (Blandino Jr et al., 2009; Frank et al., 2010; Frank et al., 2012; Kelly et al., 2018; Munhoz et al., 2006; Smyth et al., 2004) and central nervous system injury conditions (Dinkel et al., 2002; Sorrells et al., 2013). Glucocorticoids can upregulate the expression of the TLR2 and TLR4, which are activated by pathogen-associated molecular pattern molecules (PAMPS) and damage-associated molecular pattern molecules (DAMPS), and initiate signaling cascades that lead to the synthesis and release of inflammatory mediators (Hermoso et al., 2004; Weber et al., 2013). Busillo et al. (2011) also showed that glucocorticoids positively regulate the expression of the NLRP3 inflammasome in macrophages, sensitizing macrophages to

extracellular ATP and increasing the secretion of pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6 (Busillo et al., 2011). Therapies that address glucocorticoid resistance will be pivotal in the treatment of depression, and particularly in decreasing vulnerability of stress-induced relapse.

The switch from anti- to pro-inflammatory glucocorticoid activity has also been linked to other molecules, besides cytokines, upregulated by SCI. Frank et al. (2015) suggested that stress-induced elevations in central glucocorticoids result in activation of GRs and, through an unknown mechanism, increase the secretion of High-Mobility Group Box 1 (HMGB1) which then primes the NLRP3 inflammasome. HMGB1 is significantly increased in both the acute and chronic phases of SCI (Chen et al., 2011; Kigerl et al., 2018; Papatheodorou et al., 2017), and it is a potent systemic inflammatory cytokine, that can selectively bind multiple receptors (e.g. RAGE and TLRs) to activate many cell types, including macrophages and monocytes to produce cytokines (Andersson et al., 2000; He et al., 2012; Tang et al., 2014). After injury, elevations of HMGB1 may not only increase inflammation and susceptibility to depression but may also maintain the inflammatory response. Further, macrophage migration inhibitory factor (MIF), which is also elevated in rodent models and in humans in the acute and chronic stages of SCI (Bank et al., 2015; Koda et al., 2004; Stein et al., 2013; Su et al., 2017), can counter-regulate the effects of glucocorticoids (Aeberli et al., 2006; Calandra et al., 1995; Pariante et al., 1999). Intriguingly, corticotropin releasing hormone, which induces pituitary ACTH secretion, also stimulates MIF secretion from the anterior pituitary (Calandra et al., 1995; Nishino et al., 1995; Tierney et al., 2005; Waeber et al., 1999). Calandra et al. (1995) showed that

MIF then dose-dependently decreases the glucocorticoid inhibition of TNF-α, IL-1β, IL-6 and IL-8 secretion by LPS-stimulated human monocytes. Once secreted, MIF exhibits a broad range of immune and inflammatory activities, including the induction of inflammatory cytokines (Calandra and Roger, 2003). Elevated levels of MIF have also been associated with depressive symptoms (Bay-Richter et al., 2015; Edwards et al., 2010; Musil et al., 2011; Xu et al., 2018). These data suggest that the molecular changes induced by SCI may increase susceptibility to depression via glucocorticoid dysregulation and inflammation.

The apparent need for GRs to regulate inflammation after SCI, and the prevalence of glucocorticoid system dysfunction in a portion of the depressed population, presents a unique explanation for depression after SCI. SCI acts as an acute and chronic stressor that activates the immune response and engages the glucocorticoid system. Individuals with pre-existing glucocorticoid dysfunction or inflammation-induced glucocorticoid resistance, may be more susceptible to depression that results from inflammation.

Neurogenesis

Adult neurogenesis, the process by which the mature brain develops new neurons, may also play a role in depression. The neurogenesis hypothesis of depression states that neurogenesis is, at least partly, responsible for the hippocampal volume loss and gray matter structural changes found in many patients with major depression (Kempermann and Kronenberg, 2003). Neurogenesis is a slow process, so changes in cell proliferation are unlikely to be responsible for immediate/short-term decline or recovery from depression. However, neurogenesis can be affected by all of the molecular mechanisms

discussed so far (serotonin, BDNF, glucocorticoid signaling, and inflammation), and it may be indicative of reduced brain plasticity, which has been proposed as a mechanism of depression. According to this theory, depression may arise from the brain's inability to adapt and respond to changes in its environment, getting "stuck" in mental ruts. Decreased neurogenesis represents just one element of brain plasticity; full exploration of plasticity's role in depression goes beyond the scope of this work.

Decreased neurogenesis has been observed in multiple models of stress and depression, but anti-depressants consistently restore normal neurogenesis, giving rise to its validity as at least a marker, and possibly causative agent, of depression. For example, Malberg and Duman (2003) observed decreased BrdU (a marker for proliferating cells in the CNS) labeling in the hippocampi of rats susceptible to inescapable shock stress. When they administered the antidepressant fluoxetine during the inescapable shock, neuronal proliferation was protected, and the associated depression behavior was attenuated (Malberg and Duman, 2003). Many other researchers have seen similar results: Three to six weeks of physical restraint stress significantly reduces cell proliferation in the dentate gyrus of rats (Pham et al., 2003), and repeated social defeat stress elevates serum corticosterone levels, while decreasing hippocampal granule cell proliferation and survival (Czéh et al., 2002).

As mentioned, neurogenesis is closely related to serotonin and BDNF regulation in the brain. As a neurotrophin, one of BDNF's primary roles is to promote neurogenesis. It binds to cell-surface receptors, commonly TrkB, and initiates signal cascades that result in the activation of genes involved in neural plasticity, stress resistance, and cellular

survival (Bathina and Das, 2015). Overexpression of BDNF in the hippocampus has been shown to both increase neurogenesis and decrease anxiety-like behaviors in mice, similar to the activity of fluoxetine (Quesseveur et al., 2013). Conversely, reductions of BDNF, whether through genetic manipulation or other means, decrease differentiation and maturation of neuronal precursors in the dentate gyrus (Waterhouse et al., 2012). Further, BDNF itself may be an anti-depressant; in a rat model of learned helplessness, BDNF infusion directly to the dentate gyrus reduced depression-like behaviors to a degree similar to standard anti-depressants (Shirayama et al., 2002).

Similarly, serotonin, which has marked anti-depressant effects, can also influence neurogenesis. However, serotonin has a more complicated relationship with neurogenesis than BDNF. It has been shown to both increase and decrease hippocampal neurogenesis, and it has been proposed that serotonin's oppositional effects are due to its interactions with multiple types of 5-HT receptors (Brezun and Daszuta, 1999; Klempin et al., 2010; Song et al., 2016). Significantly, though, standard anti-depressants, such as sertraline, venlafaxine, and escitalopram, and especially those that work on the serotonin system, like fluoxetine, consistently promote neurogenesis while reducing depressive symptoms (Anacker et al., 2011; Jiang et al., 2005; Mahar et al., 2014; Peng et al., 2008; Santarelli et al., 2003; Sen et al., 2008; Shimizu et al., 2003). Santarelli et al. (2003) showed that multiple common antidepressants increase neuronal proliferation and reduce anxiety/depression-like behavior. They further showed that these results were blocked in 5-HT_{1A} (the serotonin receptor primarily affected by fluoxetine) knockout mice, showing

that the serotonergic effects are necessary for the restoration of neurogenesis and the reduction of depression behavior (Santarelli et al., 2003).

Finally, inflammation can decrease neurogenesis. IL-6, IL-1β, and TNF-α have all been shown to decrease hippocampal neuronal proliferation and differentiation (Kim et al., 2016). IL-1β activates NF-κB, which then blocks the transcription of genes necessary for neurogenesis. Applying an NF-κB antagonist blocks the negative effects of restraint stress *in vivo*, and it rescues neurogenesis after application of IL-1β to hippocampal neurons *in vitro* (Koo et al., 2010). Similarly, an NF-κB antagonist restores hippocampal cell proliferation after systemic inflammation, while blocking IL-6 *in vitro* promotes proliferation (Monje et al., 2011; Monje et al., 2003). From these data, it is clear that neurogenesis interacts with many of the same molecular mechanisms that we attribute to depression and that it plays an important role in recovery from and possibly resilience to depression.

Therapeutic strategies for depression after SCI

Despite the prevalence of depression after SCI, little research has been published on therapeutic strategies for these patients. One literature review, published in 2004, found only nine empirical articles addressing treatments specific to patients experiencing depression after SCI (Elliott and Kennedy, 2004). Disappointingly, more recent years have not produced many more empirical studies. This dearth of information is distressing, as depression is so widely reported in the SCI community.

Currently, there are no specific treatments for depression after SCI, beyond those available to the general population. In the general population, tricyclics, norepinephrine

reuptake inhibitors, and selective serotonin reuptake inhibitors (SSRIs) are partially effective, but for 1/3 of patients, depression is still refractory. Among SCI patients, SSRIs are used most consistently (Fann et al., 2011), although there have historically been some concerns that they may cause spasticity, possibly through interaction with the 5-HT receptors that maintain motoneuron stability (Elbasiouny et al., 2010; Harvey et al., 2006; Murray et al., 2010; Stolp-Smith and Wainberg, 1999). However, even when taking SSRIs, many patients still experience meaningful depression (Fann et al., 2011). Few antidepressant clinical trials have been conducted specifically for SCI patients. Those that have, report slight improvements but, ultimately, only partial remission of depression symptoms (Fann et al., 2015). Empirical studies of other therapies, such as cognitive behavioral therapy, psychoeducation, and coping methods, show that they are typically effective during active rehabilitation but that patients relapse after discontinuing therapy (Dorstyn et al., 2010); (Mehta et al., 2011; Perkes et al., 2014). Well-defined treatment options for depression after SCI are clearly lacking, as are effective mental health follow-up for individuals no longer in a clinical treatment facility. There is a need for alternative antidepressants for use independently or in conjunction with previously established therapies.

In sum, the data suggest that standard antidepressants still have a remarkably low success rate, indicating that SCI introduces an additional layer of complexity to depression. I hypothesize that the chronic inflammation inherent to SCI contributes to the development of depression in this model, decreasing serotonin, BDNF, and neurogenesis. Some research has already demonstrated success in managing depression, in the general

population, with anti-inflammatory drugs or cytokine inhibitors (Abbasi et al., 2012; Akhondzadeh et al., 2009; Brunello et al., 2006; Johansson et al., 2012; Maciel et al., 2013; Makunts et al., 2018). Given the high levels of baseline inflammation after SCI, this may be an effective strategy. Further understanding of the role of the inflammatory system and the molecular mechanisms mediating depression after SCI is imperative to effectively treat this affective disorder and improve quality of life for people living with spinal injury.

Specific Aims

While some elements of depression after SCI might be similar to those associated with depression in the general population, depression after SCI is complicated by both the chronic psychosocial stressors associated with the injury and ongoing alterations of the inflammatory response in a subset of individuals. Data suggest that inflammatory cytokines and the glucocorticoid system play an important role in the propagation of depressive symptoms after CNS trauma, but research has yet to elucidate a mechanism. In the aims presented in Chapters III-V, I have worked to uncover this mechanism by manipulating inflammation before and after SCI in a rat model.

We have previously shown that SCI in rats causes an increase in depression-like behavior and that a subset of SCI animals displaying depression-like symptoms have higher expression of pro-inflammatory cytokines (Luedtke et al., 2014; Maldonado-Bouchard et al., 2016). This powerful model system enables us to identify molecular changes that are critical to SCI-induced depression, beyond what is seen in SCI *per se*. I hypothesize that suprathreshold inflammation leads to the development of depression and that reducing inflammation in the acute phase of SCI will decrease the development of

depression after injury. Minocycline is an FDA-approved tetracycline antibiotic with anti-inflammatory properties, capable of crossing the blood brain barrier (Elewa et al., 2006). Importantly, it has also been shown to be neuroprotective, promote neurogenesis, and reduce depression-like behaviors in extant models (Camargos et al., 2020; Elewa et al., 2006; Li et al., 2016; Mejia et al., 2001; Ulndreaj et al., 2017). Minocycline works through a number of mechanisms, including reducing activation of microglial cells (Elewa et al., 2006; Garrido-Mesa et al., 2013; Hinwood et al., 2013). As microglial activation is a critical source of secondary inflammation immediately after SCI, reducing microglial production of inflammatory cytokines should reduce inflammation-induced depression after SCI. My first experiment (Chapter III) tests this hypothesis. Subjects were given minocycline in their drinking water for the first two weeks post-SCI, the time at which SCI-mediated inflammation is highest and may be most likely to cause depressive symptoms. I then evaluated depression behavior and serum cytokine levels for 30 days post-injury, using behavioral, molecular, and histological assays to determine the incidence of depression-like behavior and the efficacy of minocycline.

Intriguingly, I found that depression was not only associated with increased pro-inflammatory cytokine expression (particularly IL-6) post-injury, but it was also upregulated in the depressed SCI rats *before* injury, despite the fact that depression-like behavior developed only after injury. Others have also reported high IL-6 levels in the serum and plasma of patients and animals that are susceptible to developing depression. Mice that are vulnerable to developing depressive behaviors after repeated social defeat stress have elevated levels of plasma IL-6 immediately after their first defeat, whereas

resilient animals do not, and their blood monocytes produce more IL-6 when stimulated with LPS, even before the social defeat (Hodes et al., 2014). Additionally, psychological stressors increase production of IL-6 from peripheral immune cells, and CSF IL-6 levels correlate with the intensity of depression in suicide attempters (Lindqvist et al., 2009). IL-6 is a critical component in the pro-inflammatory cycle and has been strongly associated with depression. It is necessary for immune defense, but prolonged production of it contributes to numerous diseases. Given these data, I hypothesized that elevating IL-6 prior to injury would increase susceptibility to depression after a stressor such as SCI. To test this hypothesis, I administered three different doses of IL-6 systemically for a week before SCI (Chapter IV). I then evaluated depression, pain, locomotor recovery, and brain BDNF and serotonin levels for one month post-injury, comparing and determining the percent of each IL-6 dose group that developed depression-like symptoms.

Lastly, I expanded my investigation to include the glucocorticoid system. Most of the molecular elements of depression influenced by inflammation (serotonin, BDNF, neurogenesis, etc.) act in conjunction with glucocorticoids. Glucocorticoid reception is an integral part of the homeostatic response to stress. People and animals experiencing depression often have disrupted responses to glucocorticoids (cortisol in humans and corticosterone in rats). Instead of initiating the expected negative feedback loop, excessive glucocorticoids stimulate further inflammation and stress signals. This is especially relevant to spinal cord injury, because there is evidence that SCI can disrupt otherwise normal glucocorticoid receptor levels and functions (Yan et al., 1999). It is likely that pre-existing glucocorticoid receptor expression and functionality plays a major role in

predisposition for the development of depression. In my third aim, I investigated whether cortisol levels after a minor stressor were associated with inflammation and predicted disposition to developing depression after SCI. To do this I evaluated corticosterone, glucocorticoid receptor levels, and serum cytokine levels in depressed versus not-depressed animals before and after SCI. As in previous aims, I collected baseline measurements of depression, pain behaviors, and serum cytokines. Two days before SCI, I subjected half of the subjects to a forced swim stress test as a minor, acute stressor. I then evaluated their pain and depression levels for a month following SCI and determined their depression grouping.

CHAPTER II

GENERAL METHODOLOGY

Subjects

All subjects were young adult (2-3 months old, 275-300 grams), male, Sprague Dawley rats. The rats were single housed on a 12-hour light-dark cycle, with access to food and water ad libitum. Following surgery, subjects' bladders were manually expressed in the morning and evening until they regained full bladder control (operationally defined as three consecutive days with an empty bladder at the time of expression).

All of the experiments reported here were reviewed and approved by the Institutional Animal Care Committee at Texas A&M University and all NIH guidelines for the care and use of animal subjects were followed.

Surgery

Rats were given a moderate spinal cord injury with an Infinite Horizons impactor device (PSI, Fairfax Station, VA, USA). Subjects were anesthetized with 2-3% isoflurane. Once a surgical level of anesthesia was reached (absence of hindlimb pinch reflex or corneal reflex), the subject's back was shaved and disinfected with iodine and ethanol. A 5 cm incision was made through the skin on top of the spinal cord. Two incisions were made along the spinal column, spanning about 2 cm rostral to caudal, on either side of the T12 dorsal spinous process. Connective tissue and muscle surrounding the vertebrae were carefully cleared to allow room to clamp the vertebral column, and a laminectomy was performed at the T12 vertebra, exposing the L1-L2 spinal cord. The vertebral column was

stabilized and clamped in place with mounted forceps, and a 150 kiloDyne force (1 sec dwell time) was delivered to the dorsal aspect of the exposed spinal cord. The wound was closed with Michel clips. After injury, rats received 3 ml of saline subcutaneously, to replace fluid loss, and 100,000 units/kg of penicillin G (i.p.) to prevent infection. Sham surgeries also involved incisions, a laminectomy, and clearing of the connective tissue around the spinal column, but the subjects did not receive a contusion with the impactor device. All subjects recovered for 24 hours in a warm environment, with food and water available ad libitum. The Michel clips were removed approximately 14 days after injury.

Assessment of depression-like behavior

Before injury, and in the weeks following, subjects were assessed with a battery of behavioral tests for depression (Fig. 4). The tests are briefly described below.

Sucrose Preference

Anhedonia was measured with the sucrose preference test. Prior to testing, subjects acclimated to a 2% sucrose solution until 75% sucrose preference was established. For the initial acclimation, they were given access to a 2% sucrose solution instead of water overnight. Then, for two additional acclimation periods and for testing, subjects were presented with two bottles, containing 2% sucrose or water, in their home cages for 2 hours. The positions of the bottles were reversed after one hour to control for any cage-side preference. Bottles were weighed before and after the two-hour test, and sucrose preference was calculated as the percent sucrose consumed out of the total liquid (sucrose plus water) consumption. A decrease in sucrose preference is indicative of anhedonia, or a loss of interest in previously pleasurable activities (Liu et al., 2018).

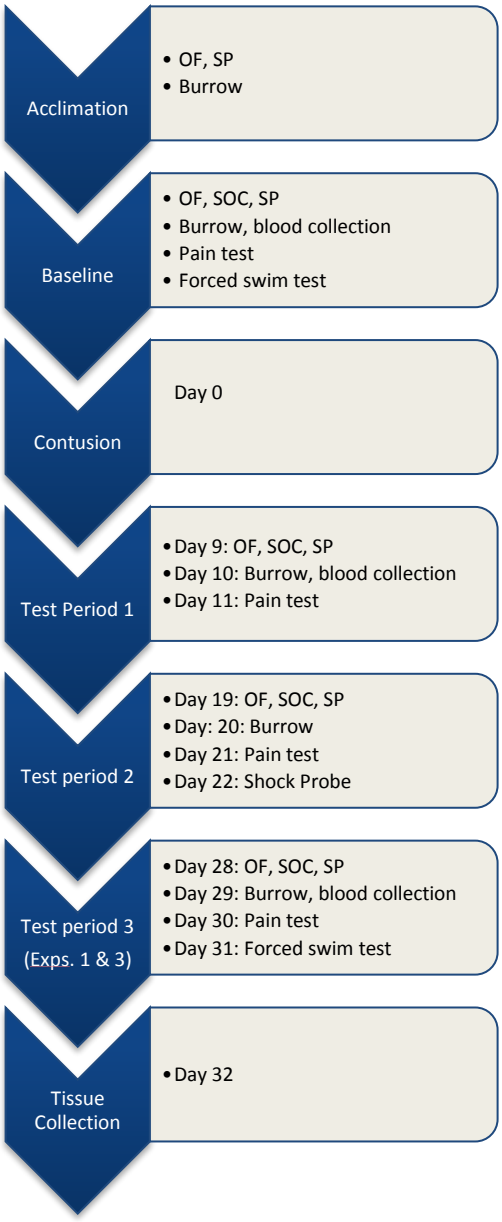

Figure 4. Behavioral testing schedule.
Behavioral testing for all experiments followed the same general schedule. Subjects were acclimated to the open field (OF)/social (SOC) arena, sucrose preference (SP) test, and burrowing tubes for a week and a half. Baseline testing was conducted the week before contusion injury. Depression behavior tests, pain sensitivity, and serum collection was conducted over 2-3 testing periods post-injury. Tissue was collected the day after the final test (forced swim test). Exp. 1: Minocycline was administered for 14 days immediately following SCI. Exp. 2: IL-6 was administered for 1 week following BL testing. Testing was repeated during IL-6 administration. Exp. 3: Forced swim stress test was administered again after BL testing, 2 days before SCI.

Social Interaction

In the social interaction test, the subjects were placed in a 90x60x20 cm black box with a novel, uninjured rat of the same age and approximate weight. The time that the SCI (or sham) subject spent initiating social contact (sniffing, grooming, or pursuing the other animal) was measured and calculated as a percent of the total 5-minute test session.

Open Field Activity

Open field activity is a common measure of psychomotor retardation or fatigue. In this test, the subject was placed in a 90x60x20 cm black box and allowed to explore for 5 minutes. Total distance traveled and total time spent in the center of the arena (defined as ≥10 cm from a wall of the arena) during the 5-minute test session was measured using video tracking software (Ethovision).

Burrowing

Burrowing was used as another measure of fatigue and anhedonia. A 10 (inner diameter) x 30 (length) cm capped PVC pipe filled with wood chips was placed into the subject's home cage for two hours. All tubes were filled to an equal level with loosely packed woodchips. The percent of the wood chips displaced by burrowing was measured as: Percent Burrowed = ([weight of the tube prior to test - weight of tube after test ÷ (weight of tube prior to test – weight of tube empty)] x 100).

Forced Swim

For the forced swim test, the rat was placed into a clear plastic container (73 cm long x 40 cm wide x 46.4 cm high) filled with 28 cm of 23°C water and video recorded for 10 min. In *post hoc* video analyses, scorers blind to the subject's treatment measured

the time the rat spent immobile, operationalized as floating in the water without struggling and making only the necessary movements to keep its head above water. Greater immobility is associated with stress-induced and anti-depressant-reversible depression (Slattery and Cryan, 2012).

Assessment of locomotor recovery

The recovery of hindlimb function was scored using the Basso, Beattie, and Bresnahan (BBB) scale (Basso et al., 1995). A numerical assignment on this scale corresponds to specific milestones in locomotor recovery. For example, 0 indicates no hind limb movement, 1 is slight movement of one or two joints, 10 is occasional stepping with no coordination, and 21 is perfect stepping with perfect coordination between the fore- and hindlimbs (i.e. uninjured stepping). After injury, the locomotor capacity (BBB) of subjects was observed for 5 minutes and scored by a trained observer on days 1-7, 9, 11, 13, 15, 18, 21, 24, and 27 post-SCI. BBB scores were transformed to help assure that the data were amendable to parametric analyses (Ferguson et al. 2004). This transformation pools BBB scores 2–4, removing a discontinuity in the scale, and scores 14–21, which are very seldom used under the present injury parameters. By pooling these scores, an ordered scale was created that is relatively continuous, with units of approximately equivalent intervals. Meeting these criteria allows for application of metric operations (computation of mean performance across legs), improves the justification for parametric statistical analyses, and increases statistical power.

