

**PSYCHOLOGICAL WELL-BEING AND SPINAL CORD INJURY RECOVERY:
A TWO-WAY STREET?**

A Dissertation

by

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ABSTRACT

Spinal cord injury (SCI) leads to increased anxiety and depression in as many as 60% of patients. Yet despite extensive clinical research focused on understanding the variables influencing psychological well-being following SCI, risk factors that decrease psychological well-being remain unclear. We hypothesized that excitation of the immune system, inherent to SCI, may contribute to the decrease in well-being.

We used a battery of established behavioral tests to assess depression and anxiety in contused rats and (1) characterized psychological well-being as a function of SCI severity, (2) examined peripheral (serum) and central (hippocampi and spinal cord) inflammation in relation to psychological well-being post SCI, and (3) explored whether social enrichment, as a modulator of psychological well-being, could improve overall recovery post SCI, by housing contused animals either alone, or with an injured or an intact cagemate.

Following SCI, the contused subjects showed one of three profiles: depression-like, depression- and anxiety-like, or no signs of decreased psychological well-being. Subjects exhibiting a purely depression-like profile showed higher levels of pro-inflammatory cytokines peripherally, whereas subjects exhibiting a depression- and anxiety-like profile showed higher levels of pro-inflammatory cytokines centrally (hippocampi and spinal cord). These changes in inflammation were not associated with injury severity; suggesting that the association between inflammation and the expression

of behaviors characteristic of decreased psychological well-being was not confounded by differential impairments in motor ability.

Social enrichment, in the form of group housing, did not improve psychological well-being post SCI. Depression- and anxiety-like signs were found in all group housing conditions. Unexpectedly, we found that the intact animals housed with contused subjects showed depression- and anxiety-like signs similar to those of contused subjects, indicating that their psychological well-being was affected by the presence of an injured cagemate. This is reminiscent of the caregiver effect in humans, specifically the manifestation of symptoms of depression in individuals who care for patients suffering with a chronic illness, such as SCI.

These experiments demonstrate that the depression and anxiety patients experience following spinal cord injury is not due solely to psychosocial factors, but may also, in part, result from increased immune activation following the injury.

DEDICATION

Ñuka ayllukunaman chinchasuyu kullasuyu kunaman, kayak kilkayta chayachipanik.

À ma famille, au nord et au sud.

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CHAPTER I

INTRODUCTION

The incidence of spinal cord injury (SCI) in the United States is reported at approximately 12,000 new cases per year, with a prevalence estimated at about 316,000 people in 2010 (National Spinal Cord Injury Statistical Center, 2011). This injury dramatically impacts the life of the affected individuals. Depending on the level and severity of the injury, SCI can result in partial or full paralysis, loss of bowel movement, loss of bladder and sexual function, and chronic pain. Moreover, the cost of treatment for a patient with SCI, in the first year post injury, can go up to \$985,774, and for the average patient, who experiences the injury at approximately 26 years of age, the lifetime cost of the injury is estimated to be around \$4,373,912 (National Spinal Cord Injury Statistical Center, 2011). Given the high costs associated with SCI, both physical and economic, it is important that we better understand the factors contributing to a successful recovery.

While recovery of lost physical function is a laudable goal, also important is improving the quality of life for people living with SCI. As many as 60% of spinal cord injured patients suffer from depression (Shin, Goo, Yu, Kim, & Yoon, 2012), anxiety (Post & van Leeuwen, 2012), and general decreased quality of life (Boakye, Leigh, & Skelly, 2012). In a longitudinal study of 1035 spinal cord injured patients, 21% of patients met the criteria for major depressive disorder at one year following injury, and

18% at five years (Hoffman, Bombardier, Graves, Kalpakjian, & Krause, 2011). Given the high percentage of spinal cord injured patients experiencing psychological symptoms, it is imperative that we better understand the mechanisms underlying decreased psychological well-being following SCI. Yet, while there is considerable research focused on restoration of physical function, there is a surprising lack of basic, empirical research on psychological well-being post SCI.