Assessment of sensory reactivity

Girdle Test

At-level allodynia was assessed using the girdle test. Animals were acclimated to the testing room for 10 minutes. During testing, the subjects were loosely held to ensure they were calm and did not vocalize due to stress. A von Frey filament (Semmes–Weinstein Anesthesiometer, Stoelting Co., Chicago, IL) with a bending force of 204.14 mN (26g force) was applied to a 4 x 11 grid across the girdle region of each subject. Because animals do not normally vocalize with this stimulus, a vocalization response indicates that a noxious stimulus was experienced. Vocalization responses were recorded and mapped onto a grid map for each animal. Number of vocalizations (Nv) were reported as a percent of the total stimulation events, given the following formula: (Nv x 100)/total number of filament applications (44).

Mechanical nociceptive reactivity

Subjects were placed into restraining tubes (7.00 cm [internal diameter]×20.00 cm [length]) to allow their legs to hang freely from the tube. After 15 min of acclimation to the tube alone, mechanical reactivity was assessed using von Frey stimulation. Nylon monofilaments (Semmes–Weinstein Anesthesiometer, Stoelting Co., Chicago, IL) of increasing strength were applied sequentially at approximately 2 s intervals to the L5 dermatome on the plantar surface of the hind paws. Stimuli were presented until subjects exhibited a motor withdrawal (spinal) and vocal (supraspinal) response. If one or both responses (motor and vocal) were not observed, testing was terminated at a force of 300 g. Each subject was tested twice on each foot in a counterbalanced ABBA order.

Thermal nociceptive reactivity

Thermal reactivity was assessed with the tail flick test. Subjects were restrained in plexiglass tubes (7.00 cm [internal diameter]×20.00 cm [length]) and placed on the tail-flick apparatus (IITC Life Science Inc., Woodland Hills, CA, USA). Their tails were positioned in the 0.5 cm deep groove on the apparatus so that the area 1.5 inches from the tip of the tail was directly under a thermal light source. The light was set at an intensity that produced a 3-4 sec latency to flick the tail away from the radiant heat source in an uninjured rat, with an 8 second cut-off in the event that the rat did not flick its tail. During testing, two tests occurred at 2-minute intervals, and the average tail flick latency across the tests was recorded.

Serum Collection

Blood was collected via saphenous vein draw during the last two hours of the light cycle for serum cytokine assays. Prior to collection, the leg (alternated across collections) was shaved to expose the saphenous vein. The animal was then gently restrained in a towel while the leg was disinfected and treated with topical lidocaine. The saphenous vein was firmly held above the collection site and punctured with a 20-gauge needle. Blood was collected in 300 µl serum microvettes (Sarsvedt) coated with clotting factors. Samples were allowed to clot for 30 - 60 min, per manufacturer instructions, and then centrifuged at 2,000 x g for 15 min at 4°C. The serum fraction was pipetted into 30 µl aliquots and stored at -20 to -80 °C for later use in cytokine assays.

Tissue collection

At the end of each experiment, subjects were euthanized with pentobarbital (100 mg/kg, i.p.). All tissue collection occurred after the animal reached deep, unresponsive level of anesthesia. Tissue was either fixed for histological spinal cord lesion analysis, or it was collected fresh for protein and RNA analysis. For fixation, subjects were perfused intracardially with 4% paraformaldehyde (PFA). A 1.5 cm section of cord, surrounding and including the contusion site, was extracted and placed in ice-cold 4% PFA for 24 hours, for further fixation. It was then transferred to 30% sucrose for cryoprotection and preservation before freezing and sectioning for histology.

For fresh tissue, the spleen was removed, weighed, and snap frozen in liquid nitrogen. Then the brain was extracted and the frontal cortex, hypothalamus, and left and right hippocampi were dissected out and snap frozen separately in liquid nitrogen. The frozen tissue was stored at -80°C until it was processed for assays.

Cytokine, BDNF, and serotonin assays

Serum collected prior to injury (baseline) and after injury was used in a multiplex assay (RECYMAG65K27PMX, Millipore) to assess 27 unique cytokines and chemokines. All samples were run in duplicate, following the manufacturer's instructions.

The frontal cortex and hippocampus samples were homogenized in lysis buffer (500 µl in Experiments 1 and 3, 400 µl in Experiment 2, composed of: 100 mM Tris, 1 M NaCl, 4 mM EDTA, 2% Triton X-100, 0.1% sodium azide, 5 µl/ml phenylmethyl-sulphonyl fluoride, and protease inhibitor cocktail [Thermo Scientific, 78442]) and allowed to sit on ice for 10 min before centrifugation at 20,000 rpm for 20-30 min. A BCA

total protein assay was performed on the resulting supernatant, and then the samples were diluted in lysis buffer to equivalent total protein levels based off of the results. The protein samples were aliquoted and stored at -80 ºC for later use.

A rat BDNF ELISA (CYT306, Millipore) was used to detect BDNF levels in the right hippocampus and frontal cortex. Samples in this assay were diluted to 0.25 mg/ml total protein with the included sample diluent (50 µg of total protein was used), and manufacturer instructions were followed.

A serotonin (5-HT) ELISA (IBL-America, IB89540) was used to detect 5-HT levels in the right hippocampus and frontal cortex. In Experiment 1, samples were diluted 1:30, and in Experiments 2 and 3, samples were diluted to 0.17 mg/ml total protein with the included sample diluent, and manufacturer instructions were followed.

Histological lesion reconstruction

Histological analyses were conducted to examine spinal lesion size and extent. Spinal cord segments stored in 30% sucrose were embedded in sectioning compound and frozen at -80 °C for cryostat sectioning. Tissue was sectioned from the rostral to the caudal end in 20 µm thick sections, and every 10th slice was preserved for staining. All sections were stained with cresyl violet for Nissl substance and luxol fast blue for myelin (Basso et al., 1995; Beattie, 1992).

The total cross-sectional area of the cord and spared tissue was assessed at the lesion center (averaged across three sections -600, 0, and +600 µm from the lesion center) using MicroBrightField software (MBF Bioscience, Williston, VT). Four indices of lesion magnitude were derived: lesion; residual gray matter (GM); residual white matter (WM);

and width. To determine the area of lesion, an experimenter blind to the experimental treatments traced around the boundaries of cystic formations and areas of dense gliosis. Nissl-stained areas that contained neurons and glia of approximately normal densities denoted residual GM. WM was judged spared in myelin-stained areas lacking dense gliosis and swollen fibers.

The total area of each cross-section was derived by summing the areas of damage, GM, and WM. Width was determined from the most lateral points along the transverse plane. These analyses yielded six parameters for each section: WM area, GM area, spared tissue (white + gray), damaged tissue area, net area (white + gray + damage), and section width. To control for variability in section area across subjects, we applied a correction factor derived from standard undamaged cord sections, taken from age-matched controls. This correction factor is based on section widths and is multiplied by all area measurements to standardize area across analyses (Ferguson et al., 2004). By standardizing area across sections, we were able to estimate the degree to which tissue was ‘‘missing’’ (i.e., tissue loss from atrophy, necrosis, or apoptosis). An accurate assessment of the degree to which a cord has been affected includes both the remaining ‘‘damaged’’ tissue as well as resolved lesioned areas (Ferguson et al., 2004). When we sum the amount of missing tissue and the measured damaged area, we derive an index of the relative lesion (percent relative lesion) in each section that is comparable across sections. We also compute the relative percent of GM and WM remaining in each section, relative to intact cords.

Immunofluorescent staining for neurogenesis

In Experiment 1 (Chapter III), neurogenesis was evaluated in the hippocampi of depressed and not depressed subjects. After perfusion and whole brain collection described above (see Tissue collection), the brains were embedded in sectioning compound and frozen. The whole brain was sectioned into twelve serial sets of free floating, coronal sections (50 µm thick), using a freezing microtome.

Four sections of the rostral hippocampus were selected for each subject and stained with goat anti-DCX C-18 and DCX N-19 (sc-8066 and sc-8067, respectively, Santa Cruz Biotechnology). The sections were washed 3 times in 0.01 M PBS and then transferred to the primary antibody solution (0.2% DCX N, 0.2% DCX C, 0.5% Tween, 5% normal horse serum, in 0.01 M PBS). They incubated at room temperature with rotation for 23 hours. The sections were washed again and incubated in secondary stain (0.5% donkey anti-goat IgG 555, 5% Tween, 5% normal horse serum, in 0.01 M PBS) for 1.2 hours. Sections were then rinsed and mounted on slides. They were allowed to dry overnight and coverslipped with Vectashield anti-fade mounting medium (H-1400, Vectashield, Burlingame, CA).

DCX positive cells in the upper and lower blades of the dentate gyrus of each section were counted using Stereo Investigator (MBF life sciences) using a 25% sampling rate, a 70x70 µm frame, and a dissector height of 15 µm. The cell count per area for upper and lower blades was calculated separately for each subject and the mean value for the upper blades and lower blades (across 4 sections) was found for each subject.

RNA extraction and rtPCR

Quantification of the glucocorticoid receptor in Experiment 3 (Chapter V) was conducted using quantitative real time rtPCR. RNA was extracted from approximately 30 mg of frozen hippocampus tissue homogenates using a RNeasy Mini kit (74104, Qiagen,). It was then DNased with 2 µl TURBO DNase, 5 µl buffer, and 38 µl water for every 5 µl of RNA (80-200 ng). During DNasing, the samples were heated to 37 °C for 30 min, then another 1 µl of DNase was added, and they were incubated for another 30 min at 37 °C.

After DNasing, the samples were cleaned with RNA Clean and Concentrate (R1013, Zymo Research, Irvine, CA). cDNA was produced from 100 ng of RNA with a qScript cDNA synthesis kit (101414-100, QuantaBio), using 4 µl of the included buffer, 1 µl of reverse transcriptase (replaced by water in negative controls), and water to bring the total volume to 20 µl after the appropriate volume of RNA was added. The cDNA was amplified using thermocycler settings: 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C hold.

PCR primers for the alpha and beta isoforms of the glucocorticoid receptor, as well as a common sequence to identify all forms of the glucocorticoid receptor, were obtained from Life Technologies (DuBois et al., 2013). See Table 1 for the primer sequences. qPCR reactions were prepared in triplicate with 0.5 µl of the forward primer, 0.5 µl of the reverse primer, 5 µl of PerfeCTa SYBR Green FastMix (101414-284, QuantaBio), 2 µl of water, and 2 µl of sample cDNA. The reaction was run on a ViiA7 qPCR machine (Applied Biosciences) at 50 °C for 2 min, 95 °C for 10 min, ([95 °C for 15 sec, 57 °C for 30 sec, 65 °C for 30 sec] x 50 cycles), 95 °C for 15 sec, 60 °C for 1 min, and 95 °C for 15 sec.

Table 1. Glucocorticoid receptor primer sequences

Primer	Sequence
GR alpha Forward	GCGACAGAAGCAGTTGAGTCAAC
GR alpha Reverse	CCATGCCTCCACGTAACTGTTAG
GR beta Forward	GCGCTTGAGGCTAAGATAGTC
GR beta Reverse	CCCATGTTTCTGCCTCTTTCTTTG
GR common Forward	GCCCTGGGTTGGAGATCATAC
GR common Reverse	CATGCAGGGTAGAGACATTCTC

Statistics

To identify changes in depressive behavior caused by SCI, in each experiment, we first calculated change from baseline scores. For each of the behavioral tests of depression, scores collected from all post-injury testing periods (days 9-10, 19-20, 28-30) were subtracted from the baseline scores collected immediately prior to the spinal contusion injury. A hierarchical cluster analysis (HCA) was then used to group subjects into cohorts based on their change from baseline scores, from either the last testing period (Aim 1) or an average of the last two testing periods (Aims 2 and 3), across the tests of depression-like behavior described. As described in Luedtke et al. (2014), an HCA was performed using Ward's method, applying squared Euclidean distance as the distance measure. The number of appropriate clusters was obtained by looking for a break in the agglomeration coefficient change and by observing the dendrogram, which visually depicts the distance between linked clusters. After identifying the number of clusters depicted in the dendrogram, the HCA was repeated using the same parameters but requesting a single

solution of two clusters. A new variable, cluster membership, was generated for all subjects.

Analysis of variance (ANOVA), 2-way ANOVAs, or t-tests (as appropriate) were used to compare the change from baseline scores across the identified clusters, on each of the behavioral tests of depression and sensory tests. Repeated measures ANOVAs were used to compare BBB scores, as a measure of locomotor recovery, across days, as well as corticosterone levels across time (Aim 3). A repeated measures ANOVA was also used to examine the effects of depression or drug doses on spared or damaged tissue across the lesion site (Aim 1).

CHAPTER III

MINOCYCLINE AND DEPRESSION AFTER SPINAL CORD INJURY

Introduction

SCI is an inflammatory disease, marked by an increased immune reaction in the spinal cord, periphery, and even the brain (Davies et al., 2007; Popovich et al., 1997; Wu et al., 2014a; Wu et al., 2014b). This immune reaction is primarily mediated by macrophages and microglia, the immune cells of central nervous system. While the immune response is critical for the resolution of the primary injury after SCI, an extended inflammatory response can lead to secondary tissue damage and immune dysregulation throughout the entire body, which has been associated with elevated pain, decreased locomotor recovery, and even depression (Allison and Ditor, 2015a; Anisman, 2009; Beumer et al., 2012; Cruse et al., 2000).

Numerous lines of evidence implicate inflammation in the development of depression. As discussed previously (see Chapter I), elevated levels of inflammatory cytokines are not only observed in depressed patients, cytokines can also induce depression when administered therapeutically for other illnesses, such as cancer and hepatitis infection (Capuron et al., 2009; Capuron et al., 2002). Further, elevated inflammation has been observed in rodent models of SCI-induced depression, indicating that its mechanism is likely ubiquitous across species (Boadas-Vaello et al., 2018; do Espírito Santo et al., 2019b; Farrell and Houle, 2019; Li et al., 2017; Maldonado-Bouchard et al., 2016).

Recognizing the potential of targeting inflammation as an antidepressant therapy, researchers have begun to investigate the efficacy of anti-inflammatory treatments (Allison and Ditor, 2015b; Farrell and Houle, 2019; Raison et al., 2013). For example, in a rat model of depression after SCI, Farrell and Houle (2019) targeted tumor necrosis factor (TNF), an inflammatory factor often found elevated in depression, with XPro1595, a soluble TNF inhibitor. Contrary to the expected decrease in depressive behavior, they found that the incidence of depression increased with TNF inhibition (Farrell and Houle, 2019). They suggested that inhibition of soluble TNF in the periphery may shift signaling from the TNF receptor to TNFR2, which could result in Th17 lymphocyte infiltration into the CNS and subsequent depression (Farrell and Houle, 2019). The relationship between depression and inflammation is clearly nuanced, necessitating more research.

In a clinical trial, Allison and Ditor (2015) investigated the effects of an anti-inflammatory diet on depression in people with SCI. One month after eliminating inflammation-inducing foods and introducing anti-inflammatory supplements to their diets, patients had lower serum IL-1β expression and lower scores on the Center for Epidemiological Studies Depression Scale than they did before the start of treatment. However, this study had a small sample size (20 individuals) and did not directly target any specific inflammatory pathways. Further, they found quite small effects of inflammation on depression, with only IL-1β being significantly correlated to depression scores. It is likely that multiple cytokines act in concert to induce depression. Despite these limitations, this study suggests that reducing overall inflammation after SCI may have a positive impact on psychological wellbeing.

To test this hypothesis, the current experiment used minocycline, a tetracycline antibiotic known for its anti-inflammatory properties and ability to cross the blood-brain barrier, to reduce inflammation in a rodent model of SCI and evaluate its effects on depression. Minocycline has been heavily investigated as a neuroprotectant after stroke, traumatic brain injury, and SCI, with promising results in all three fields (Camargos et al., 2020; Elewa et al., 2006; Li et al., 2016; Mejia et al., 2001; Ulndreaj et al., 2017). Minocycline produces an anti-inflammatory effect in the CNS by reducing microglial activation, preventing microglia from contributing to deleterious inflammation. Because minocycline targets inflammation in the CNS, and because it is well tolerated, it is a potential anti-inflammatory agent for SCI-induced inflammation and depression. We hypothesized that oral administration of minocycline for two weeks after spinal cord injury, the time period of the greatest inflammatory response, would reduce the incidence of depression-like symptoms and decrease the expression of pro-inflammatory cytokines in the serum of rats with SCI.

Methods and results

Male Sprague-Dawley rats were given a moderate contusion injury and assessed with the tests of depression-like behavior, motor function and pain reactivity, as described in Chapter II (General Methodology). On the day following the spinal contusion injury, the subjects' home-cage water bottles were replaced with bottles containing 0, 0.33, or 1 mg/ml of minocycline (n=11 in each group, minocycline hydrochloride; Sigma-Aldrich, St. Louis, MO, USA) dissolved in filtered water. Minocycline treatment lasted for 14 days,

and the minocycline was replaced every 7 days, to maintain its stability at room temperature (Pearson and Trissel, 1993).

As outlined in Figure 4, serum, depression behavior, and pain behavior were collected before injury and for 32 days post-injury. At the end of the 32-day assessment period, subjects were humanely euthanized with pentobarbital (100 mg/kg, i.p.), and their brains and spinal cords were collected postmortem, as described in Chapter II (General Methodology). In this experiment, half of the subjects (n=18) were perfused intracardially with 4% paraformaldehyde (PFA) for histological spinal lesion reconstruction and assessment of neurogenesis in the hippocampi.

Fresh brain and spinal tissue were collected from the other half (n=15) of the subjects. The frontal cortex, hypothalamus, and left and right hippocampi were dissected out of the brain and snap frozen separately in liquid nitrogen. The frozen tissue was stored at -80°C until it was processed for serotonin and BDNF ELISAs.

Statistical testing was conducted as described in the General Methodology. Three subjects were dropped from the analyses for failure to reach minimum baseline sucrose preference (70%).

One-third of SCI rats develop depression

Based on the hierarchical cluster analyses (Fig. 5) and *post hoc* t-tests, 33% of the subjects exhibited depression-like behavior. Depression-like behaviors did not differ across clusters prior to injury (Fig. 6F). However, after injury, the depressed subjects displayed lower open field activity compared to their not-depressed counterparts (Days 20

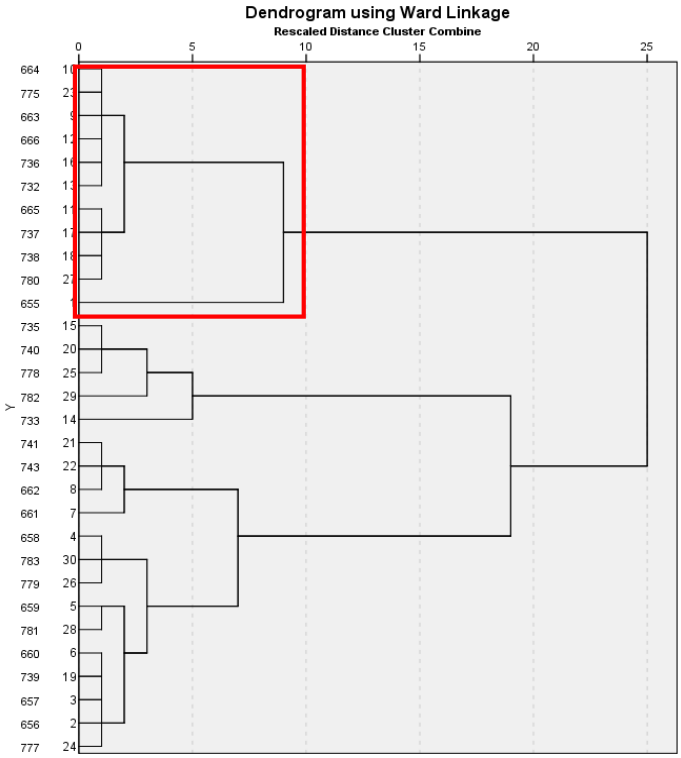

Figure 5. Dendrogram of depression clusters.
Hierarchical cluster analysis separated the subjects into two groups, using their scores from sucrose preference, open field activity, and social activity in the third testing period (days 29-30 post injury). The depressed cluster is outlined in red.

and 29 post-injury, t(28)= -2.52, p=0.018; t(28)= -4.38, p<0.001, respectively, Fig. 6A). They also showed lower social interaction on days 9 and 29 (t(28)= -3.14, p=0.004; t(28)= -5.17, p<0.001, respectively, Fig. 6B). There was a trend for higher immobility on the forced swim test, but it was not statistically significant (Fig. 6E).

Minocycline does not impact water consumption or incidence of depression

There was no significant difference in the amount of water (with or without minocycline) consumed across the 14 days of administration (F(2,27)=1.97, p=0.159).

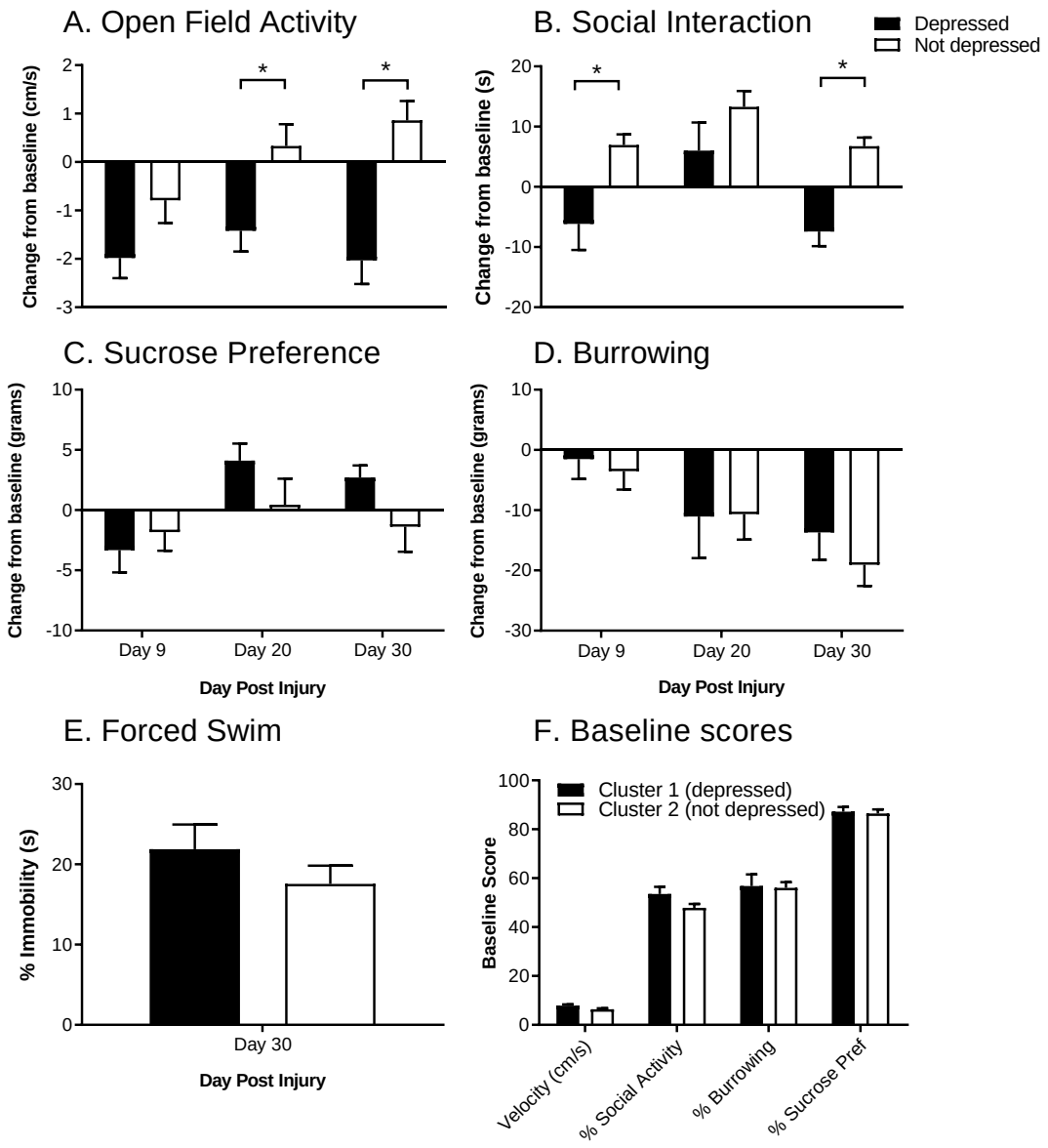

Figure 6. Depression-like behaviors are apparent in a subset of SCI animals.
Throughout recovery, animals that clustered in the depressed group explored an open field arena less than their not-depressed counterparts (A). They also initiated social interaction with a novel conspecific less than their not-depressed group (B). While they did not differ significantly on any other behaviors (C-E), there was a trend towards higher immobility on the forced swim test (E). All behaviors but forced swim are presented as change-from-baseline. There were no behavioral differences between the groups before SCI (F). * $p<0.05$

During the first week of minocycline administration, all minocycline dose groups consumed similar amounts of liquid, resulting in an average of 10.5 ± 1.2 mg of minocycline per day for the low dose group and 28 ± 3.9 mg/day for the high dose. Similarly, in the second week post-injury, the average minocycline intake was 13.5 ± 0.4 mg/day for the low dose group and 33 ± 4.1 mg/day for the high dose.

Despite adequate consumption of minocycline, there was no substantial effect of treatment on depression-like behavior. Subjects in the high dose group did burrow more than either of the other two groups on Day 9 post-injury ($F(2,27)=6.85$, $p=0.004$, Fig. 7D), and they exhibited higher social activity than the controls on Day 20 post-injury ($F(2,27=4.28$, $p=0.024$, Fig 7B). However, after hierarchical clustering, which used the scores collected by the time the drug had cleared the system at Day 30, subjects from the three drug dose groups split evenly between the "depressed" and "not depressed" groups, indicating that, overall, minocycline treatment did not have a lasting effect on the development of depression-like behavior (Fig. 7F).

Locomotor recovery

Minocycline treatment did not affect locomotor recovery across the 30 days post injury ($F(2,27)=0.92$, $p=0.409$, Fig. 8A). Similarly, there was no effect of depression on recovery as assessed with the BBB scale ($F(1,28)=1.94$, $p=0.175$, Fig. 8B).

Minocycline treatment increased pain reactivity thresholds

Minocycline administration decreased pain in the weeks following SCI, and the high dose of minocycline had a long-term analgesic effect. Animals that received the higher dose of minocycline had a longer latency to flick their tail away from a heat source

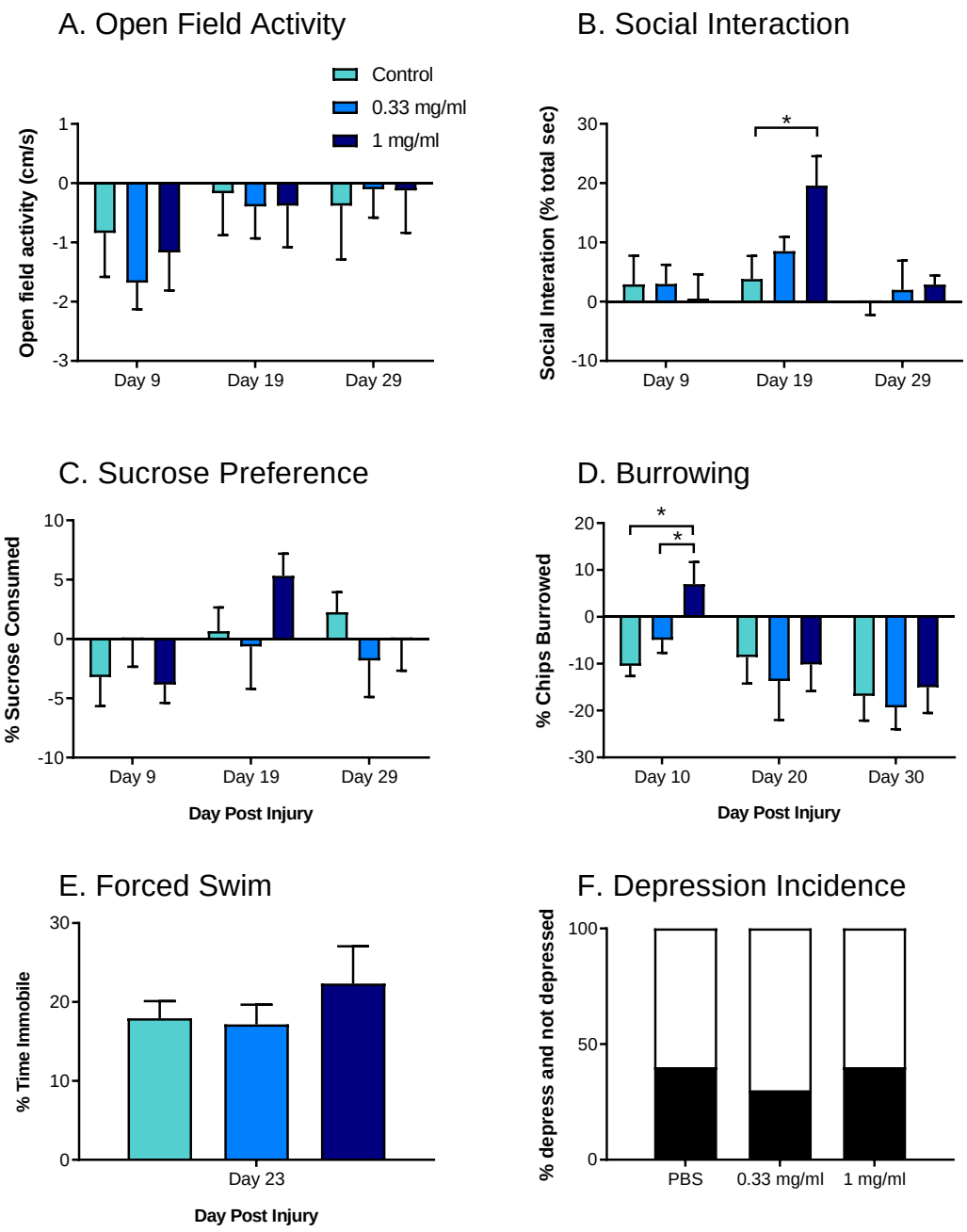

Figure 7. Minocycline does not affect depression-like behaviors.
The high dose minocycline group exhibited more social interaction on Day 19 (B) and more burrowing on Day 10 (D). There were no differences between the dose groups on other indices of depression (A,C,E) or on incidence of depression grouping (F). * $p<0.05$

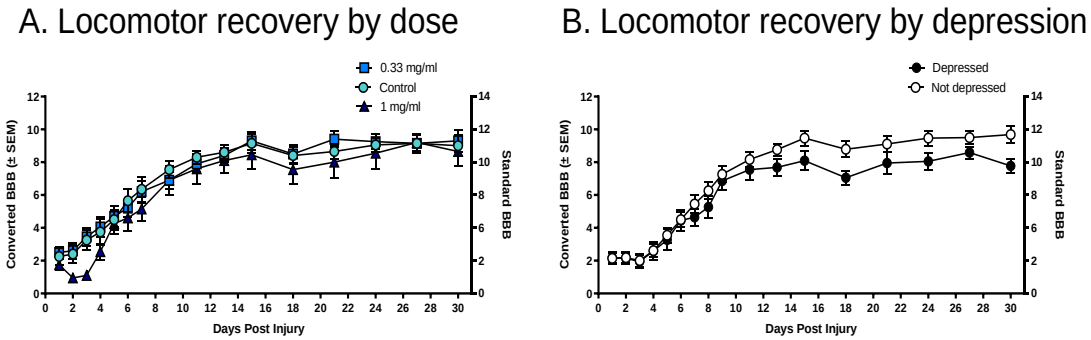

Figure 8. Locomotor recovery in minocycline and depression groups.
BBB locomotor scoring shows little difference among minocycline dose groups (A) or between depressed and not-depressed SCI rats (B). All groups achieved stepping by Day 9, when behavioral testing began.

(measured as change from baseline) compared with the low dose minocycline or controls (Days 11, 22, 28; $F(2,27)=9.86$, $p=0.001$; $F(2,27)=10.09$, $p=0.001$; $F(2,27)=5.44$, $p=0.01$, respectively, Fig. 9B). The vocalization and motor response thresholds on the tactile reactivity test also increased for the high dose group on Days 4 (vocal), 11 (motor), and 22 (motor), indicating lower sensitivity to mechanical stimuli ($F(2,27)=4.79$, $p=0.017$; $F(2,27)=7.64$, $p=0.002$; $F(2,27)=3.99$, $p=0.03$, respectively, Fig. 9C-D).

Sensory and pain scores did not differ between depressed and not-depressed animals, indicating that pain was unlikely to have played a role in the development of depression-like symptoms (Fig. 10).

Neither depression nor minocycline affected lesion size

Commensurate with the locomotor data, there were no differences in lesion size, gray matter, and white matter sparing between the depressed and not-depressed groups.

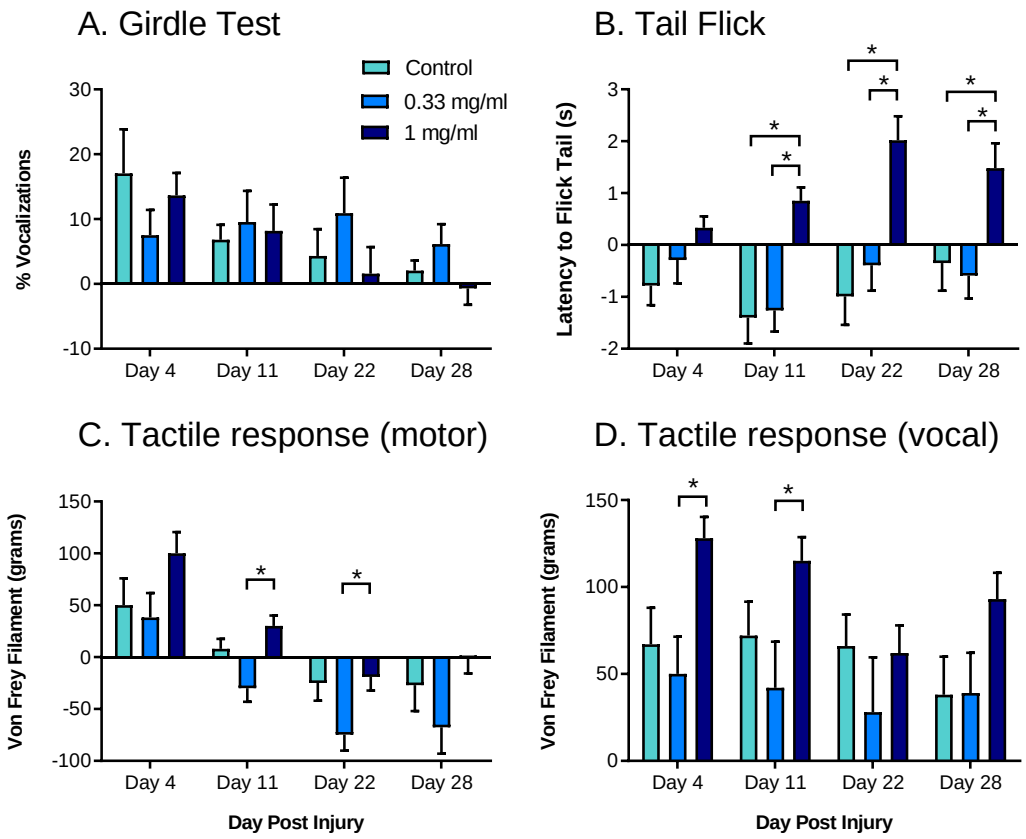

Figure 9. Sensory reactivity in minocycline groups.
All scores are presented as change-from-baseline. The high dose minocycline group increased their latency on the tail flick test, on days 11, 22, and 28 post-injury, compared to both the low dose and controls (B). The low dose minocycline group withdrew their paws at lower levels of pressure on the Von Grey tactile test (C), and the high dose group vocalized at higher pressures than did the low dose group (D). *p<0.05

Additionally, despite its potentially neuroprotective properties, minocycline did not impact lesion size or spared white matter. However, the high dose minocycline group had less spared gray matter than the vehicle controls, across the center of the lesion ($F(2,14)=6.06$, $p=0.013$, Fig. 11).

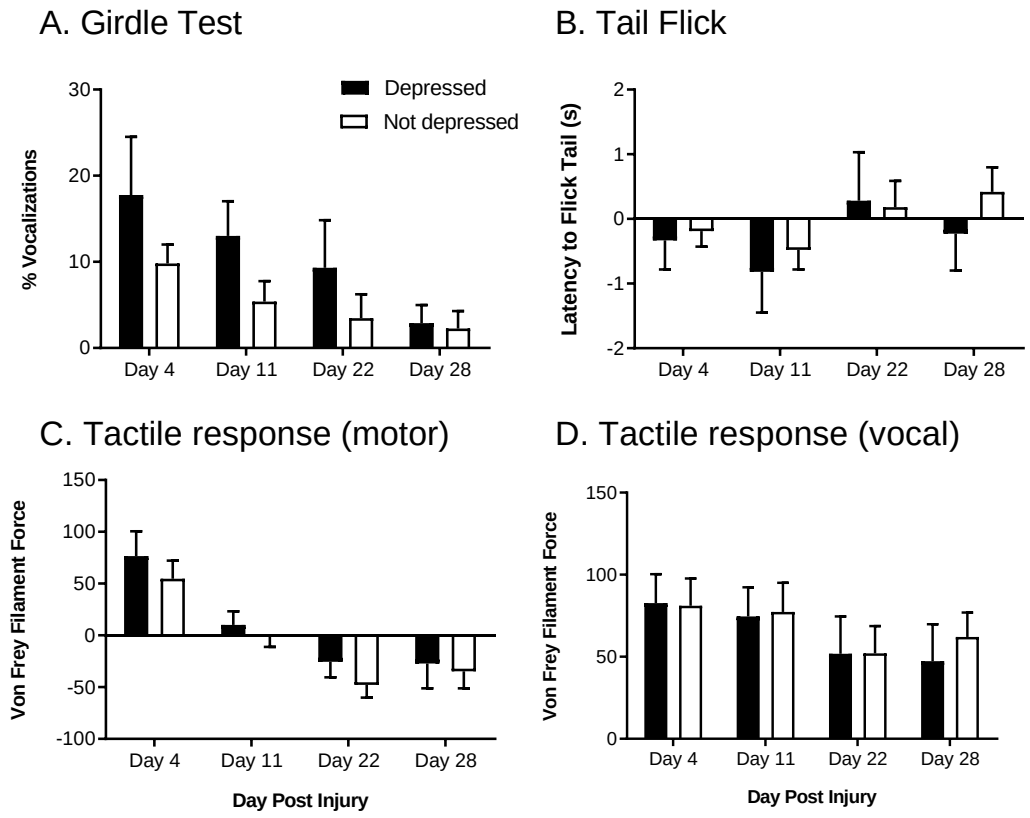

Figure 10. Sensory reactivity in depression groups.
All scores are presented as change-from-baseline. Sensory and pain scores did not differ between depressed and not depressed groups

Subjects that develop depression have elevated pro-inflammatory cytokine profiles

To explore the relationship between inflammation, depression, and minocycline activity, serum cytokine levels were measured before, and 10 days after, injury using a 27-plex magnetic bead assay (RECYMAG65K27PMX, EMD Millipore, Billerica, MA). None of the cytokine levels in the minocycline groups differed from those of the vehicle controls at either timepoint, indicating that oral minocycline did not impact peripheral inflammation after injury.

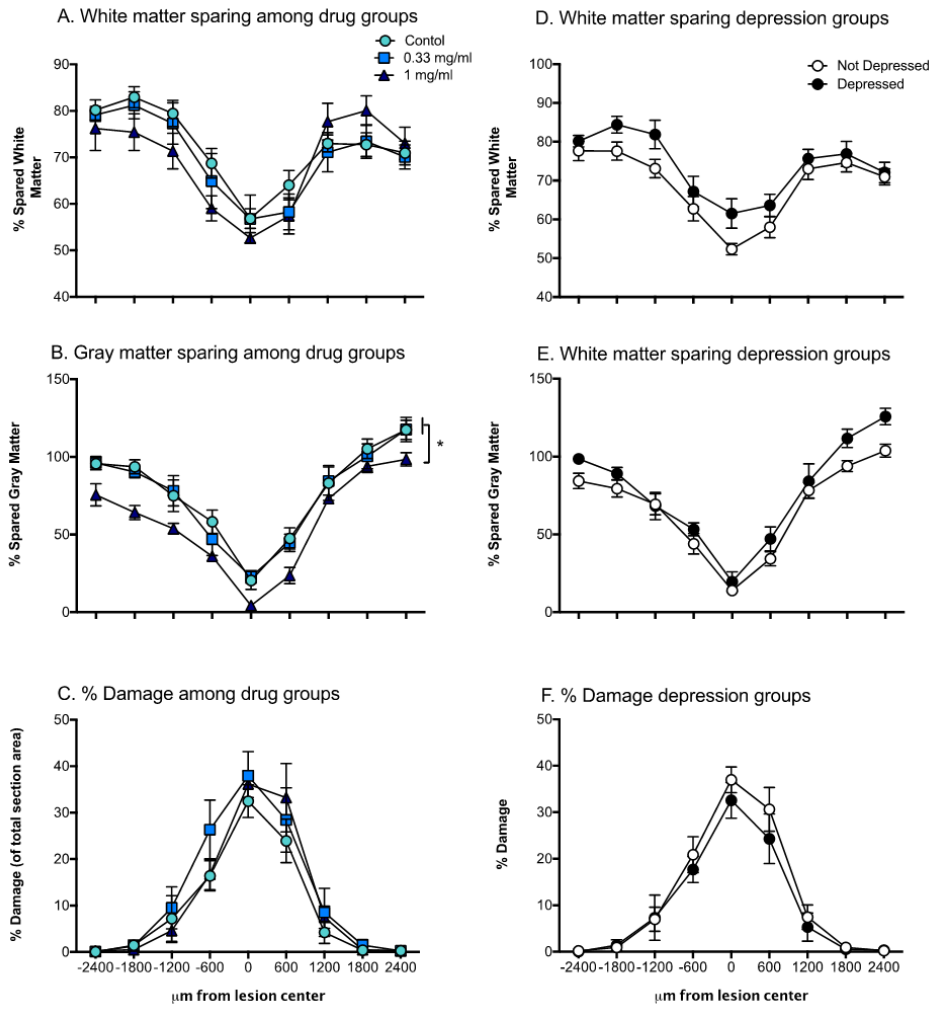

Figure 11. Lesion analysis in minocycline and depression groups.
Panels A-C show spared white matter, gray matter, and percent damage across the extent of the lesion, among the minocycline groups. The high dose minocycline group had less gray matter sparing than the the dose (B). They did not differ in white matter sparing or percent damage (A, C). Panels D-F show the same measures for depressed and not depressed groups. Depressed and not-depressed groups did not differ in histological indices of spinal cord damage. *$p<0.05$