Extensive research has been conducted in the clinical setting to understand the factors influencing psychological well-being following SCI. Clinical studies suggest that predictive factors include individual traits such as resilience, optimism, and neuroticism, as well as psychosocial factors, such as quality of life, loss of independence, social isolation, and financial hardships (Boakye et al., 2012; Boekamp, Overholser, & Schubert, 1996; Bombardier, Richards, Krause, Tulskey, & Tate, 2004; Botticello, Chen, Cao, & Tulskey, 2011; Botticello, Chen, & Tulskey, 2012; Chan, Lee, & Lieh-Mak, 2000a; Chang, Wang, Jang, & Wang, 2012; Elliott et al., 1991; Geyh et al., 2013; Guilcher et al., 2012; Jensen et al., 2014; Khazaepour et al., 2014; Lohne & Severinsson, 2005; Peter, Rauch, Cieza, & Geyh, 2012; Post & van Leeuwen, 2012; Saunders, Krause, & Acuna, 2012; Schöenberg et al., 2012). Importantly, however, although all spinal cord injured patients experience these hardships, to varying extents, not all spinal cord injured patients report decreased psychological well-being. Studies repeatedly find that it is a sub-group of spinal cord injured patients who develop symptoms of depression or anxiety. For example, a 10-year longitudinal study of 87 people with SCI found that a only a subset of participants showed signs of depressions throughout the years, and the

common characteristics of this sub-group were unclear (Pollard & Kennedy, 2007). In fact, even when taking into account individual traits and psychosocial factors, psychological well-being following SCI cannot be clearly explained, or predicted.

A compelling proposal is that, in addition to psychosocial stressors, the activation of the immune system resulting from the SCI itself may be contributing to the development of depression and anxiety following injury. Support for this hypothesis is provided by both rodent and human studies (Maes, 1999; Miller, Haroon, Raison, & Felger, 2013; Smith, 1991; van West & Maes, 1999; Vogelzangs, Beekman, de Jonge, & Penninx, 2013). In animal models for example, stressed rats were found to show increased spleen and brain (hippocampus, hypothalamus, and cortex) mRNA levels of pro-inflammatory cytokines relative to non-stressed rats (You et al., 2011). Furthermore, in rodent models, central or systemic administration of pro-inflammatory cytokines produces sickness behavior characterized by behavioral and physiological changes resembling depression (Anisman, Merali, Poulter, & Hayley, 2005), which can be alleviated with anti-depressants (Merali, Brennan, Brau, & Anisman, 2003). A number of human studies have also found an association between elevated levels of pro-inflammatory cytokines and depression (Dowlati et al., 2010; Howren, Lamkin, & Suls, 2009; Liu, Ho, & Mak, 2012) as well as anxiety (Miller et al., 2013; Pace & Heim, 2011). Given that pro-inflammatory cytokines are up-regulated following SCI (Yip & Malaspina, 2012), it is possible that immune system activation plays a pivotal role in the development of depression and anxiety following the trauma.

In this dissertation, we present experiments designed to test the hypothesis that changes in cytokine expression following SCI contribute to the development of an anxiety- and depression-like profile in contused rodents. The use of a rodent model of SCI is ideal for this purpose, as confounding psychosocial factors found in post SCI human studies, such as financial difficulties and loss of independence, are avoided. In the animal model, therefore, we are able to focus on the specific effects that pathophysiological changes inherent to SCI have on psychological well-being. Furthermore, the rodent model of SCI allows us to study the effects of SCI on psychological well-being both at a behavioral level and a molecular level, examining cytokine expression in the periphery as well as in the brain post SCI.

In this introduction, we first review the definition of psychological well-being, depression and anxiety, and how immune changes are known to affect psychological well-being. Next, we discuss mechanisms through which psychological well-being is in turn thought to affect the immune system. Then, we review physiological changes following SCI which could potentially alter processes involved in psychological well-being, with particular attention to the role of inflammation following the trauma, the focus of this dissertation. Finally, we outline the specific aims of this dissertation.