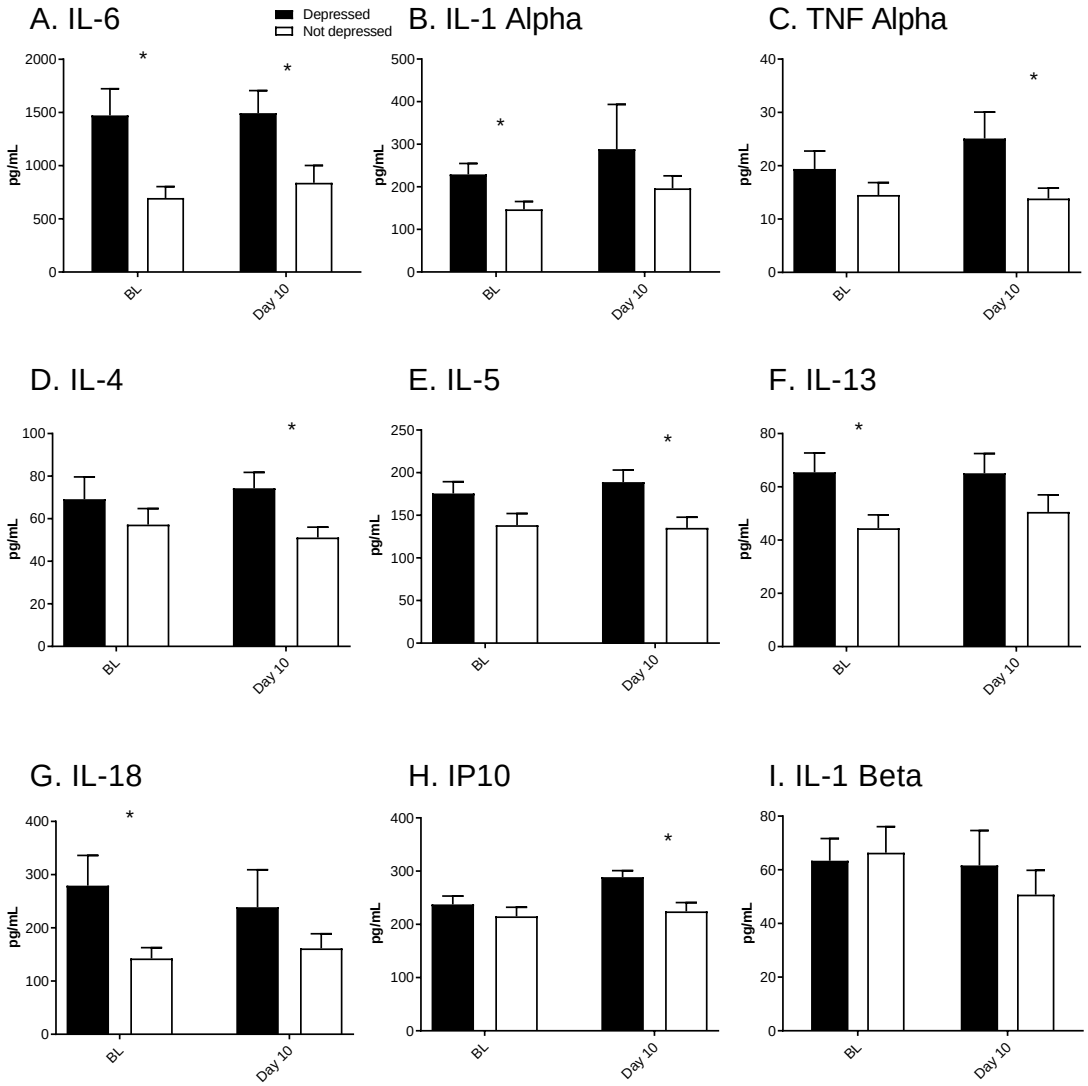

Figure 12. Inflammation is elevated in depressed patients.
Cytokine levels are significantly higher in depressed compared with not-depressed subjects prior to and 10 days after SCI. IL-6, IL-1α, IL-13, and IL-18 are elevated before injury (A-B, F-G). IL-6, TNF-α, IL-4, IL-5, and IP10 are elevated 10 days after injury (A, C-E, H). Individual differences in baseline cytokine levels (1.5-2.2X greater in depressed subjects) may predict vulnerability to the development of an affective disorder. * $p < 0.05$

However, we saw differences in cytokine levels between the depressed and not-depressed groups *both before and after injury*. Of the 27 cytokines and chemokines measured, 6 cytokines and chemokines were upregulated at 10 days post-injury in the depressed group: IL-6, TNF-α, IP-10, IL-4, and IL-5 ($t(19)=2.62$, $p=0.017$; $t(21)=2.39$, $p=0.026$; $t(22)=3.23$, $p=0.004$; $t(22)=2.59$, $p=0.017$; $t(22)=2.63$, $p=0.015$, respectively, Fig. 12). Intriguingly, these animals also exhibited pro-inflammatory profiles before injury, with upregulated IL-6, IL-1 α, IL-13, and IL-18 ($t(15)=3.24$, $p=0.006$; $t(20)=2.55$, $p=0.019$; $t(20)=2.35$, $p=0.029$; $t(16)=2.61$, $p=0.019$, respectively, Fig. 12).

Serotonin is decreased in brains of depressed subjects

Commensurate with behavior, there was a significant effect of depression group on serotonin levels in the frontal cortex ($t(11)= -2.33$, $p=0.007$). As shown in Figure 13,subjects that displayed depression-like behavior had lower serotonin expression levels than their not-depressed counterparts. There was a similar trend toward decreased serotonin in the hippocampus, but it was not significant with the small sample size (Fig. 13). BDNF levels did not differ between depressed and not-depressed groups (Fig. 13).

Neurogenesis

Neurogenesis was assessed in the upper and lower blades of the dentate gyrus by stereological counting of immature neurons stained for doublecortin (DCX). One subject was removed from the analysis because of its poor tissue quality and staining. One-tailed t-tests comparing depression groups showed that the depressed subjects had fewer DCX

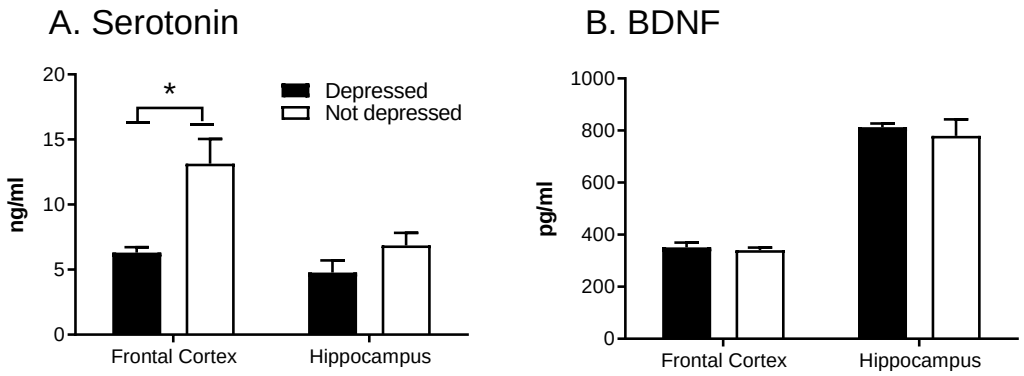

Figure 13. Brain serotonin and BDNF levels in depression groups.
Depressed subjects displayed lower serotonin in the frontal cortex and trended towards lower serotonin in the hippocampus (A). There were no differences in BDNF levels. * $p<0.05$

positive cells in the upper blade of the dentate gyrus than the not-depressed subjects ($t(13)= -1.77$, $p=0.0494$, Fig 14A).

Additionally, there was a clear trend for higher neurogenesis in the minocycline treated animals, with the high minocycline dose subjects exhibiting the greatest number of DCX stained immature neurons ($F(2,12)=2.02$, $p=0.175$, Fig. 14B).

Discussion

The experiments reported here assessed the incidence of depression after SCI and the efficacy of minocycline for protection against depression and inflammation. In an effort to reduce acute, SCI-associated inflammation, minocycline was orally administered for 14 days post-SCI. Approximately one-third of the subjects displayed depression-like behavior in the month following injury. This is commensurate with both human data (Williams and Murray, 2015) and our previous findings in the rodent model of depression after SCI (Brakel et al., 2019; Luedtke et al., 2014; Maldonado-Bouchard et al., 2016).

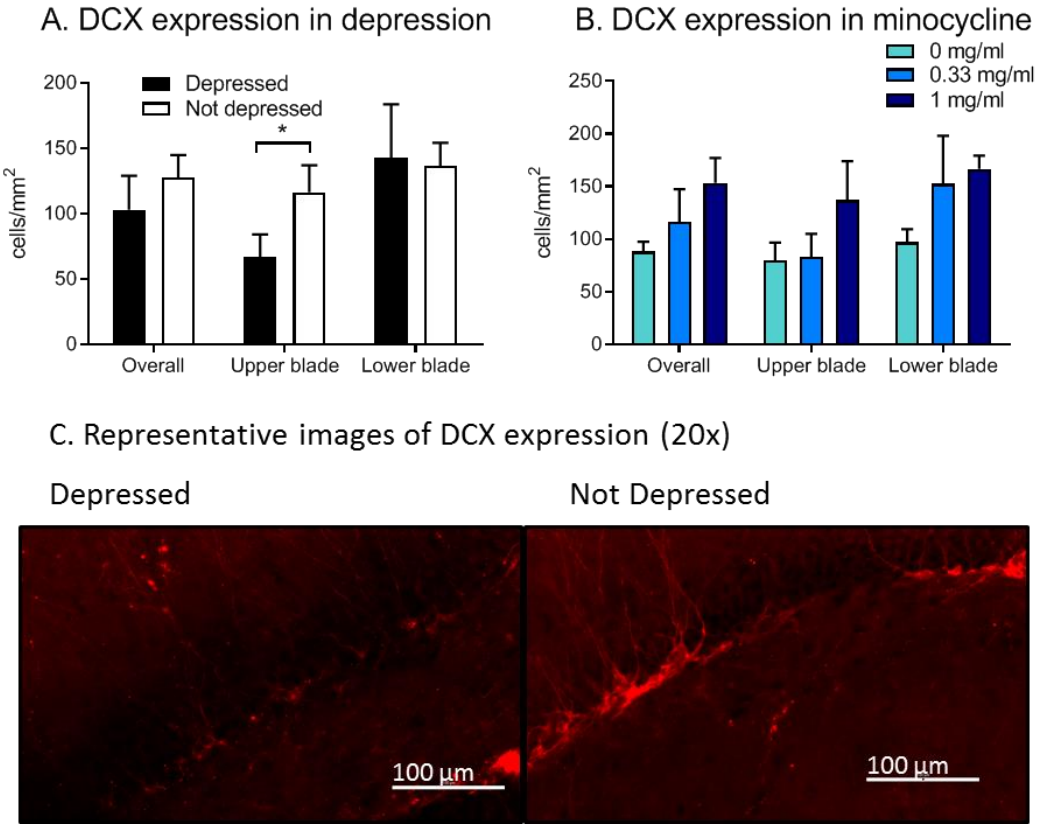

Figure 14. Neurogenesis is decreased in depressed subjects.
Depressed subjects had lower DCX expression in the upper blade of the dentate gyrus (A). There was a trend for greater DCX expression in the 1 mg/ml minocycline dose group (B). Representative images from the upper blade of the dentate gyrus illustrate the differences between the depressed and not depressed groups. * $p<0.05$

Also consistent with the literature, depression was associated with increased peripheral inflammation post-injury and decreased serotonin levels in the prefrontal cortex. Intriguingly, pro-inflammatory cytokine expression was not only elevated post-injury in rats that developed depression-like behaviors, but also *prior* to injury. Inflammation may be a cause of, or a biomarker for, predisposition to depression. Contrary to my original

hypothesis, however, minocycline treatment did not affect peripheral cytokine levels or protect against the development of depression-like behavior.

The anti-inflammatory efficacy of the minocycline treatment may have been affected by the dose or route of administration. Because the subjects received the minocycline through their drinking water, we could not control the exact dose each animal received or whether they drank immediately before behavioral testing and serum collection. As the half-life of minocycline in rats is only a few hours (Elewa et al., 2006), it is possible that the subjects did not ingest a biologically relevant amount in an appropriate time frame to have anti-inflammatory effects. Moreover, although sufficient to reduce inflammation in milder inflammatory conditions, the dose of minocycline used may not have been sufficient to reduce the robust inflammation inherent to SCI. This hypothesis is supported by the spinal lesion analysis. Although minocycline has been repeatedly shown to be neuroprotective (Camargos et al., 2020; Elewa et al., 2006; Li et al., 2016; Mejia et al., 2001; Ulndreaj et al., 2017), we saw no effect of minocycline on lesion size or locomotor recovery after SCI, indicating that our dose or route of administration may not have been biologically meaningful. An alternate intraperitoneal route of administration may be more appropriate in this model, using doses that have been reported to reduce lesion size in an SCI model.

Indeed, while others have seen anti-depressant effects of chronic, oral, minocycline administration, at doses consistent with those used in the current study, they have also found limited effects on inflammation (Burke et al., 2014; Camargos et al., 2020; Hinwood et al., 2012; Hinwood et al., 2013; Molina-Hernández et al., 2008). In a rodent

stress model, Hinwood et al. (2013) found that chronic, oral minocycline reduced microglial branching, size, and process length in the frontal cortex. They did not, however, find any alterations in pro-inflammatory markers or cytokines in their stressed groups, indicating that the interaction between inflammation and minocycline was quite low (Hinwood et al., 2013). In an olfactory bulbectomy (OB) rat model of depression, combined with spinal nerve ligation, Burke et al. (2014) also found that chronic (14 days), oral minocycline treatment decreased depression behavior and decreased pain reactivity (increased paw withdrawal latency in an acetone reactivity test). Intriguingly, the minocycline treatment did not decrease microglial expression, and, in fact, appeared to increase both pro- and anti-inflammatory cytokine levels in the prefrontal cortex of the OB rats following the spinal nerve ligation (Burke et al., 2014). This finding contrasted with the effects seen in non-OB rats, who also underwent spinal nerve ligation, and in whom minocycline reduced pro-inflammatory markers. Measuring minocycline levels in the prefrontal cortex with mass spectrometry, Burke et al. (2014) verified that supraspinal levels of the minocycline were similar in sham and OB rats. Collectively, therefore, neither Hinwood et al. (2013), Burke et al. (2014), nor ourselves found strictly anti-inflammatory effects of oral minocycline in any of our stress/depression models. Burke found that chronic minocycline treatment actually increased inflammation, Hinwood found that it prevented microglial activation, but did not impact cytokine expression, and we found no impact on cytokine expression. One potential explanation is that while minocycline may decrease microglial activation, it may increase the activity of other immune cells. Minocycline has been shown to increase cytokine release from monocytes pretreated with

lipopolysaccharide, but not phytohemagglutinin, indicating that minocycline's action may depend on the type of cellular stressor it is paired with (Kloppenburg et al., 1996). These data suggest that minocycline may have differential effects in the presence of a depression-like phenotype.

In the SCI depression phenotype, extended minocycline treatment may also have counterintuitively contributed to inflammation. Because minocycline is an antibiotic, extended oral administration may disrupt the natural gut flora. Disrupted gut flora, combined with SCI-induced gut dysbiosis, can lead to more inflammation (Brakel and Hook, 2019). While not statistically significant, serum cytokines collected at Day 10, which was while minocycline was present, were slightly higher in the minocycline groups than in the controls, indicating that we may have inadvertently reduced natural gut flora and increased inflammation in the treated animals. Further, these rats (and those in the following experiments) also received penicillin immediately following SCI and for at least one day following injury. Even brief doses of antibiotics can significantly deplete the gut microbiota (Becattini et al., 2016; Park et al., 2019). It is possible that our antibiotic administration, though short, disrupted the gut microbiota enough to produce inflammation and depression. In fact, SCI itself may have impacted gut dysbiosis and inflammation. Others have shown that SCI patients have lower microbiota diversity, and mice with spinal contusion injuries exhibit significantly modified taxa in their gut microbiota compared to uninjured controls (Kigerl et al., 2016; Zhang et al., 2018). Further, gut dysbiosis is associated with inflammation for over a month after SCI (Myers et al., 2019). Superimposing antibiotic treatment onto an already disrupted gut microbiome

may have further exacerbated intestinal and peripheral inflammation. Research on the impact of long-term minocycline treatment on gut dysbiosis is still incipient, but it is highly worth pursuing.

Interestingly, although the effects of minocycline on depression or peripheral inflammation were minimal, this treatment did appear to reduce pain in the weeks following SCI. The high dose of minocycline increased the rat's latency to flick their tails away from aversive heat, and it increased the forces at which they vocalized on the Von Frey tactile response test. The difference in tail flick response persisted for a month after injury, indicating that minocycline may have a chronic effect. Others have also found that minocycline reduces long-term pain, though most deliver the drug i.p. rather than orally (Cho et al., 2011; Li et al., 2016; Marchand et al., 2009). Alternatively, the protection against pain may come from loss of gray matter. The high dose minocycline subjects had lower amounts of spared gray matter. If sensory neurons from the dorsal horn were completely lost, afferent pain signaling from the spinal cord would also be reduced.

Despite the absence of effects of minocycline, we did replicate and extend our previous findings on depression in the rodent SCI model. We found that subjects that displayed depression-like behavior had higher pro-inflammatory cytokine expression before and after SCI, had decreased serotonin in the frontal cortex and decreased neurogenesis in the hippocampus. Depressed subjects exhibited less neurogenesis in the upper blades of their hippocampi than not-depressed subjects. This finding concurs with previous literature showing that stress and depression correlate with decreased hippocampal neurogenesis (Czéh et al., 2002; Malberg and Duman, 2003; Pham et al.,

2003). Malberg et al. (2003), for example, found that inescapable foot shock produced depression behaviors and decreased neurogenesis in rats. Because decreased neurogenesis has been observed in multiple models of stress and depression, it is likely that stress universally decreases neurogenesis, and the depression elicited from SCI may be quite similar to that caused by other stressors. Additionally, we saw a trend for a dose-dependent effect of minocycline on neurogenesis. Oral minocycline, at a dose similar to ours, has been previously shown to increase hippocampal neurogenesis in adult mice (Kohman et al., 2013). Minocycline has also improved neuronal proliferation and differentiation in other models of stress and depression. Intriguingly, we saw increased neuronal proliferation in minocycline-treated rats, despite the fact that moderate to severe SCI *per se* causes decreased hippocampal neurogenesis (Jure et al., 2017). These facts indicate that, while minocycline may not have decreased proinflammatory cytokine expression, it may have still been an effective supraspinal neuroprotectant.

Replicating our previous studies (Maldonado-Bouchard et al., 2016), we also observed elevated TNF-α and IL-6 in the depressed subjects compared to not-depressed subjects after injury, but, surprisingly, we saw no effect of injury itself on cytokine levels. Peripheral proinflammatory cytokines were not elevated ten days after injury compared to baseline levels. This is particularly interesting, because the general consensus in the literature is that SCI causes persistent inflammation. Elevated levels of TNF-α, IL-6, IL-1RA, and IL-2 have been seen in serum of SCI patients years after their injuries (Davies et al., 2007; Hayes et al., 2002). We suggest that in the absence of confounding secondary infections, pressure sores, and other SCI-related inflammatory complications, elevations

in peripheral inflammation may be transient. Indeed, in the spinal cord, elevations in pro-inflammatory cytokines seem to be transient. Time-course studies of SCI-induced inflammation in rodents has shown an increase in TNF-α, IL-6, and IL-1β during the first few hours after an injury, but levels return to baseline after 24 hours (Yang et al., 2005). Altinors et al. (2009) also found elevated TNF-α, IL-6, and IL-1β in serum 24 and 48 hours after spinal ischemia, but they did not explore later time points (Altinors, 2009). Serum IL-6, especially, elevates 24 hours after contusion injury in rats but returns to BL levels after 7 days (Yang et al., 2018). In the current study, it is likely that by 10 days post-injury, peripheral inflammation had returned to baseline in most subjects, resulting in no distinct differences between the levels of pro-inflammatory cytokines. The chronically high inflammation that Davies et al. and Hayes et al. observed in their human patients may be due to long-term physiological modifications brought about by living with an SCI. Future studies would benefit from comparing peripheral inflammation in the hours after SCI with that of the later days.

The current study also revealed an unexpected finding of elevated baseline pro-inflammatory cytokine expression in depressed subjects, *before SCI and before the subjects exhibited depression-like symptoms*. Prior to injury, there were no behavioral differences between the depressed and not-depressed subjects, but IL-6, IL-1α, IL-13, and IL-18 were elevated in the depressed group. Similar evidence has also been presented in human and animal studies. In fact, in a landmark study (The Whitehall Study), Gimeno et al. (2009) found pre-existing differences in IL-6 expression among people that were susceptible to the development of depression. The Whitehall Study (Gimeno et al., 2009)

followed hundreds of white-collar office workers for twelve years, collecting data on their mental health status as well as their peripheral blood cytokine profiles. The study found that baseline levels of C reactive protein and IL-6 predicted depressive symptoms that manifested up to 12 years later. Studies looking at both juvenile and aged populations have found similar results: IL-6 measured in 9 year-old children was predictive of depression and other mental disturbances 9 years later, and both IL-6 and CRP measured in adults over 60 were predictive of depression identified 5 years later (Khandaker et al., 2014; Zalli et al., 2016). These inflammatory molecules are clearly ubiquitous across populations and are important to the development of depression. In a mouse model of social defeat stress, Hodes et al. (2014) also found that leukocytes from mice susceptible to mild social defeat stress, which does not typically produce depression-like behaviors, produced more IL-6 when stimulated with lipopolysaccharide (LPS) than those from resilient mice. Additionally, when IL-6 activity was blocked with a monoclonal IL-6 antibody or eliminated through an IL-6 knockout, mice subjected to social defeat stress were less likely to develop depression-like behaviors.

These data suggest that ongoing IL-6 activity is necessary for susceptibility to depression. Collectively, these results raise an intriguing hypothesis that pre-existing peripheral inflammation might predispose an individual to develop depression symptoms after a traumatic event. Minocycline may not be sufficient to reduce depression or inflammation after a traumatic CNS event, such as SCI. However, the current experiment shows that inflammation prior to injury may predispose individuals to depression post-injury.

CHAPTER IV

IL-6 PRE-TREATMENT INCREASES DEPRESSION FOLLOWING SPINAL CORD INJURY[2]

Introduction

Our previous results (Chapter III) suggest that inflammation post-injury may not be critical for the expression of depression-like symptoms. While depressed subjects had higher pro-inflammatory cytokine levels at Day 10 post-injury, they also had increased cytokine levels prior to injury, and cytokine expression levels were not substantially different from pre- to post-injury. In fact, our data suggest that in many studies, the inflammation associated with post-stress depression may actually reflect pre-existing differences in inflammation. Pre-existing inflammation may in turn be a cause, or consequence, of molecular changes that increase susceptibility to depression after exposure to a significant stressor.

A number of studies suggest that there is an interaction between resilience and inflammatory processes. For example, when compared with individuals that adopt active coping strategies, individuals with passive coping strategies have greater plasma concentrations of interleukin-6 (IL-6) following a 3 min simulated public speaking challenge (Carroll et al., 2011). Feelings of helplessness and anxiety during a social stress

[2] Portions of this chapter are reprinted with permission from "SCI and depression: Does inflammation commandeer the brain?" by Kiralyn Brakel & Michelle Hook, 2019. Experimental Neurology, 320, Article 112977, Copyright 2019 by Elsevier Inc.

test are also associated with sensitization to common allergens (e.g., house dust mite, ragweed, tree mix, grass mix) and greater release of IL-6 from stimulated primary blood leucocytes (Kiecolt-Glaser et al., 2009). Further, Maes and colleagues found that psychological stressors increase the production of IL-6, IL-1ra, and IFN-γ from cells in the peripheral blood of a student population, particularly those who identified as stressed (Maes et al., 1997; Maes et al., 1995; Maes et al., 1993; Maes et al., 1998). In susceptible people, stress seems to increase inflammation.