Defining psychological well-being

Psychological well-being, for the purposes of this dissertation, is defined as the absence of depression and anxiety. According to the DSM-IV-TR, the diagnostic criteria for major depressive disorder include “*depressed mood and/or loss of interest or*

pleasure in life activities for at least 2 weeks” and at least five of a list of symptoms including fatigue, inability to concentrate, suicidal ideation, depressed mood, inability to experience pleasure, noticeable psychomotor changes, changes in appetite or low self-esteem (Center for Substance Abuse Treatment, 2008). Our operational definition for depression-like behavior in rats is intended to mirror most of these symptoms and is taken from a previous study in the laboratory. It includes anhedonia, psychomotor retardation, lack of interest in social interactions, and helplessness (Luedtke et al., 2014). According to the APA, “*Anxiety is an emotion characterized by feelings of tension, worried thoughts and physical changes like increased blood pressure*” (American Psychological Association, 2014). Our operational definition for anxiety-like behavior in rats includes active anxiety, which is characterized by fight or flight behavior, and passive anxiety, which is characterized by withdrawal behavior (Steimer, 2011). Throughout this dissertation, we use tests designed to assess these behaviors in order to evaluate psychological well-being.

At a molecular level, major depressive disorder is commonly defined as a monoamine dysregulation disorder in which brain levels of serotonin, norepinephrine, and dopamine levels are altered (Saveanu & Nemeroff, 2012). Post-mortem studies of suicide victims and positron emission tomography studies of depressive patients have shown that serotonin depletion in the central nervous system is strongly associated with the development of depression (Drevets et al., 1999; Mann et al., 1996). Furthermore, the most common and generally successful treatment for depression is the administration of selective serotonin-reuptake inhibitors (SSRIs), which increase the availability of

serotonin at synaptic clefts. However, it is now accepted that decreased serotonin levels do not explain depressive symptoms in all patients (aan het Rot, Mathew, & Charney, 2009). Indeed, research also suggests a role for norepinephrine in depression (Ressler & Nemeroff, 2001). Patients suffering from major depressive disorder exhibit altered levels of cerebrospinal fluid norepinephrine (Saveanu & Nemeroff, 2012), and post-mortem studies have found increased expression of adrenergic receptors in suicide victims (Sastre, Guimon, & Garcia-Sevilla, 2001). In addition, some studies suggest that patients who do not respond well to the common SSRIs can benefit from antidepressants that act both on serotonin and norepinephrine (Horst & Preskorn, 1998). Furthermore, dopamine is also considered to be involved in the pathophysiology of clinical depression. For example, a positron emission tomography study comparing dopamine binding potential in the striatum of depressed and healthy participants found that depressed individuals show reduced dopamine binding potential (Meyer et al., 2001). It has been suggested that treatments promoting dopamine neurotransmission may provide positive results in patients who do not respond well to SSRI treatment (Saveanu & Nemeroff, 2012). Finally, Nutt (2008) has suggested that these three neurotransmitters may be responsible for different symptoms of depression. Low levels of serotonin may explain anxiety; low levels of norepinephrine may be linked to reduced energy and interest in pleasurable activities; and low levels of dopamine may explain decreased motivation and interest in rewards (Nutt, 2008). These studies underscore the complexity of the mechanisms underlying the development and maintenance of depression.

Over the last decades, in addition to serotonin, norepinephrine, and dopamine, other signaling molecules implicated in the pathophysiology of depression have been identified, such as the brain-derived neurotrophic factor (BDNF) and the nerve growth factor (NGF) (Banerjee, Ghosh, Ghosh, Bhattacharyya, & Mondal, 2013; de Azevedo Cardoso et al., 2014; Satomura et al., 2011). Both central and peripheral decreases in BDNF levels have been found in depressed individuals. Banerjee et al. (2013) compared postmortem levels of BDNF and NGF in the hippocampi of depressed individuals who committed suicide to those of individuals who died from natural causes. The results were conclusive: ELISAs, Western Blots and rt-PCR all indicated that BDNF and NGF levels were decreased in the suicide victims relative to the comparison group. Likewise, serum levels of BDNF and NGF have been found to be decreased in individuals suffering from major depressive disorder, relative to healthy individuals (de Azevedo Cardoso et al., 2014; Satomura et al., 2011). It is important to note, however, that these studies were conducted with individuals who were already depressed at the time of analyses. Given these experimental constraints, we cannot determine with certainty whether these neurotrophic factors are a cause, or a consequence of depression. Nonetheless, their association with depression is clear, and these data provide evidence that molecules beyond the monoamines are involved in major depressive disorder. Similarly, another family of molecules implicated in depression, and for which perhaps even stronger evidence exists, are the cytokines and inflammation-related molecules. The following sections review the evidence implicating cytokines in the development of

both depression and anxiety, and discuss the implications of an inflammatory-mediated sub-type of depression in the context of SCI.

The role of the immune system in psychological well-being

There is a growing body of literature, both human and animal, pointing to a role for chronic peripheral and central inflammation in major depressive disorders, anxiety, and even schizophrenia (Anisman, 2009; Black & Berman, 1999; Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Felger & Lotrich, 2013; Maes, 1999; Muller, 2013; Pace & Miller, 2009; Rosenblat, Cha, Mansur, & McIntyre, 2014; Uddin et al., 2011; Wager & Gianaros, 2014; Walker, Kavelaars, Heijnen, & Dantzer, 2014; Zunszain, Hepgul, & Pariante, 2012). The literature on the association between inflammation and depression specifically, includes both experimental and clinical evidence. For example, a double-blind, crossover study of 20 healthy male participants found that immune activating agents such as endotoxins can induce transient depressive-like symptoms (Reichenberg et al., 2001). Likewise in rats, administration of LPS or other immune agents has consistently been shown to induce depression-like signs, such as increased learned helplessness (Makino, Kitano, Komiyama, & Takasuna, 2000) and anhedonia (De La Garza, 2005) in the hours following the treatment. Furthermore, in the clinical setting, immunotherapy has been repeatedly been found to induce depression symptoms in cancer patients (Dantzer et al., 2008). Up to 30 to 50% of patients undergoing interleukin-2 (IL-2) or interferon- α (IFN- α) therapy for cancer or hepatitis C develop marked symptoms of depression and anxiety (Capuron, Ravaud, Miller, & Dantzer, 2004; Musselman et al., 2001). An emerging literature also highlights an association

between inflammation and anxiety (Salim, Chugh, & Asghar, 2012). For example, in a mouse model of premature aging, high levels of anxiety have been found to be associated with chronic inflammation (Vida, Gonzalez, & Fuente, 2014). Activation of the innate immune system, particularly increases in brain interferon- 1β (IL- 1β) levels, augment anxiety-like behaviors in the open-field test and the elevated plus maze test (Chiu et al., 2014). Similar evidence exists in humans. For example, Pitsavos et al. (2006) demonstrated, in a cross-sectional study, that high levels of C-reactive protein levels (a biomarker of inflammation) are positively correlated with anxiety in healthy individuals. These findings highlight the critical relationship between altered immune function and symptoms of depression and anxiety.

Although the exact mechanism through which inflammation may affect mood is not known, several potential mechanisms have been suggested. Three major pathophysiological mechanisms are the cytokine hypothesis, the hypothalamic-pituitary-adrenal (HPA) axis hypothesis, and the microglial hypothesis.

The cytokine hypothesis

The cytokine hypothesis proposes that cytokines are directly involved in inducing depression. Cytokines such as IL-2, IFN- γ , tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6) are known to increase the enzymatic activity of indolamine 2,3-dioxygenase (IDO) (Maes, Leonard, Myint, Kubera, & Verkerk, 2011; Roman, Kreiner, & Nalepa, 2013). This enzyme, in turn, acts as a catalyst for the conversion of tryptophan, the precursor for serotonin, into kynurenine, whereby levels of tryptophan

available are decreased, and, consequently too, the levels of serotonin that can be produced (Capuron et al., 2003; Capuron et al., 2001). By promoting the conversion of tryptophan into kynurenine, therefore, cytokines contribute to serotonin depletion and, if not to the development of depression, at the very least to its maintenance. In addition, cytokines can also facilitate the breakdown of existing serotonin. Cytokines such as IL-6 and TNF- α , assist in the breakdown of 5-Hydroxytryptophan (5-HT) into 5-Hydroxyindoleacetic acid (5-HIAA). In sum, cytokines not only reduce the amount of serotonin produced, but can also increase the amount of serotonin degraded, thus contributing to the development of depression through two pathways.