Susceptibility to depression may also be predicted by inflammatory cytokines, particularly IL-6. In white collar workers with no serious health complications, serum IL-6 and C-reactive protein (CRP) levels predicted symptoms of depression that developed 12 years after the serum samples were collected (Gimeno et al., 2009; Zalli et al., 2016). Similarly, Khandaker and colleagues (2014) measured serum IL-6 and CRP levels in 9-year-old children and found that those with the top-third of IL-6 values were more likely to be depressed at 18 years of age. In an aging population (>55 years old), these same inflammatory markers predicted depression 5 years after sample collection (Gimeno et al., 2009; Zalli et al., 2016). Interestingly, others have shown that baseline inflammatory biomarkers also correlate with the effectiveness of antidepressant treatments. Patients with high baseline levels of CRP respond better to the TNF-α inhibitor infliximab or the selective serotonin reuptake inhibitor (SSRI) escitalopram, while those with lower CRP respond better to the tricyclic nortriptyline (Raison et al., 2013; Uher et al., 2014). These results indicate that a patient's inflammatory profile not only impacts their susceptibility

to depression, but also their response to treatment. This suggests a connection between inflammation and neuromodulation.

Similar associations between depression susceptibility and IL-6 have been seen in animal models. Mice that are vulnerable to developing depression-like behaviors 10 days after experiencing repeated social defeat stress have elevated levels of plasma IL-6 immediately after their first defeat, whereas resilient animals do not (Hodes et al., 2014). This suggests that minor stressors can expose pre-existing differences in individuals. These pre-existing differences are seen in immune reactivity, as whole blood monocyte cultures collected from susceptible rats before social defeat produce more IL-6 when exposed to an immune stressor, such as lipopolysaccharides, than do cultures from resilient mice (Hodes et al., 2014). Strikingly, if bone marrow from a stress-susceptible mouse is transplanted into a naive recipient, that mouse also becomes susceptible to even a mild stressor (Hodes et al., 2014). These data suggest that IL-6 may not only be a biomarker of susceptibility but may also drive an individual's response to a stressor.

In the previous experiment (Chapter III), rats that developed depression after SCI also had elevated serum IL-6 before injury. These data, added to the reviewed literature, support the hypothesis that pre-existing inflammation, particularly IL-6, is associated with depression following a stressor. Based on the studies of Hodes et al. (2014), I hypothesize that elevated IL-6 prior to SCI may not only predict depression, but may also cause depression. In the following experiment, I elevated IL-6 levels in a group of animals by administering exogenous IL-6 intraperitoneally (i.p.) for a week prior to spinal contusion

injury. I then assessed post-injury depression in the IL-6 treated animals and vehicle-treated controls.

Methods and Results

In this experiment, rats received a moderate contusion (n=54) or sham (n=15) injury and were assessed with the tests of depression-like behavior, motor function, and pain reactivity, described in Chapter II (General Methodology). To increase pre-injury IL-6 levels, the rats were given an i.p. injection 0, 1.6, or 3.2 μg of recombinant rat IL-6 protein (506-RL/CF, R&D Systems, Minneapolis, MN), suspended in 1X PBS, daily for 7 days immediately prior to injury.

Depression behavior and pain reactivity were assessed before IL-6 administration, after IL-6 administration, and for 23 days post-injury, as shown in Figure 4. At 24 days post-injury, subjects were humanely euthanized with pentobarbital (100 mg/kg, i.p.). Fresh brain tissue was collected postmortem and snap frozen for BDNF and serotonin ELISAs, as described in Chapter II (General Methodology).

Statistical testing was conducted as described in the General Methodology. Eight subjects were removed from the study. One subject died after surgery, 4 contused subjects were removed from the analyses for failure to reach minimum baseline sucrose preference (80%), 2 for BBB scores >8 on Day 1 post-injury, and 1 for failure to recover above a BBB score of 1.

IL-6 increases incidence of depression

As might be expected, injury condition significantly affected open field activity. There was a main effect of injury on days 9 and 19 post-surgery ($F(1,55)=7.06$, $p=0.01$;

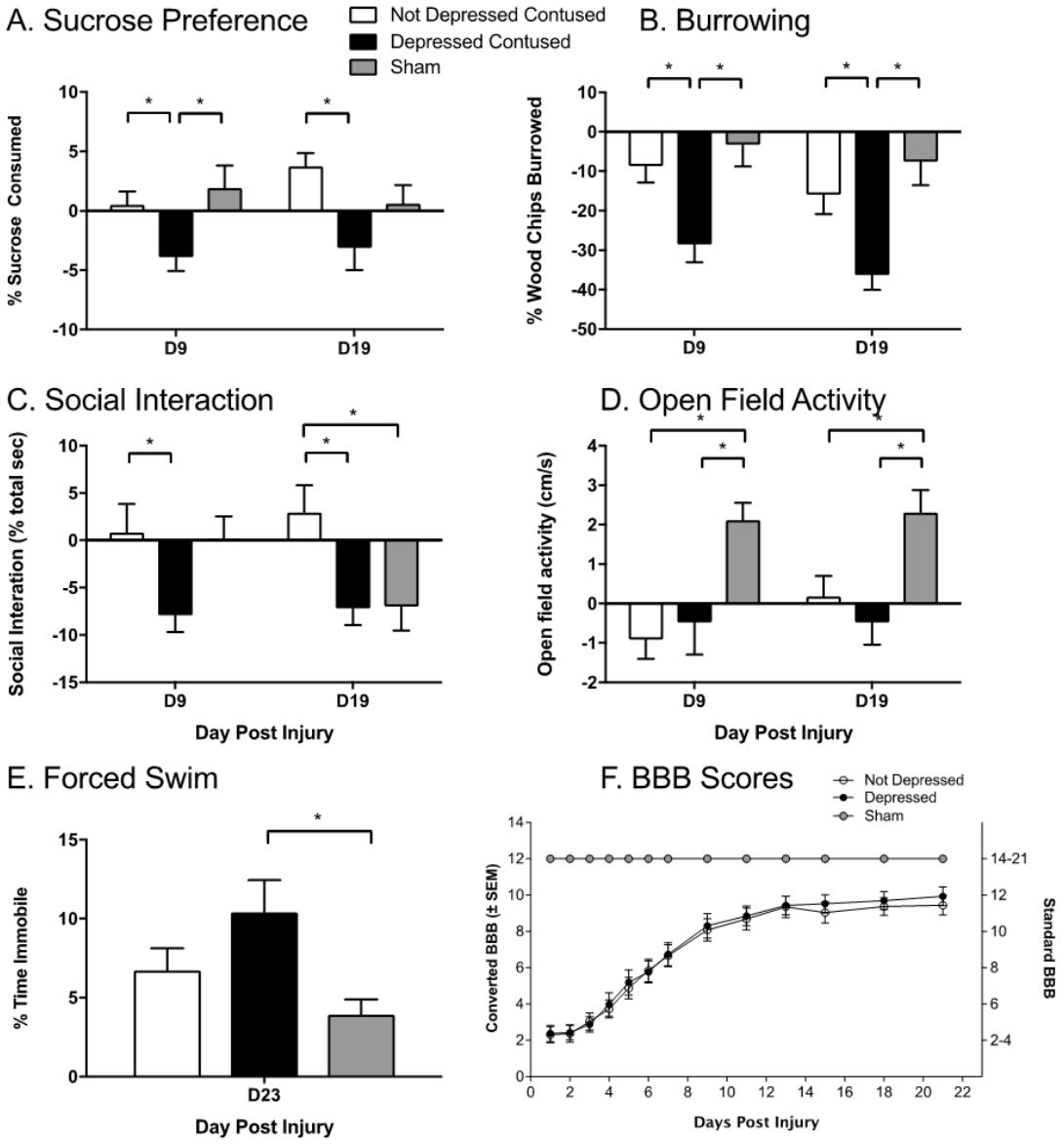

Figure 15. Depression behaviors in sham and contused rats.
Throughout recovery, animals that clustered together as "depressed" had lower sucrose preference than not-depressed or sham animals (A). They also burrowed less and initiated less social interaction than the not-depressed and sham groups (B-C). While they did not differ significantly on open field activity, there was a significant effect of surgery (D). The depressed group also exhibited higher immobility than the shams on the forced swim test (E). All behaviors are presented as change-from-baseline. There was no difference in locomotor recovery between the depressed and not-depressed contused groups (F). * $p<0.05$

F(1,55)=6.69, p=0.01, respectively). On both days, the contused subjects moved less than the shams (Fig. 15D). There was also a main effect of surgery on burrowing behavior on days 10 and 20 post-injury (F(1,55)=5.50, p=0.02; F(1,55)=4.91, p=0.03). Again, the contused subjects displayed decreased burrowing activity compared to shams (Fig. 15B).

Because the sham subjects differed from the contused animals so definitively, only contused subjects were included in depression clustering. Additionally, because open field activity was associated with locomotor recovery, it was not included as a factor in the cluster analysis. These measures ensured that the hierarchical cluster analysis divided subjects based on their depression-like behavior only and not their recovery of locomotor function. Using the subjects' scores averaged across the last two testing periods (Days 9-10 and Days 19-20) from the array of depression tests (social activity, sucrose preference, burrowing, and forced swim test), hierarchical cluster analysis divided the contused subjects into two groups. Comparisons of the clusters and the sham subjects across the individual tests revealed differences in sucrose preference, social interaction, burrowing, and immobility on the forced swim test (Fig. 15). One cluster of SCI subjects (depressed) displayed increased depression-like behavior relative to the other SCI cluster (not-depressed) and sham controls. ANOVAs and *post hoc* Tukey tests determined that the depressed group had lower sucrose preference than the not-depressed group at Days 9 and 19 post-injury (F(2,57)=5.04, p=0.01; F(2,57)=5.73, p=.005, respectively, Fig. 15A). They also had lower sucrose preference than the shams on Day 9 post-injury (F(2,57)=5.04, p=0.01, Fig. 15A). On the burrowing task, ANOVAs showed differences among the three groups (F(2,57)=7.98, p=0.001; F(2,57)=8.76, p<0.001, Fig. 15B). Post hoc tests

determined that subjects in the depressed cluster burrowed less than the not-depressed subjects on Days 10 and 20 and less than the shams on the same days. The depressed group also had lower social interaction than the not-depressed group on Days 9 and 19 ($F(2,57)=3.68$, $p<0.03$; $F(2,57)=5.76$, $p=0.005$, respectively, Fig. 15C), and spent more time immobile on the forced swim test than the shams ($F(2,57)=3.59$, $p=0.03$, Fig. 15E). Open field activity and locomotor recovery did not differ across the depression groups.

Comparing the incidence of depression across the IL-6 dose groups, 67% of the moderate-dose IL-6 subjects clustered in the depressed group. This incidence is significantly higher than the predicted 30% ($X^2(1,8)=6$, $p<0.05$, Fig. 16F) that we have previously observed in SCI rats (Brakel et al., 2019; Luedtke et al., 2014; Maldonado-Bouchard et al., 2016). Similar to previous results, 35% percent of the PBS-treated and 46% of the high-dose IL-6 animals clustered in the depressed group (Fig. 16F). Further comparisons revealed a main effect of IL-6 dose on social behavior by 9 days post-injury ($F(2,55)=5.325$, $p=0.008$, Fig. 16C). *Post hoc* tests showed that the moderate IL-6 dose group (1.6 µg/day for 7 days) displayed decreased social behavior compared to the control or high-dose (3.2 µg/day for 7 days). There was also a trend for both IL-6 dose groups to have higher immobility on the forced swim test by the end of the experiment (Fig. 16E), but it was not statistically significant. Locomotor recovery did not differ among the IL-6 dose groups (Fig. 17).

Sensory recovery

Two-way ANOVAs were conducted evaluating the effects of surgery and IL-6 dose on pain and sensory recovery. There was a main effect of surgery on at-level pain

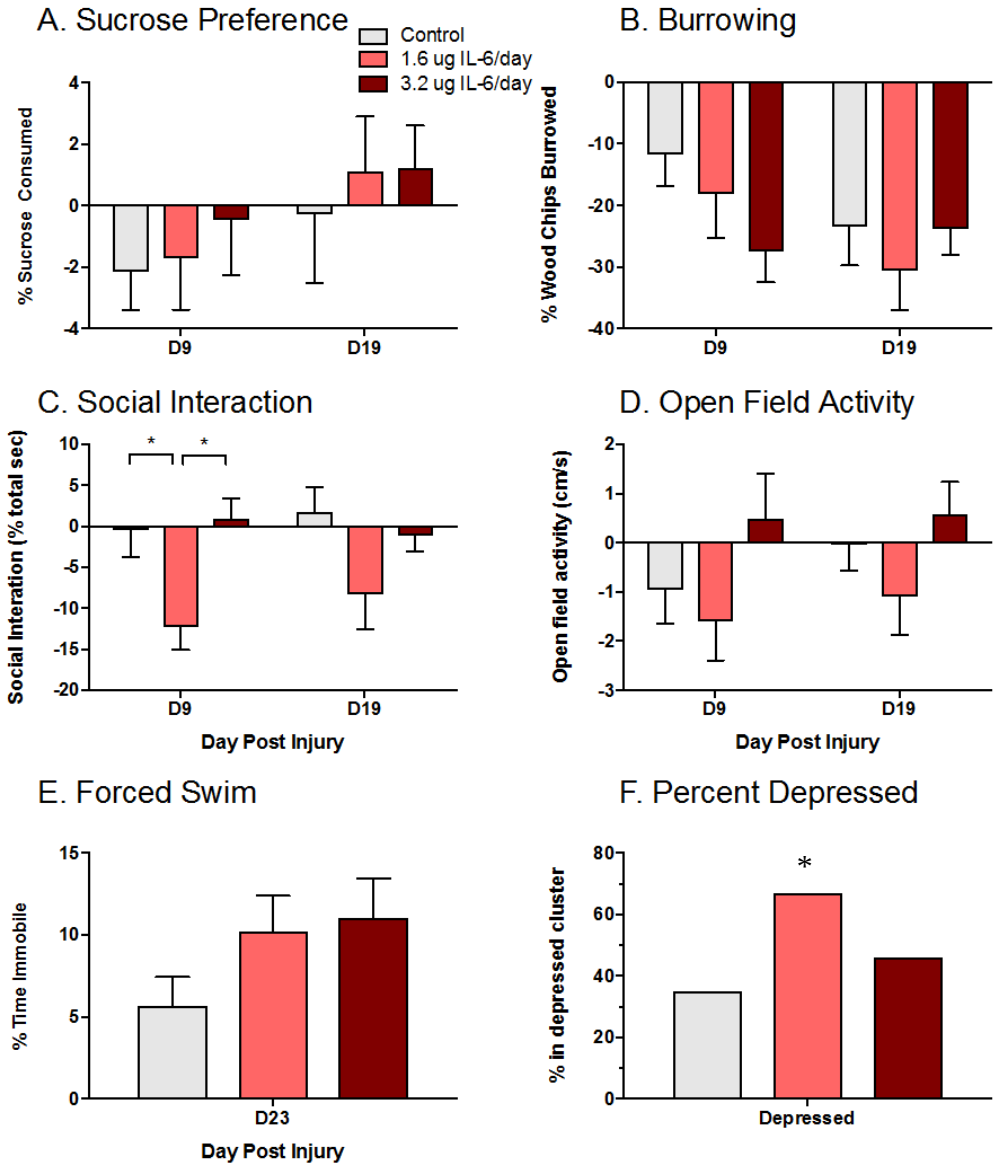

Figure 16. Depression behavior among IL-6 pre-treatment groups.
The subjects that received the moderate dose of IL-6 exhibited decreased social activity compared to the high dose and control groups (C). While there were not significant differences among the groups on the other behavioral tests, there was a trend for the IL-6 treated animals to have higher immobility on the forced swim test (E). Sixty-seven percent of the moderate dose IL-6 animals clustered in the depressed group, which was significantly more than the predicted 33% (F). * $p<0.05$

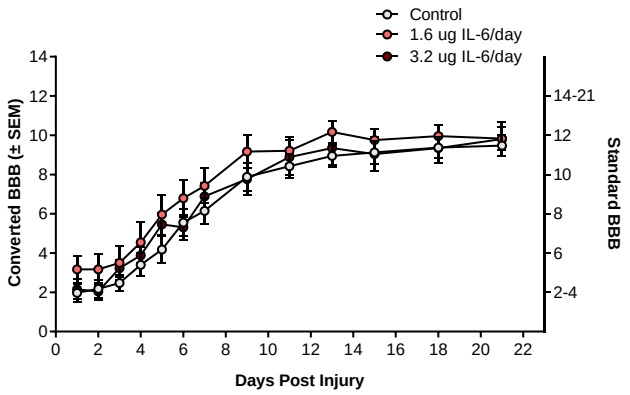

Figure 17. Locomotor recovery among IL-6 pre-treatment groups.
There is no difference of locomotor recovery among the IL-6 dose groups. Both groups achieved stepping by Day 9, when behavioral testing began.

throughout recovery; the shams vocalized less than the contused subjects on the girdle test (Day 4: $F(1,55)=12.9$, $9=0.001$; Day 11: $F(1, 55)=8.1$, $p=0.006$; Day 21: $F(1,55)=4.1$, $p=0.05$; Fig. 18A). There was also an effect of surgery on motor and vocal response in the Von Frey tactile testing on Days 4 and 21 post injury: the contused subjects had increased mechanical reactivity thresholds relative to the shams, indicating loss of hindlimb motor control and sensitivity (Day 4 motor: $F(1,55)=67.8$, $p<0.001$; Day 4 vocal: $F(1,55)=26.3$, $p<0.001$; Day 21 vocal: $F(1,55)=7.1$, $p=0.01$, Fig 18C-D).

Neither depression, nor IL-6 pre-treatment greatly impacted at-level pain or hindlimb tactile reactivity, although the depressed group vocalized less on the girdle test on Day 4 than the not-depressed group ($F(2,57)=11.96$, $p<0.001$, Figs. 18C-D and 19C-D). Two-way ANOVAs showed a main effect of IL-6 dose on tail flick latency on day 11 ($F(2,55)=8.4$, $p=0.001$, Fig. 19B). *Post hoc* comparisons showed that the animals with the medium dose of IL-6 (1.6 μg/day) had a higher latency to flick their tails compared to the high dose group and controls.

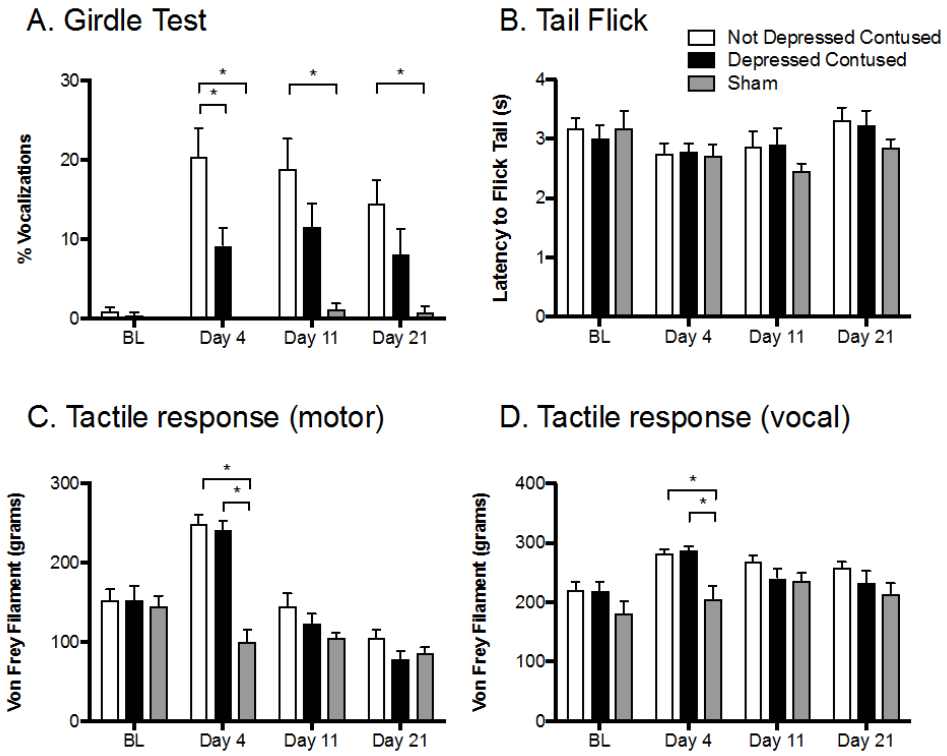

Figure 18. Sensory reactivity of sham and contused rats.
There was a main effect of surgery on at-level pain throughout recovery, measured by the girdle test, (A). There was also a main effect of surgery on acute tactile sensitivity, measured by paw withdrawal and vocalization on the Von Frey tactile test (C-D). * $p<0.05$

Cytokines

Serum cytokine levels were measured before injury, during IL-6 administration, and 10 days after injury (Fig. 20). IL-6 levels seemed to elevate after systemic IL-6 administration in the medium dose (1.6 μg/day) and then remained elevated after injury, although there was no statistical significance across the groups.