Cytokines can also alter the synthesis of catecholamines. For example, pro-inflammatory cytokines such as IFN- γ generally trigger the synthesis of 5,6,7,8-tetrahydrobiopterin (BH4), a co-factor for enzymes (phenylalanine and tyrosine) that synthesize the precursors for dopamine, norepinephrine, and epinephrine. However, IFN- γ also prompts macrophages to release reactive oxygen species, which degrade BH4. Therefore, some have suggested that chronic inflammation may cause a decreased availability of BH4, which would result in lower levels of catecholamine production in the long-term (Sperner-Unterweger, Kohl, & Fuchs, 2014). This could explain altered epinephrine and norepinephrine levels in patients with some forms of depression.

The hypothalamic-pituitary-adrenal axis hypothesis

Whereas the cytokine hypothesis supposes a direct relationship between cytokines and serotonin levels, the HPA axis hypothesis suggests that increased levels of

cytokines such as interleukin-1 (IL-1), IL-6, TNF- α and IFN- γ contribute to the development of depression via activation of the HPA axis and the alteration of glucocorticoid levels. It is known that individuals with depression exhibit increased activation of the HPA axis as well as high levels of circulating glucocorticoids (Holsboer, 2000; Pariante & Miller, 2001). Given the usual anti-inflammatory role of glucocorticoids, this initially appears contradictory to the argument that inflammation is involved in depression. In reality, however, the two are supporting evidence for the role of cytokines in depression.

Cytokines activate the HPA axis, and this results in increased production of glucocorticoids (Rosenblat et al., 2014). Chronically increased levels of cytokines can, in this manner, induce glucocorticoid resistance, which is characterized by the decreased expression of glucocorticoid receptors, as well as decreased sensitivity and function of the glucocorticoid receptors (unable to translocate from the cytoplasm to the nucleus) (Pace, Hu, & Miller, 2007). This decrease in glucocorticoid receptor function in turn prevents glucocorticoids from exerting their anti-inflammatory effects (Pace & Miller, 2009). Therefore, as a result of chronically elevated circulating glucocorticoids and the glucocorticoid resistance that ensues, cytokine levels continue to increase, thus further activating the HPA axis and increasing levels of glucocorticoids produced by the adrenal glands, perpetuating this cycle. Furthermore, glucocorticoids normally function in a negative neuroendocrine feedback loop; high plasma levels signal the hypothalamus to stop producing corticotrophic-releasing hormone (CRH), and signal the anterior pituitary to stop producing adrenocorticotropic hormone (ACTH). With glucocorticoid

resistance, this normal feedback inhibition is lost, and glucocorticoids continue to be produced. The increase in glucocorticoids due to glucocorticoid resistance not only activates the stress response (Maldonado Bouchard & Hook, 2014) but also plays a direct role in depression. Specifically, glucocorticoids can increase levels of the hepatic enzyme tryptophan 2,3-dioxygenase (TDO). TDO is a powerful catabolic enzyme of tryptophan and, similar to IDO, increased levels of TDO lead to tryptophan depletion, which results in decreased levels of serotonin production, and increased levels of tryptophan catabolites (Maes et al., 2011; Wolf, 1974). Chronic HPA axis activation can therefore both result from depression, and contribute to perpetuating depression (Figure 1).