Serotonin and BDNF

BDNF levels in the frontal cortex and hippocampus were compared among depressed groups and IL-6 dose groups using ELISA assays, as described in the General

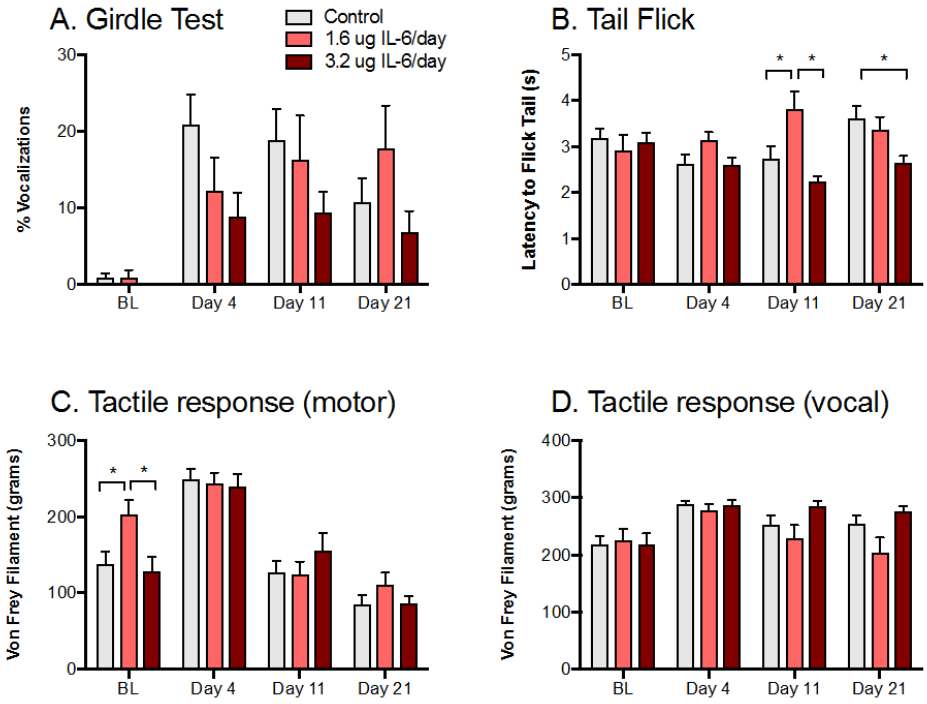

Figure 19. Sensory reactivity of IL-6 pre-treatment groups.
There was no effect of IL-6 on at-level pain (A). The moderate IL-6 dose group showed elevated latency to flick their tails away from a painful light by day 11 post-injury, and the high dose group decreased their latency by the end of the experiment (B). There were no differences in tactile reactivity post-injury (C-D). * $p<0.05$

Methodology (Chapter II). Due to the large number of subjects in this study and the longitudinal demands of behavioral characterization, the subjects were run in multiple cohorts, spaced a few weeks to a few months apart. Unexpectedly, there were large differences in BDNF and serotonin between cohorts, even when the assays were run on the same day using ELISA plates and kit components from the same lot. The values were converted to $\log_2$ values to reduce the variability, but ANOVAs still detected significant differences between the third and the first two cohorts in the BDNF assays and between

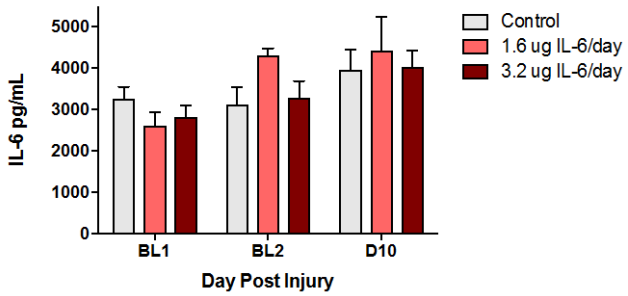

Figure 20. IL-6 levels in pre-treated rats.
While not statistically significant, the moderate IL-6 dose resulted in elevated serum IL-6 levels after one week of administration (BL2). All dose groups had slightly elevated IL-6 at 10 days post-SCI.

the second cohort and the other two in the serotonin assays. Because of this complication, the significantly different cohorts were analyzed and graphed separately (Fig. 21).

A depression effect was seen on BDNF in the frontal cortex; the depressed group had lower BDNF expression than the not-depressed group ($t(36)=2.85$, $p=0.007$, Fig. 21A). There were no differences in BDNF expression among shams and contused subjects (Fig. 21B). The high-dose contused IL-6 animals also exhibited lower BDNF expression in the hippocampus than the other two contused dose groups ($F(2,35)=5.04$, $p=0.012$, Fig. 21C).

Surprisingly, there were no differences in serotonin levels between depressed and not-depressed groups in either cohort set in the hippocampus or frontal cortex. However, both depressed and not-depressed contused subjects displayed lower serotonin in the hippocampus than the shams ($F(2,43)=12.62$, $p<0.001$, Fig. 22A). Two way ANOVAs showed no main effects of dose or injury across the IL-6 groups (Fig. 22C-D).

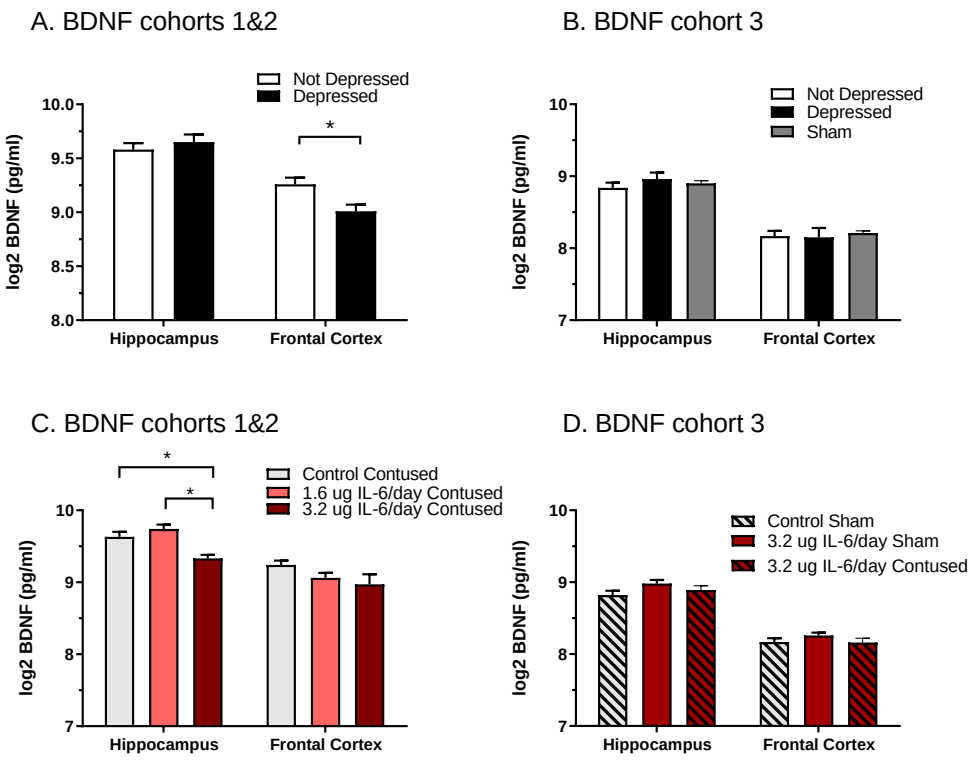

Figure 21. BDNF expression in depression and IL-6 pre-treatment groups.
Depressed subjects from cohorts 1&2 displayed decreased BDNF in their frontal cortex compared to not-depressed subjects (A). High IL-6 pre-treatment was associated with lower hippocampal BDNF (C). *p<0.05

Discussion

Elevation of serum IL-6 prior to SCI was sufficient to increase the incidence of depression in this rodent model. SCI rats treated with a moderate dose of IL-6 for 7 days prior to injury had elevated serum IL-6 expression relative to vehicle-treated controls. Post-injury hierarchical clustering, based on an array of depression behaviors, also revealed that two-thirds of the moderate-dose IL-6 group were characterized as depressed. In previous experiments, about one-third of SCI rats developed depression-like behaviors

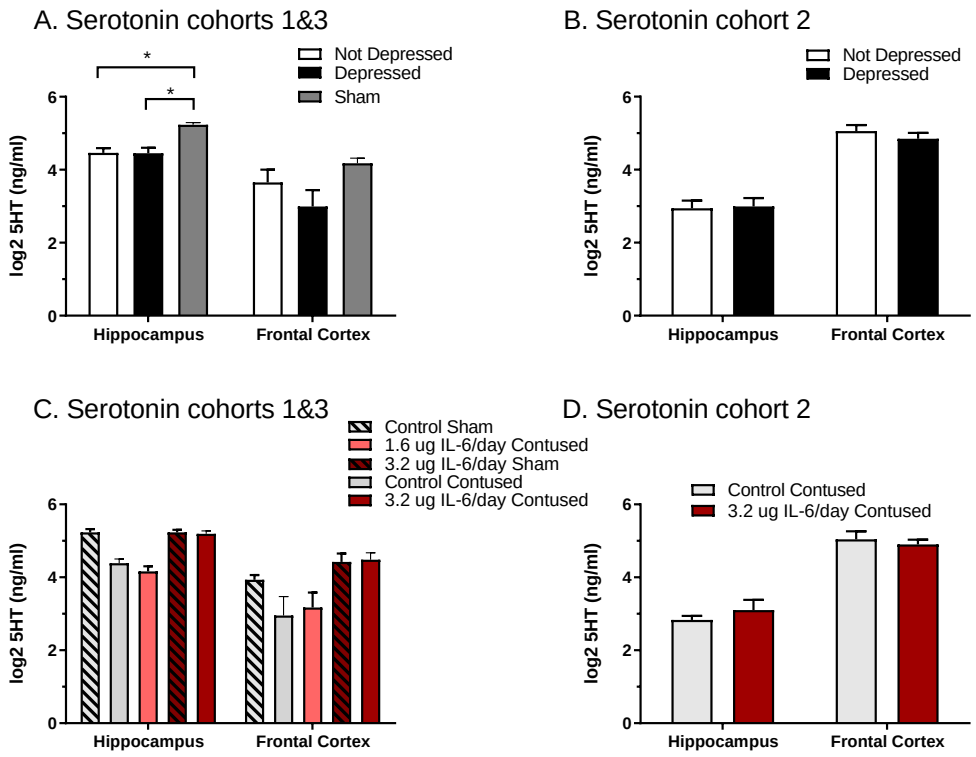

Figure 22. Serotonin expression in depression and IL-6 pre-treatment groups.
There was an effect of surgery on serotonin levels in the hippocampus in cohorts 1&3 (A). No other changes in serotonin were observed. *p<0.05

(Brakel et al., 2019; Luedtke et al., 2014; Maldonado-Bouchard et al., 2016), which corresponds with the incidence in the human population (Williams and Murray, 2015). A two-fold increase in the percent depressed represents a meaningful deviation from the expected incidence, and it indicates that IL-6 does play a role in establishing a predisposition for depression.

As previously discussed, associations between elevated IL-6 before a stressor and depression following the stressor have been observed in both humans and animals (Gimeno et al., 2009; Hodes et al., 2014). In large populations, IL-6 has been predictive

of present and future depression (Baune et al., 2012; Gimeno et al., 2009; Khandaker et al., 2014; Zalli et al., 2016). In a 6-year study in the Netherlands, Lamers et al. found that baseline IL-6 levels were positively correlated with recurring depression in previously depressed patients, but not with onset of major depression in previously healthy individuals (Lamers et al., 2019). Another study evaluated depression symptoms and inflammation in middle-aged twins (Huang et al., 2019). They found that IL-6 levels at the first testing timepoint predicted depression symptoms at follow-up seven years later (Huang et al., 2019). These studies indicate that IL-6 may be a meaningful predictor of future depression.

The existing literature, combined with the results presented in this chapter, also indicates that IL-6 alone, or combined with a stressor, is capable of producing depression. Hodes et al. (2014) administered IL-6 to mice before mild social defeat stress, which increased their vulnerability to depression after the test. Similarly, Rizzo et al. (2012) noted elevated depression-like behavior in mice that were centrally administered IL-6. Further, these effects were reversed with the addition of an IL-6 monoclonal antibody, showing that it was, indeed, the exogenous IL-6 administration that caused the observed behavioral changes (Rizzo et al., 2012).

While IL-6 activation before a stressor is sufficient to increase susceptibility to depression, it does not necessarily explain the increased incidence of depression after SCI specifically. The proportion of people with elevated cytokines is likely the same in people with SCI as exposed to any other stressor. Indeed, even a subset of people without injury or infection have cytokine levels comparable to those of people with chronic SCI (Davies

et al., 2007). It is possible that injury-induced IL-6 elevations may pre-dispose SCI patients to develop depression with subsequent (physical or non-physical) stressors. In the acute phase of SCI, when pro-inflammatory cytokines are upregulated, people with SCI experince significantly more stressors than members of the uninjured population (Arango-Lasprilla et al., 2011; Fann et al., 2011; Migliorini et al., 2009). Everything from navigating daily tasks, to socializing or maintaining economic stability introduces greater stress for a person with SCI than the average healthy person. This increased stress also extends into the chronic phase of injury, when is could synergize with infection-induced inflammation. The intriguing possibility that heightened IL-6 increases susceptibility to depression, when paired with a stressor post injury, needs further investigation in the animal model.

At the supraspinal level, both depression and IL-6 were associated with reduced BDNF levels in the brain. This finding is in keeping with extant literature (Jehn et al., 2015; Mondelli et al., 2011.; Shirayama et al., 2002). BDNF has been closely associated with depression: elevating it or it's receptor, TrkB, reduces depression-like behaviors (Quesseveur et al., 2013; Shirayama et al., 2002). Rat models of chronic stress, which result in depression-like behavior, exhibit elevated IL-6 and BDNF (You et al., 2011), Interestingly, there is also negative relationship between IL-6 production and BDNF in human patients. Jehn et al. (2015) found that IL-6 predicted BDNF levels in cancer patients, and that IL-6 and BDNF were strongly associated with depression and cognition. Further, patients experiencing first-time psychosis exhibit higher levels of inflammatory

cytokines (including IL-6 and TNF-α) and lower BDNF, which are predicted by recent psychosocial stressors (Mondelli et al., 2011).

Strangely, in the current experiment, we did not see an effect of depression on serotonin. This is unusual, because decreased serotonin is commonly associated with depression, and because we observed a reduction in serotonin in the frontal cortex in the previous experiment (Chapter III). It is possible that the stratification of the groups simply obscured the results (there is a trend for the depressed group to have lower serotonin in the frontal cortex), but it is also possible that other factors influenced serotonin production instead. The majority of research investigating the effect of spinal cord injury on serotonin primarily involves the serotonergic projections necessary for motor movement. Our results suggest that spinal cord injury *per se* causes reductions in supraspinal serotonin levels, which may be why further reductions associated with depression are harder to isolate.

Finally, while the incidence of depression was increased in the moderate-dose IL-6 group, it was not significantly increased in the high-dose IL-6 group. Similarly, IL-6 expression was elevated in the moderate-dose IL-6 group after administration but was not in the high-dose group. While it seems odd that the high-dose group had lower levels of serum IL-6 after administration than the moderate-dose group, it may be that the higher dose of IL-6 initiated anti-inflammatory reactions that reduced total IL-6 levels. IL-6 is a pleiotropic cytokine that influences a number of systems and mediates both pro- and anti-inflammatory mechanisms (Scheller et al., 2011). IL-6 levels concomitant with those caused by a strenuous workout can increase IL-10 and IL-1ra, both anti-inflammatory cytokines, in plasma in humans (Steensberg et al., 2003). IL-10 acts as a swift and potent

inhibitor of pro-inflammatory cytokine production, including IL-6 (Wang et al., 1994). If our introduction of large quantities of IL-6 quickly initiated production of IL-10, it is possible that IL-10 reduced IL-6 levels to baseline by the time serum was collected. This would explain the differences we see between the cytokine levels in the dose groups and their behavior: if IL-6 has to be within a certain range to elicit its negative effects, then the high dose group,which initiated counterintuitive suppression of IL-6, would be the least affected. Another, more direct, pathway by which high levels of IL-6 can regulate themselves is through the JAK/STAT pathway. This pathway ultimately leads to the modulation of transcription of many cytokines and other signaling molecules. One class of these signals, suppressor of cytokine signaling (SOCS) proteins, inhibits IL-6, completing a negative feedback loop. Excessive IL-6 should trigger SOCS activity, which would in turn reduce IL-6 activity (Alexander and Hilton, 2004). Future studies would benefit from a more detailed investigation of IL-6 activity, outlining its production throughout the body and its routes of activity. In the presence of tissue damage or infection, monocytes, macrophages, and microglia (in the CNS) are the primary producers of IL-6 (Tanaka et al., 2014). However, in IL-6 is also produced by fibroblasts, endothelial cells, and mesenchymal cells (Tanaka et al., 2014). Further, IL-6 action is mediated by method of activation, dose, and duration, all of which should be taken into account when investigating its impact on recovery outcomes after SCI.

Overall, though, IL-6's role in depression symptomology after SCI is clearly connected to a myriad of other processes. Understanding the role that IL-6 plays in the

susceptibility to stress may be crucial to our understanding depression that occurs after extreme stressors such as SCI.

CHAPTER V

STRESS BEFORE SCI AND THE GLUCOCORTICOID RESPONSE

Introduction

The previous chapters have shown that pre-existing inflammation, specifically elevated IL-6, increases the incidence of depression after SCI. Others have also shown that the IL-6 response is heightened in individuals susceptible to stress (Hodes et al., 2014), but the reason for this response is unknown. I propose that pre-existing inflammation works with the glucocorticoid system to move the HPA axis into a state of hyperexcitability. Typically, the HPA axis reduces stress response and inflammation via glucocorticoid (cortisol in humans, corticosterone in rats) release. In response to stress the hypothalamus produces corticotropin releasing hormone, which causes the pituitary gland to produce adrenocorticotropic hormone (ACTH). ACTH then stimulates the synthesis of cortisol in the adrenal glands, which release cortisol into circulation. Cortisol can have direct effects on the hypothalamus, as well as travel to targets throughout the body, where it binds to the glucocorticoid receptor (GR) in the cell cytoplasm. Once cortisol binds to it, the GR translocates into the cell nucleus, where it dimerizes and acts as a transcription factor for many regulatory genes, modulating neural input into the hypothalamus and reducing ACTH production. However, when the glucocorticoid receptor (GR) is dysfunctional, glucocorticoids fail to initiate negative feedback, and cortisol builds up, causing hyperactivity of the HPA axis (also referred to as glucocorticoid resistance), depression, and inflammation.

Glucocorticoid receptor dysfunction has been seen ubiquitously in humans experiencing depression and in rodents exhibiting depression-like symptoms (Pariante, 2017). In a popular test of GR functionality, the exogenous glucocorticoid dexamethasone is administered, and the time it takes for it to clear from circulation is measured. Depressed patients have significantly higher dexamethasone levels in these tests, and they take longer than healthy controls to metabolize and remove the glucocorticoid from circulation (Heuser et al., 1994; Pariante, 2017). These results have been linked to dysfunctions of the glucocorticoid receptor itself. For example, Bet et al. (2009) found that GR gene polymorphisms, combined with childhood adversity, put people at risk for developing depression as adults. Additionally, both people and rodents expressing lower levels of functional GR are more likely to develop depression (Bet et al., 2009; Chiba et al., 2012; Pariante, 2017; Ridder et al., 2005). Two isoforms of the GR have been identified in humans and rats: GRα and GRβ. The GRβ isoform is inactive and is more prevalent in depressed individuals, leading to decreased GR functionality (Carvalho et al., 2014; DuBois et al., 2013). These data indicate that GR expression and function are good indicators of depression susceptibility.

Glucocorticoid receptor sensitivity is easily altered by inflammation, stress, or illness, all characteristics of spinal cord injury (Maldonado Bouchard and Hook, 2014). Pro-inflammatory cytokines prompt cascades that interact with the GR and prevent it from initiating an anti-inflammatory feedback response. For example, IL-1 activates the p38 MAPK pathway, which prevents GR translocation into the cell nucleus via phosphorylation (Pariante et al., 1999), as shown in Figure 3. Another common

inflammatory marker, tumor necrosis factor alpha (TNF-α), activates the JNK pathway, which inhibits GR function via protein-protein interactions (Pace et al., 2007). Pre-existing inflammation may cause low-grade glucocorticoid resistance that magnifies when a physical or psychological stressor is introduced into the system.

Chronic stress also contributes to glucocorticoid resistance, which, in turn, promotes inflammation and increases susceptibility to illness (Cohen et al., 2012). People with SCI are already at a high risk for chronic stress and inflammation, making them more likely to experience disrupted GR function. Surprisingly, little research has been conducted on glucocorticoid receptor function after SCI, despite the fact that acute glucocorticoid steroid administration has been used as an emergency therapy for decades (Xu et al., 1998; Young et al., 1994). In a rat model of SCI, elevated GR expression has been seen for up to 1 day post-injury, but there have been no studies on the long-term effects of SCI on GR expression (Yan et al., 1999). It is likely that chronic stress and the immune activation from SCI work synergistically to inhibit GR function, as has been seen in other illness models.

Based on the connection between GR function and depression, and on the findings that pre-existing inflammation predicts depression, I hypothesize that animals that have high glucocorticoid levels after a mild stressor will be more likely to develop depression after SCI. I also hypothesize that animals that develop depression after SCI will have lower GR expression, higher pre-existing inflammation, and higher corticosterone levels than those that do not. To test these hypotheses, serum corticosterone levels were measured in rats before and after a stressor as well as after SCI. These rats were evaluated for

depression using an array of behavioral tests, and their inflammatory responses were assayed.

Methods and results

Rats were given a moderate contusion injury (n=30) and assessed with tests of depression-like behavior, motor function, and pain reactivity as described in Chapter II (General Methodology). To assess the basal stress response, half of the subjects underwent a mild stressor, in the form of a 10-min forced swim test, two days before injury. This mild stressor has been shown in other models to cause a temporary stress (glucocorticoid) response (Gong et al., 2015). Serum was collected 40 min after the forced swim stress, as described in General Methodology.

Serum corticosterone and cytokine levels were measured before and after injury; depression-like behavior and sensory reactivity were assessed before injury and for 30 days post-injury, as generally described in Figure 4. Thirty-two days after SCI, subjects were humanely euthanized with pentobarbital (100 mg/kg, i.p.), and fresh brain tissue was collected postmortem, as described in Chapter II (General Methodology). The frontal cortex and hippocampus were dissected out of the brain and snap frozen separately in liquid nitrogen. The frozen tissue was stored at -80°C until it was processed for rtPCR or ELISAs, as described in Chapter II (General Methodology).

Statistics were conducted as described previously (General Methodology). Three subjects were removed from analyses for BBB scores greater than or equal to 8 on Day 1 post-injury, and one was excluded for failure to achieve 75% sucrose preference by baseline testing.

Depression occurred in 50% of contused animals regardless of stressors

Hierarchical cluster analysis was conducted using the depression scores averaged across, the last two testing periods. Two animals had very high forced swim scores, driving any cluster that included the forced swim test. Additionally, the previous experiment showed that velocity tends to drive clustering toward recovery of function rather than depression behavior *per se*. Therefore, both the forced swim test and velocity were removed from the cluster analysis. Sucrose preference, social activity, and burrowing were used to determine the clusters. The resulting analysis produced two clusters, one of which exhibited distinct reductions in sucrose preference and social activity across the recovery period (sucrose preference: Day 19, $t(24)=3.46$, $p=0.002$; Day 29, $t(24)=4.40$, $p<0.001$; social activity: Day 19, $t(24)=3.51$, $p=0.002$; Day 29, $t(24)=2.57$, $p=0.017$, Fig. 23).

Forty-two percent of the group that underwent forced swim stress before SCI, and 58% the group that did not, displayed depression-like behaviors after SCI. T-tests revealed no differences between the stressed and unstressed groups on the depression tests following SCI, indicating that this mild stressor did not produce lasting behavioral effects.

Glucocorticoid response in depressed animals

Serum corticosterone levels increased 40 minutes after forced swim stress in the stressed group compared to the unstressed group, indicating that the forced swim was an effective mild stressor. Repeated measures tests revealed main effects of time and stress ($F(2,48)=6.41$, $p=0.003$; $F(1,24)=18.79$, $p<0.001$, respectively, Fig. 24). Corticosterone in

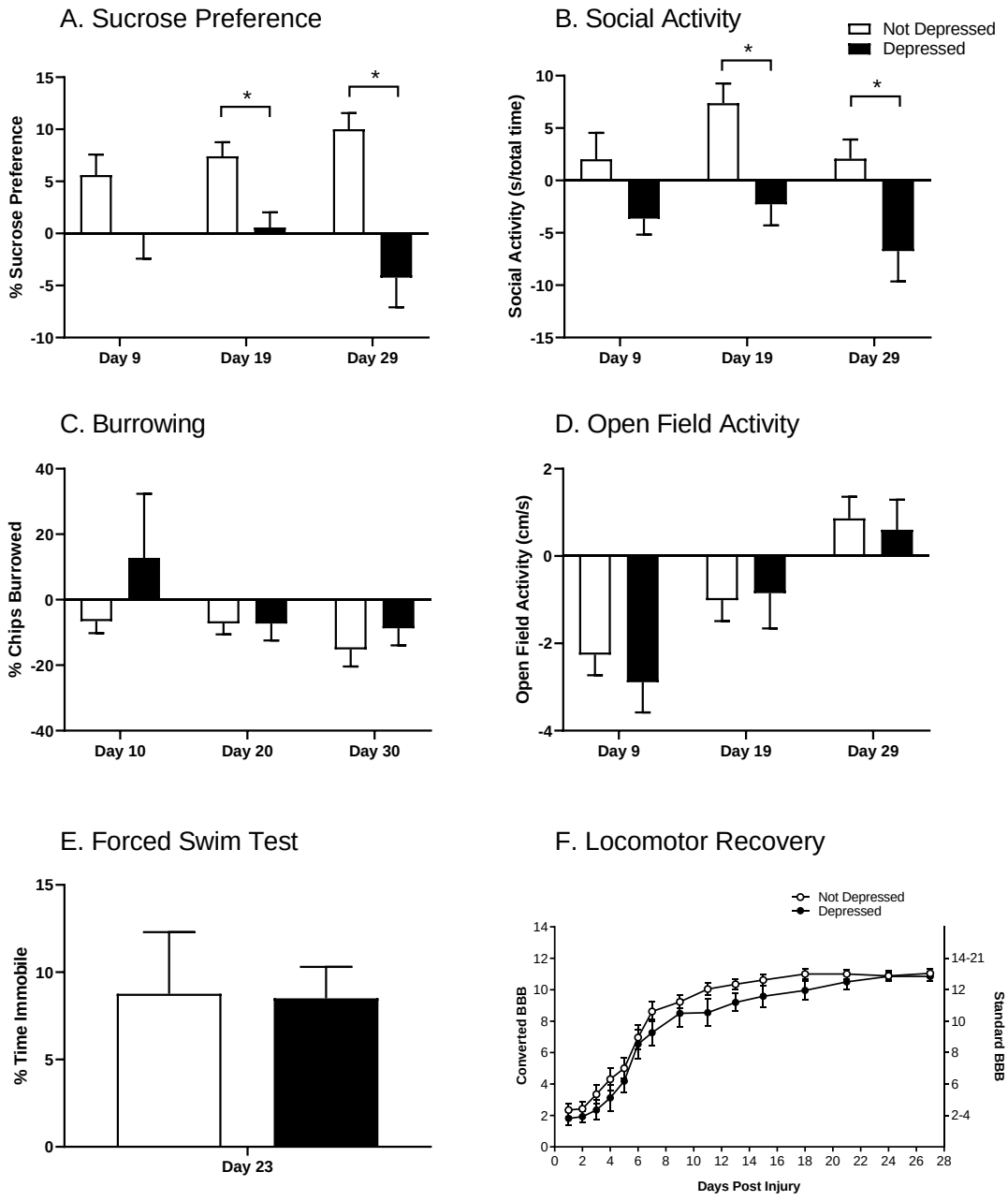

Figure 23. Depression behavior after SCI.
Throughout recovery, animals that clustered together as depressed had lower sucrose preference than not-depressed or sham animals (A). They also engaged in less social activity than the not-depressed group (B). There were no differences in burrowing, open field activity, time spent in the center of the open field arena, or locomotor recovery (C-F). All behaviors are presented as change-from-baseline. * $p<0.05$

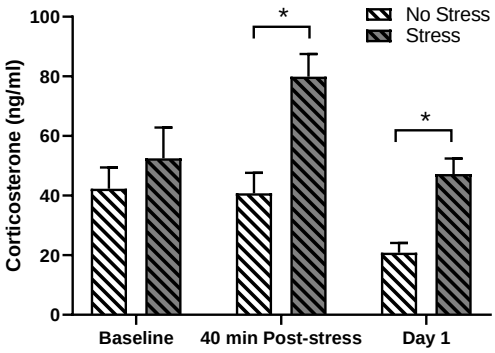

Figure 24. Stress increases corticosterone.
Rats' corticosterone levels increased 40 min. after a forced swim stress test, and rats stressed before injury released more corticosterone 1 day after SCI than unstressed subjects (A). * $p<0.05$

the stressed group was also elevated 24 hours after SC1 (3 days after forced swim stress) compared to the unstressed group.

Serum corticosterone and hippocampal glucocorticoid receptor (GR) expression levels were also compared between depressed and not-depressed animals. High post-stress corticosterone levels did not predict depression, but the animals that exhibited depression-like behavior had slightly higher corticosterone at baseline (*before* the forced swim stress), compared to the not-depressed group. They also had lower change-from-baseline corticosterone levels post-injury compared to the not depressed group ($t(24)=1.99$, $p=0.05$, Fig. 25), possibly indicating that they released less corticosterone following SCI than their not-depressed counterparts.

Contrary to my hypothesis, there were no statistically significant differences in glucocorticoid receptor mRNA expression between depressed groups, although the depressed group tended to have lower receptor expression across both the alpha and beta

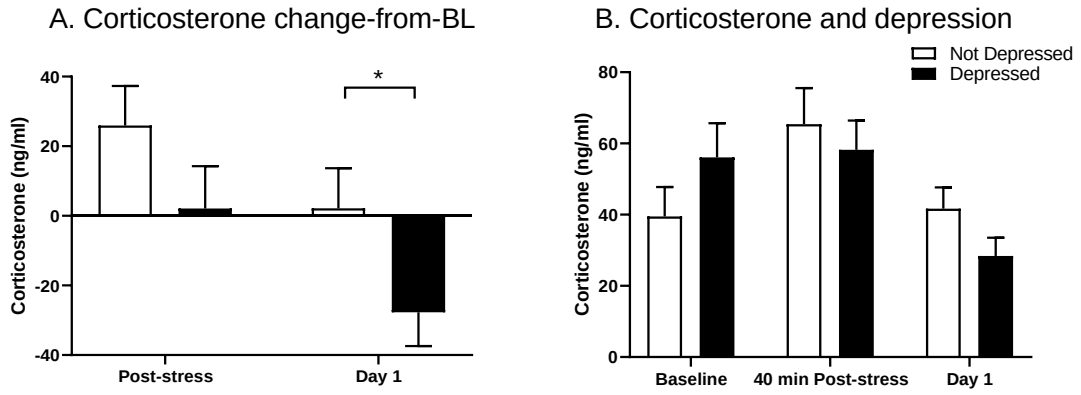

Figure 25. Corticosterone in depressed rats.
Depressed rats' serum corticosterone decreased on day 1 post injury compared to the not-depressed group (A). * $p<0.05$

receptor isoforms (Fig. 26). There was also no significant correlation between corticosterone levels and GR expression and no differences in the GRα:GRβ ratio between the depressed and not depressed groups.

Depressed rats exhibit less BDNF

BDNF and serotonin were assayed in the hippocampus and frontal cortex of depressed and not-depressed rats using ELISAs. The depressed subjects had lower hippocampal BDNF than the not-depressed subjects ($t(24)=2.16$, $p=0.041$, Fig. 27). There were no differences in serotonin levels in the hippocampus or frontal cortex. Interestingly, hippocampal BDNF positively correlated with hippocampal serotonin ($r=0.439$, $p=0.025$, Fig. 27). Hippocampal serotonin, in turn, positively correlated with baseline serum corticosterone ($r=0.413$, $p=0.036$, Fig. 27).

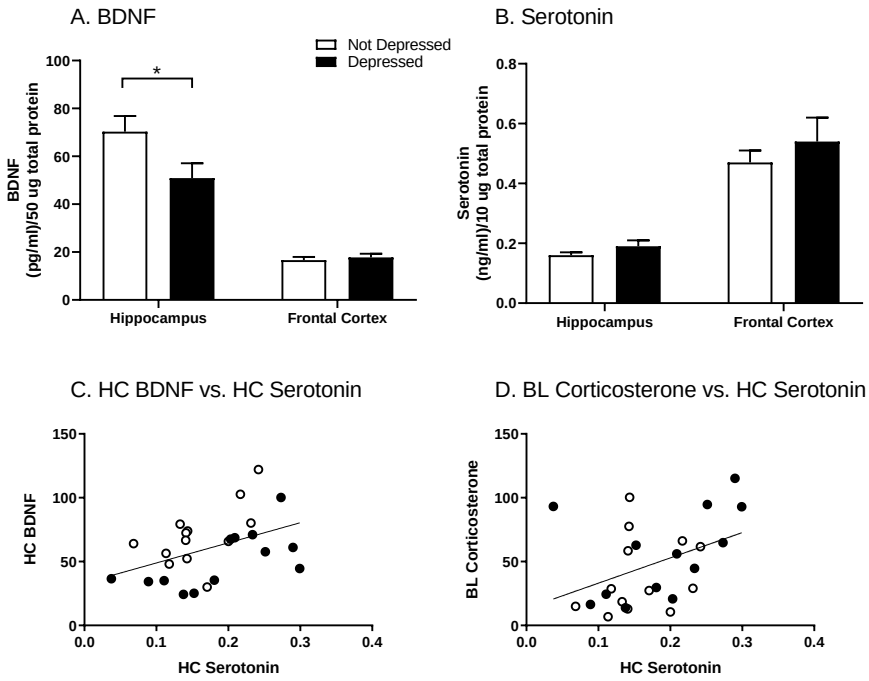

Figure 26. BDNF and serotonin in depressed rats.
BDNF levels were lower in the hippocampi of depressed animals compared to not depressed (A). No changes were seen in serotonin levels (B). Hippocampal BDNF was positively correlated with hippocampal serotonin (C). Additionally, hippocampal serotonin was positively correlation with baseline corticosterone (D). * $p<0.05$

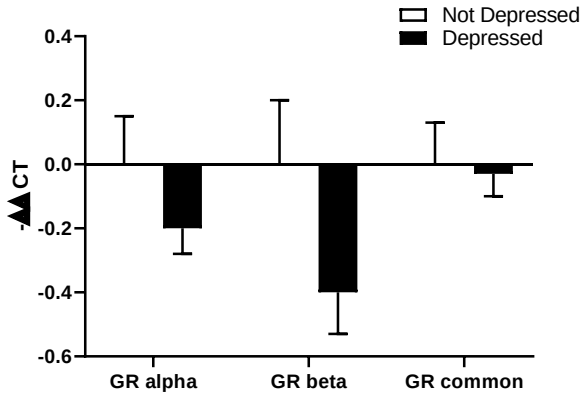

Figure 27. Glucocorticoid receptor expression in depressed rats.
Depressed rats exhibited slightly lower negative delta CT scores, a measure of mRNA levels, across the alpha and beta isoforms of the glucocorticoid receptor.

Elevated pro-inflammatory cytokines in depression

Cytokines levels from serum collected before stress, after stress, and 10 days after SCI were measured using 9-plex magnetic bead assays (RECYTMAG-65K, EMD Millipore). Depressed subjects displayed elevated IL-6, IL-1α, IL-1β, IL-10, and IL-4 *before* injury ($t(24)= -2.51$, $p=0.019$; $t(24)= -2.23$, $p=0.035$; $t(24)= -2.02$, $p=0.05$; $t(24)= -2.77$, $p=0.011$; $t(24)= -2.19$, $p=0.04$, Fig. 28), replicating our previous experiments, but there were no differences between the groups by ten days post-injury. Repeated measures tests showed a significant effect of time for all cytokines, but, intriguingly, only IL-1α and TNF-α were elevated in response to SCI at ten days post-injury (Fig. 28B, D). Additionally, TNF-α levels from Day 10 negatively correlated with glucocorticoid receptor expression at the end of the experiment (Fig. 29). Linear regression showed that elevated TNF-α predicted decreased glucocorticoid receptor expression ($r^2=0.324$, $p=0.043$).

Sensory reactivity

Pain and sensory reactivity tests were conducted for four weeks following SCI. There was a trend for at-level pain, measured with the girdle test, to increase three weeks following injury, but it did not differ between depressed and not-depressed groups (Fig. 30). There were also no differences between groups on latency to flick their tails away from a hot light or reactivity to Von Frey filaments, but there was an effect of time: both groups increased motor reactivity to mechanical stimulation across time, responding to

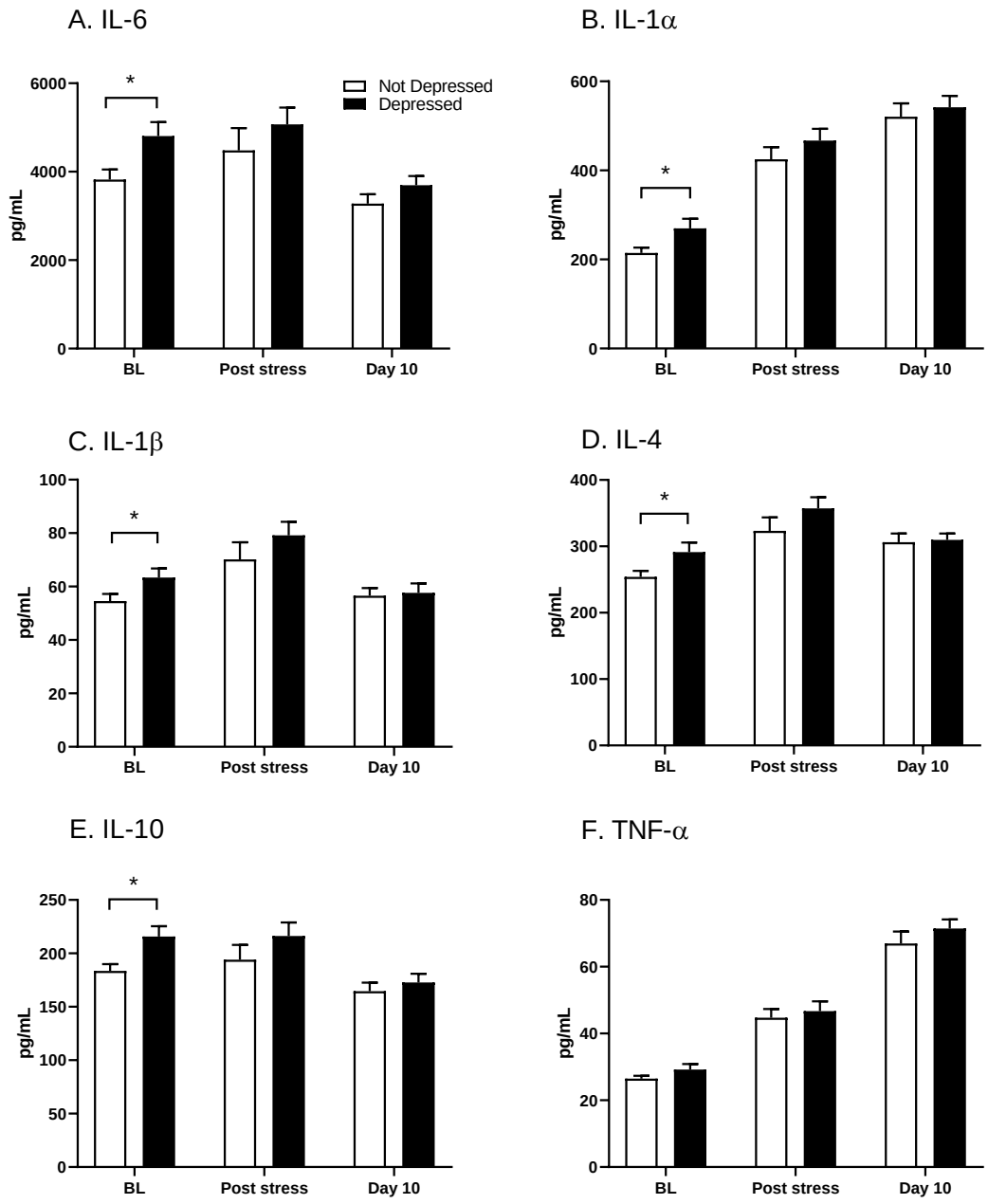

Figure 28. Depressed subjects exhibit higher inflammation before SCI.
Serum cytokines were elevated in depressed subjects at baseline (BL), *before* SCI and the development of depression-like behaviors (A-E) TNF-α and IL-1α were also elevated in response to injury (B, F). * $p<0.05$

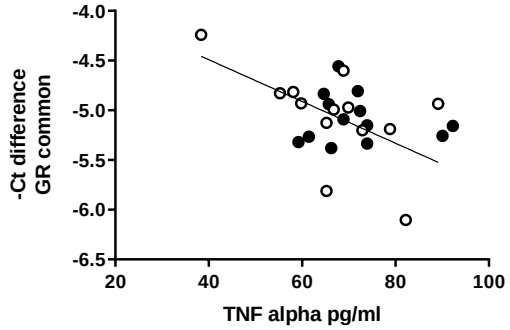

Figure 29. TNF correlates with glucocorticoid expression.
Serum TNF-α at 10 days post-injury negatively correlated with hippocampal glucocorticoid receptor expression. R^2=0.324, p=0.043

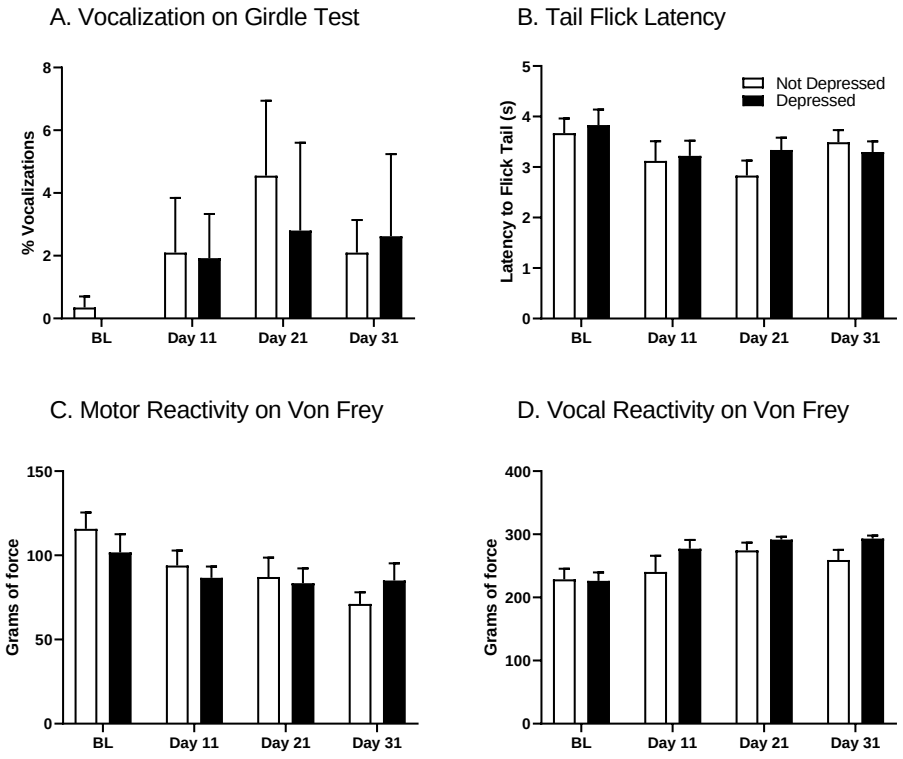

Figure 30. Sensory reactivity in depressed rats.
Serum TNF-α at 10 days post-injury negatively correlated with hippocampal glucocorticoid receptor expression. R^2=0.324, p=0.043

lower force stimulations, but vocal reactivity decreased ($F(3,72)=4.8$, $p=0.004$; $F(3,72)=7.7$, $p=0.001$, respectively, Fig. 30).

Discussion

Contrary to my hypothesis, higher corticosterone did not predict future depression, indicating that the immediate stress response (or at least corticosterone release) is not an indicator of susceptibility to depression. However, the 10-minute forced swim stress successfully elevated corticosterone levels, and the stressed group had elevated corticosterone compared to the unstressed group at least one day after SCI (3 days after the stress test). Nonetheless, the distribution of depression was similar across the stressed and unstressed groups.

In fact, a high number of the subjects in this experiment were susceptible to depression. Nearly 50% of all subjects clustered in the depressed group, exhibiting signs of anhedonia (reduced sucrose preference and social activity). This is a larger percentage than we have seen in most of our depression studies (Brakel et al., 2019; Luedtke et al., 2014), but it is not unprecedented in this model of depression after SCI. Using the same hierarchical cluster model of depression analysis, Maldonado et al. (2016) found three behavioral phenotypes (healthy, depressed, and depressed/anxious) after SCI, and the two depressed groups made up 57% of the total population. Similarly, Farrell et al. (2019) found that 58% of their rats developed a depression phenotype after SCI. Those rats were female, however, which may influence the depression incidence. Women are more likely to develop depression in the human population, and unpublished data from our own lab has shown sex differences affect the development of depression in our rat model of SCI.

What is apparent in the current study is that the higher incidence of depression was not due to pre-injury stress. The incidence of depression was actually slightly lower in the group exposed to stress, with 43% of the group displaying depression-like behavior, compared with 54% in the unstressed group. This indicates that the stressor was either not large enough to work synergistically with the SCI, or that it may have even been somewhat protective. There is some evidence that cortisol pre-treatment may protect against negative affect after stress test in healthy humans (Het and Wolf, 2007), but the majority of research shows that stressors and elevated glucocorticoids contribute to depression in humans and animals (Bet et al., 2009; Chiba et al., 2012; Fleshner et al., 1995; Malisch et al., 2009; Zhao et al., 2008). The two-hit hypothesis of psychiatric illnesses posits that an adverse event during development prepares (genetically, physiologically, and/or psychologically) an individual to be susceptible to a later stressor in adulthood. This hypothesis has been widely applied to schizophrenia and moderately investigated in regard to depression. Early adversity in people or rodents predisposes to develop depression after another stressor later in life. This experiment applied the two-hit hypothesis to adult stress, but we found that a mild stressor did not produce a meaningful impact even when combined with SCI. Stressors in adulthood may also predispose individuals to developing depression after a second, stronger "hit," but this hypothesis will need to be more completely explored.

Intriguingly, depressed subjects produced less corticosterone on the day after injury, compared to their baseline production (before stress), than the not-depressed subjects. The significance of this change-from-baseline measurement was partially driven by slightly higher corticosterone production from depressed subjects at baseline and

slightly lower production at Day 1 (Fig. 25B). Nevertheless, it is interesting, because depression is often associated with *elevated* corticosterone production. However, spinal cord injury has also been shown to disrupt the diurnal glucocorticoid cycle, which may be affecting our results (Gaudet et al., 2018). This combination of depression-related corticosterone elevations and SCI-associated disruptions likely accounts for the variability across days in the glucocorticoid levels in these subjects. Depression is generally paired with elevated glucocorticoid levels, in both humans and animals. Higher cortisol levels have been detected in currently depressed patients and in patients who have relapsed after anti-depressant treatment (Appelhof et al., 2006; Keller et al., 2017). In rodents, administration of corticosterone induces depression-like behaviors, and mice genetically bred for higher corticosterone are more likely to exhibit depression-like behaviors (Malisch et al., 2009; Zhao et al., 2008). It is clear that corticosterone is associated with the onset of depression; it is also possible that elevated baseline corticosterone levels could predict susceptibility to depression, independent of pre-injury stress.

In addition to slightly elevated baseline corticosterone in the depressed subjects, we also saw slightly lowered glucocorticoid receptor (GR) expression. While the differences between the depressed and not-depressed groups were not statistically significant, they do align with what others have reported. Human studies tend to find lowered GR in depressed patients, though some have found no differences (Pariante and Miller, 2001). In animal models, researchers have seen a causal link between decreased GR and depression-like behavior. Mice bred to express low GR are more susceptible to stress tests (Ridder et al., 2005), and rats subjected to chronic restraint stress exhibit more

depressive behaviors and much lower GR production (Chiba et al., 2012). Additionally, people experiencing depression exhibit a lower ratio of the active α form of the GR to the inactive β form, which may account for their glucocorticoid resistance. We did not see differences in α:β ratios among our depressed and not-depressed animals. It is possible that greater distinction of the α:β ratios were obscured by the many cell types in the hippocampus homogenate from which the GR was measured. GR expression has been repeatedly demonstrated in neurons, microglia, and astrocytes, but it seems that the isoform ratios in all of these cell types have not been explored (Liposits and Bohn, 1993; Tanaka et al., 1997; Unemura et al., 2012). Higher α:β ratios have been seen in circulating monocytes of depressed patients with elevated inflammation, so it is likely that individual cell types in the brain would also render more specific results (Carvalho et al., 2014). Sorting neurons, microglia, and astrocytes before extracting GR RNA may produce clearer and more meaningful results in the future.

Interestingly, post-injury TNF-α expression negatively correlated with overall GR expression after SCI. TNF-α is an inflammatory cytokine involved in the acute inflammatory response and also responsible for activating multiple pathways that suppress the GR during glucocorticoid resistance. This cytokine not only elevates with depression and SCI (Liu et al., 2012; Wang et al., 2002), but administration of exogenous glucocorticoids reduces TNF-α expression after SCI (Xu et al., 1998). As discussed in the Chapter I (Fig. 3), TNF-α activates NF-κB, which inhibits the GR, resulting in higher cytokine expression and further activation of the stress response. The correlation between high TNF-α and low GR in our subjects suggests that inflammation is, indeed, impacting

GR response. This has also been seen in human depression studies, where TNF mRNA expression negatively correlates with GR-α mRNA expression in depressed patients (Carvalho et al., 2014). Looking at our cytokine data, in this experiment, TNF-α was one of the few inflammatory factors that was elevated both after stress and after SCI. Because it is related to SCI, stress, and depression, it could very well be a key player in SCI-induced depression. Farrell and Houle (2019) investigated this relationship in female rats. They administered a TNF inhibitor to rats after SCI in an effort to reduce TNF activity and thus reduce depression (Farrell and Houle, 2019). Counterintuitively, animals that received the inhibitor exhibited more depression-like behaviors (Farrell and Houle, 2019). Given that there are known differences between male and female inflammatory responses (Klein and Flanagan, 2016; Rainville et al., 2018), it is possible that TNF's impact on depression would be different in male rats. This molecule, and its interaction with the HPA axis after SCI, is worth exploring further in male and female models of SCI.

In addition to TNF-α, a number of other serum cytokines were elevated at baseline in the depressed subjects, replicating the results from Chapter III. As seen previously, we demonstrated that IL-6 and IL-1α were elevated at baseline in the depressed subjects. This replication underscores the assertion that inflammation before SCI predicts subsequent depression-like behaviors. As mentioned previously, others have found that pre-existing inflammation predicts susceptibility to depression following a stressor (Gimeno et al., 2009; Hodes et al., 2014; Zalli et al., 2016). Our data strongly support the published studies. Contrary to our expectations, though, we did not see that SCI greatly increased inflammation. While TNF-α and IL-1α did increase e after SCI, neither IL-6 nor IL-1β

were elevated at 10 days post-SCI. As discussed in Chapter 3, it is likely that SCI-induced inflammation has subsided by Day 10 post injury and earlier assessment of cytokine levels may be more informative of an interactive relationship between inflammation and depression after SCI. However, it is also possible that, while IL-6 is predictive of depression susceptibility, TNF-α and IL-1α are more associated with the inflammatory response after SCI. Investigating this relationship may provide insight into inflammation's interaction with depression after SCI.

Finally, as in previous experiments, BDNF was decreased in the brains of depressed subjects. As discussed in Chapter IV, this finding supports our phenotypic categorization of depression based on behavior and is consistent with the extant literature. Lower BDNF has been observed in the hippocampus and plasma of both rodents and humans exhibiting signs of depression (Jehn et al., 2015; Martinowich et al., 2007; Mondelli et al., 2011; Quesseveur et al., 2013; Shimizu et al., 2003). Interestingly, BDNF was also positively correlated with serotonin, even though serotonin itself was not associated with depression in this experiment. There is an abundance of evidence showing that BDNF and serotonin reciprocally regulate each other (Martinowich and Lu, 2008). Among other things, BDNF promotes the development and function of serotonergic neurons. In turn, serotonin upregulation promotes CREB phosphorylation, which positively regulates BDNF transcription (Martinowich and Lu, 2008). BDNF and serotonin are both also controlled by inflammation through the kynurenine pathway (discussed in Chapter I), which diverts tryptophan away from serotonin production and produces neurotoxic quinolinic acid (Fig. 2). However, BDNF's regulation of serotonin

predominantly relies on neurogenesis, so BDNF's influence on serotonin availability would likely have a delayed biological effect. Thus, BDNF-related differences in serotonin expression may be a chronic, rather than acute, feature of depression, and changes in serotonin may emerge at later timepoints than those assessed in the current study.

Overall, mild stress before SCI did not increase susceptibility to depression or help identify subjects that were prone to developing depression, but baseline inflammation and corticosterone were again elevated in depressed subjects. Pre-existing individual differences clearly play a role in susceptibility to the development of depression after SCI.

CHAPTER VI

DISCUSSION AND CONCLUSIONS

In these experiments, I used behavioral and molecular measures to determine the effects of inflammation on depression after spinal cord injury. Commensurate with previous studies from our laboratory, I found that SCI increases the incidence of depression relative to non-SCI rats (Brakel et al., 2019). Additionally, across experiments, I saw that depression after SCI was associated with elevated serum cytokines, including IL-6 and IL-1α, before injury. With further investigation, I found that exogenous IL-6, administered before SCI, predisposed individuals to develop depression after SCI. How IL-6 predisposes a subject to depression remains unknown. I proposed that pre-existing inflammation may lead to glucocorticoid resistance, leading to dysregulation of the immune response after injury, and subsequently to depression. Refuting this hypothesis, I found that subjects with pre-existing inflammation did not have higher corticosterone levels post-injury, although they did have elevated depression-like behaviors. In the following sections, I will discuss the effects of inflammation on the development of depression and show that a physical and emotional stressor such as SCI puts patients at greater risk for impaired psychological health.

Depression in a rodent model of SCI

The results from each of my experiments showed that a subset of rats developed depression-like behaviors after SCI, further validating our model of SCI-induced depression. Hierarchical clustering based on an array of depression tests is a unique

method of determining depression in a rat model of SCI, because it takes into account many aspects of behavior. In my experiments, we identified depression through increased immobility on the forced swim test and decreased social activity, sucrose preference, burrowing, and open field activity. Hierarchical clustering identified approximately 33-50% of untreated animals as depressed, replicating our previous studies of depression in a rat model of SCI (Brakel et al., 2019; Luedtke et al., 2014; Maldonado-Bouchard et al., 2016).

Notably, we saw markers of depression not only at the behavioral level, but also at the neurotransmitter level. Supraspinal serotonin was downregulated in the depressed subjects in the first experiment (Chapter III), and BDNF was downregulated in the depressed subjects in the other two experiments (Chapters IV and V). These data are meaningful for validation of this rodent model of SCI because they are concomitant with changes found in the human population. Decreased BDNF and serotonin are associated with major depressive disorder (Fakhoury, 2016; Kambeitz and Howes, 2015; Mahar et al., 2014; Martinowich and Lu, 2008; Martinowich et al., 2007; Mondelli et al., 2011; Shimizu et al., 2003). Serotonin is one of the most commonly known neurotransmitters associated with depression. Its depletion results in depressive symptoms, and some of the most effective antidepressants, selective serotonin reuptake inhibitors (SSRIs), work by inhibiting pre-synaptic reuptake of serotonin, increasing its availability for post-synaptic receptors. BDNF, which can modulate serotonin, is also downregulated in depression. When BDNF levels are low, neurogenesis is inhibited, resulting in a loss of brain plasticity (Kempermann and Kronenberg, 2003; Taliaz et al., 2010). Supporting this, in Chapter III,

we found reduced hippocampal neurogenesis in the depressed subjects. These data are similar to those of Taliaz et al. (2010), who saw both increased depression-like behaviors and decreased neurogenesis in mice with knocked down hippocampal BDNF. Together, these data highlight the importance of molecular measures in any animal model of depression.

Importantly, the behavioral and molecular changes that develop in the depressed subset of subjects after SCI are independent of locomotor recovery, lesion size, or pain. Across experiments, we saw no differences in locomotor recovery in the depressed and not-depressed groups. Spinal cord lesion analysis in Chapter III confirmed that there were no differences in injury severity between the depression groups. Each of the experiments also showed that depression in this rat model was not associated with the development of neuropathic pain. However, as shown in Chapter IV, locomotor function and pain response are altered considerably in SCI compared to sham injury. By assessing depression in SCI subjects only, rather than comparing them with shams, we control for these injury-specific, confounding factors. In doing so, we have created a model that is capable of differentiating individual differences among animals after injury.

This is meaningful because, despite the fact that all SCI rats have injuries that impact their performance on behavioral tasks, a subset of these subjects develop definable depression-like behaviors. This indicates that there is something unique about these animals' responses to spinal cord injury, which lead to depression. I originally thought that diverse inflammatory responses to SCI led to the differences in depression behavior after injury. That hypothesis was proved incorrect when cytokine profiles of the SCI rats

showed little chronic inflammation after SCI and when inflammatory markers in the depressed subjects were not consistently upregulated during that time. Interestingly, the inflammatory data instead indicated that the immune profile before injury may play a crucial role in the predisposition towards depression.

Inflammation and depression

There is growing evidence that inflammation is associated with depression. In animal models of depression, IL-6, IL-1β, and TNF-α are elevated after four weeks of chronic mild stress (Zhu et al., 2006). Administration of antibodies against inflammatory molecules also decrease both inflammation and stress-induced depression behaviors (Hodes et al., 2014). Similarly, in humans, individuals with depression tend to have higher inflammatory cytokines and immune responses (Lindqvist et al., 2009; Liu et al., 2012; Maes et al., 1997), and administration of inflammatory cytokines increases the incidence of depression in the clinical population. For example, interferon-alpha therapeutically administered to cancer patients significantly increases their depression symptoms (Capuron et al., 2002; Capuron and Ravaud, 1999). Conversely, IL-6 neutralizing antibodies have entered clinical trials for treatment of depression in patients with inflammatory diseases, and they have successfully attenuated depressive symptoms (Sun et al., 2017).

Surprisingly, our data indicate that inflammation following SCI may not be the proximate cause of depression. In Chapter III we saw that inflammation was elevated in the depressed subjects compared to the not-depressed after SCI, but this finding was not replicated in Chapter V. Additionally, we saw little inflammation in response to SCI itself.

In both Chapters III and V, few inflammatory cytokines or chemokines rose higher than their baselines by 10 days post-injury. If chronic SCI-induced inflammation were responsible for the maintenance of depression, the subjects from these experiments would have had greater cytokine production after injury compared to their baseline levels. The literature also shows mixed results regarding inflammation after rodent SCI. Popovich et al. (1999) evaluated inflammatory cells surrounding the lesion site for weeks after SCI, and they found activated microglia and macrophages up to 28 days post injury. They did not, however, evaluate peripheral inflammation. In fact, surprisingly few people have measured peripheral cytokine levels after rodent SCI. Yang et al. (2018) observed that peripheral IL-6 levels rose quickly after SCI and returned to baseline levels by 7 days post-injury. Similarly, Maldonado et al. (2016) found elevated serum cytokines at Day 1 post-injury in depressed SCI rodents, compared to not-depressed and uninjured animals, but there was little effect of SCI *per se*. By Day 24 post-injury, nearly all serum cytokine levels had returned to baseline levels (Maldonado-Bouchard et al., 2016). These data indicate that measuring serum cytokines at Day 10 post-injury in our studies was likely too late to see meaningful differences associated with SCI. Immediate changes in inflammation are most obvious in the hours to days after SCI.

To further explore the relationship between SCI-induced inflammation and depression, we could evaluate depression and inflammation at a much later period (6 months or more). In humans, inflammation and depression are seen months to years after injury (Arango-Lasprilla et al., 2011; Davies et al., 2007; Dryden et al., 2005). Chronic SCI may alter an individual's physiology through long-term changes such as gut dysbiosis

or neurogenesis. These changes may take months to fully manifest themselves, and they may explain why depression and inflammation are harder to detect in the weeks following SCI. Additionally, our method of detecting depression using hierarchical clustering may give inconsistent clusters. Eliminating one behavior from the clustering model can sometimes drastically change the results. This is not ideal, because, occasionally, baseline differences or outliers within one behavioral test necessitate that test's removal from the cluster analysis. This is one of the reasons that molecular confirmations, such as BDNF, serotonin, and cytokine levels are important; they provide secondary levels of validation to the behavioral phenotype. However, in the future, it may be more convenient to create a quantitative measure of depression. This is possible through a discriminant function analysis, in which each behavioral test is weighted and inserted into an equation (Luedtke et al., 2014). Each animal would then receive a quantitative "depression score" that could be used as continuous measurement in statistical analyses. A depression "cut off" would be established based on the scores of animals already identified as depressed. This would provide a more quantitative and objective method of determining depression, possibly emulating depression questionnaires administered in clinical settings.

Even without a quantitative measure, I was able to successfully identify depression after SCI and associate it with molecular markers of depression, including markers of inflammation before injury. While others have identified depression after SCI, they have yet to associate it with baseline molecular predictors for susceptibility.

Inflammation's influence before injury

In my experiments I found that inflammation before injury was predictive of susceptibility to depression after injury. In experiments 1 and 3 (Chapters III and V), subjects that developed depression-like behaviors a month after injury also had elevated IL-6 levels prior to injury. As discussed previously in those chapters, these results are commensurate with findings in other animal models of depression and the clinical population. Hodes et al. (2014) reported greater IL-6 produced, before a stressor, from the leukocytes of animals that were later susceptible to repeated social stress. In humans, an increasingly large number of studies have reported a longitudinal relationship between elevated IL-6 and the subsequent development of depression, even when IL-6 was not related to depression cross-sectionally (Baune et al., 2012; Gimeno et al., 2009; Khandaker et al., 2014; Lamers et al., 2019; Zalli et al., 2016). Further, pre-existing IL-6 levels can predict the success of antidepressant treatments. IL-6 levels are higher in depressed patients resistant to SSRI treatment, but electroconvulsive therapy is more effective in treatment resistant patients with higher IL-6 levels before the therapy (Kruse et al., 2018; Yoshimura et al., 2009).

The pre-existing IL-6 differences observed in these studies are interesting, given that they were observed in genetically similar rats raised, housed, and handled under similar conditions. Humans experience incredible genetic and environmental diversity, which easily explain the predictive inflammatory differences seen in many large, longitudinal studies. The reduced diversity in my rat studies indicates that even small differences my have a larger inflammatory impact. Firstly, the Sprague Dawley strain is

outbred, so there will be some genetic differences among individuals. Some of the subjects may have had polymorphisms that contribute to glucocorticoid receptor dysfunction or other inflammatory processes. Additionally, prenatal and neonatal stressors may have impacted adult inflammation, causing the baseline differences we observed. Neonatal stressors can impact inflammation in adulthood (Kentner et al., 2010). For example, in rodents, maternal separation has been shown to increase susceptibility to parasitic and viral infections, disrupt hippocampal development, and activate inflammatory microglia (Barreau et al., 2006; Meagher et al., 2010; Roque et al., 2016). Any inadvertent stressors introduced during development, before we received the rats or conducted our experiments, could have caused long-lasting immune changes and elevated serum cytokines.

In Chapter IV, I saw that administering IL-6 systemically before injury resulted in a greater incidence of depression-like behaviors after injury. This finding was especially important because it shows that inflammation before injury in not just associated with depression, it may be causal to depression. Hodes et al. (2014) also saw a causal effect of peripheral IL-6 on the development of depression-like behaviors. Systemic administration of an IL-6 monoclonal antibody significantly reduced depression-like behaviors in mice exposed to repeated social defeat stress (Hodes et al., 2014). Further, IL-6 knockout mice display greater resilience to both social defeat stress and witnessed social defeat than wildtype mice (Hodes et al., 2014). Together, these experiments provide compelling evidence that inflammation, and specifically IL-6, plays an important role in predisposing individuals to the development of depression.

Mechanisms of pre-existing inflammation-associated depression

There has been much research investigating the role that inflammation may play in causing depression, but there has been little regarding the mechanisms of inflammation-related *predisposition* to depression. The strongest theory for the relationship between depression and pre-existing inflammation is that the inflammation works on the HPA axis to make it vulnerable to major stressors. IL-6 is related to many processes, and it is not surprising that it has also been found to impact the HPA axis and the glucocorticoid system, both of which are driving forces behind depression. IL-6 has been associated with reduced expression of glucocorticoid-inducible genes and reduced hippocampal volume in depressed patients, compared to healthy controls (Frodl et al., 2012). IL-1α, another inflammatory cytokine upregulated both before and after SCI in our depressed subjects, also influences the glucocorticoid receptor (GR) (Pariante et al., 1999). It reduces GR translocation into the cell nucleus and promotes cytosolic GR binding, preventing the GR from enacting its typical transcriptional effects (Pariante et al., 1999). TNF-α also modulates the HPA axis via multiple pathways that inhibit GR function. Through one of its pathways, TNF-α activates NF-κB, which binds to the GR in the cell nucleus. NF-κB then inhibits the GR's trascriptional activity, preventing the transcription of genes necessary for downregulating the stress response (Pace et al., 2007). If an inflammatory mileu such as this were present, it could generate a perpetually hyperactive HPA axis. This would create glucocorticoid resistance (a common trait in depressed patients). When a major stressor, such as SCI, was introduced to the system, it would then initiate a major

glucocorticoid response, which, if unchecked, would produce a number of negative psychological effects, including depression.

Clinical significance

Knowing that basal inflammation can affect mood after SCI is useful for controlled, animal studies, but it is difficult to apply directly to SCI patients. Very rarely do we have serum or plasma samples from patients before they receive an injury. However, pre-existing inflammation may explain why depression incidence is much higher among the SCI population. We and others have repeatedly shown that inflammation before a stressor predicts depression after a stressor. It may be that elevated inflammation from the SCI combines with elevated physical and emotional stressors after the SCI to create the conditions needed to induce depression. If this is the case, clinicians may be able to identify high-risk patients through their serum cytokine levels immediately post-injury and intervene before exposure to ongoing stressors.

Additionally, clinicians may be able to use knowledge of pro-inflammatory profiles to influence their choices for antidepressant treatment. We already know patients with higher levels of circulating cytokines are more likely to be treatment-resistant (Maes et al., 1997). We also know that IL-6 and other cytokines are associated with resistance and responsiveness to specific antidepressants (Raison et al., 2013; Uher et al., 2014; Yoshimura et al., 2009). Given these findings, I would propose that we produce individualized therapies for SCI patients. For example, patients with high inflammation may be able to go on anti-inflammatory medications in conjunction with standard antidepressants.

Non-pharmaceutical methods of reducing inflammation are also available. Diet and exercise greatly influence inflammation, corticosterone, and BDNF in the brain and may help reduce susceptibility to depression. Maintaining a healthy diet and sufficient exercise are challenges that the SCI community already face. Many people with SCI have to be careful about what they eat and when, because they experience autonomic dysreflexia or gut disruptions. Exercise can also be a challenge, even for patients with mild SCI. Paralysis, low blood pressure, and autonomic dysfunction are obvious disruptions to normal exercise, but patients with even a mild SCI may find that pain or exhaustion inhibits their ability to exercise. Any antidepressant regimen for people with SCI would do well to incorporate many methods of inflammation reduction, including healthy lifestyle changes.

Conclusion

Here, I proposed that inflammation during, and even before, SCI increases the likelihood of developing depression after SCI. I found that this was, indeed, the case, and that inflammation is also related to glucocorticoid receptor function and hippocampal changes in the brain. Elevated inflammatory cytokines are a predictor of future depression and should be a target for depression therapy. Overall, these experiments underscore the importance of a deeper understanding of the relationship between stress, inflammation, and how pre-existing differences should influence psychiatric health care.

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