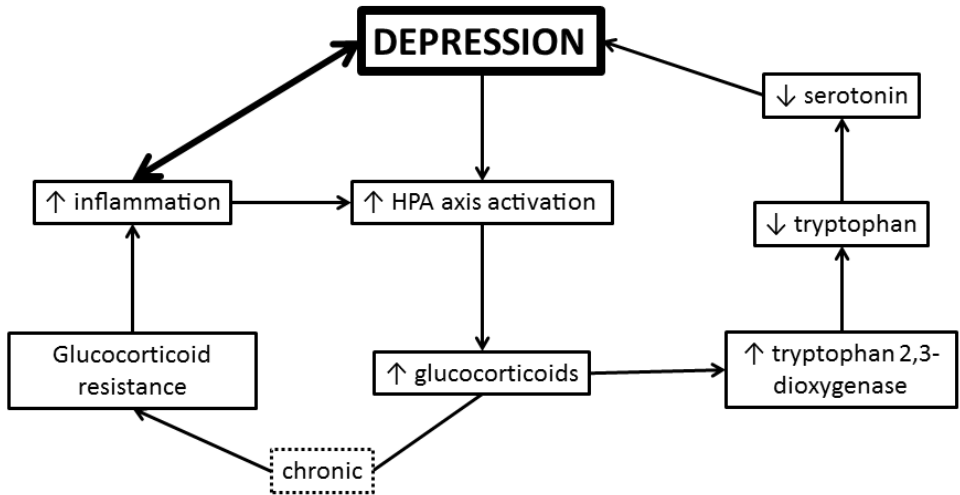


Figure 1- Suggested mechanism for role of inflammation and HPA axis in depression.

The microglial hypothesis

Finally, cytokines are also thought to be involved in depression indirectly, via microglial activation. Studies have recently uncovered the role of quiescent microglia in the phagocytic engulfment of neuronal synapses in healthy brains (Tremblay & Majewska, 2011). Using electron microscopy and two-photon in vivo imaging, Tremblay et al. (2011) demonstrated that even normal visual stimuli influence microglial behavior in the visual cortex of mice, increasing phagocytic engulfment of synapses. These types of changes in plasticity are accentuated following an innate immune response. Cytokines, such as TNF- α and IL-1 β activate microglia, resulting in increased phagocytic engulfment of synapses (Park & Bowers, 2010). This is thought to lead to synaptic pruning, a regulatory process whereby neurons and synapses are eliminated to the benefit of fewer, more efficient connections (Miyamoto, Wake, Moorhouse, & Nabekura, 2013). When inflammation becomes chronic, however, microglia remain activated, constantly producing cytokines. The constant production of pro-inflammatory cytokines can inhibit neurogenesis, lead to neurotoxicity, and further microglial activation (Ekdahl, 2012; Rosenblat et al., 2014; Stertz, Magalhaes, & Kapczinski, 2013). Consistent with this hypothesis, increases in microglial activation has been found in the prefrontal cortex of individuals with bipolar disorder (Stertz et al., 2013). Furthermore, individuals with major depressive disorder frequently show decreased hippocampal volume (Bremner et al., 2000; Campbell, Marriott, Nahmias, & MacQueen, 2004; Duman, Malberg, & Thome, 1999; Frodl et al., 2002; MacQueen et al., 2003; Mervaala et al., 2000; Paradise, Naismith, Norrie, Graeber, & Hickie, 2012; Stockmeier

et al., 2004). Comparing the hippocampal volume of individuals with major depressive disorder versus normal controls, Stockmeier et al. (2004) found that depressed individuals showed decreased hippocampal volume overall, with up to a 35% increase in glial cells in the same region. This decrease in hippocampal volume is suggested to be due to a decrease in synaptic connections, which, possibly, is due to increased microglial activation. Through chronic activation of microglia, cytokines can establish a vicious circle, in which continued cytokine production results in further activation of microglia, in addition to affecting the HPA axis, and serotonin levels, as seen in the two previous sections.

The effect of psychological well-being on the immune system

Not only can immune system dysregulation play an important role in modulating psychological well-being, but psychological well-being can in turn influence the immune system. A variable frequently used to study this relationship is chronic social stress. It is well established that chronic social stress can lead to a blunted glucocorticoid response and an elevated immune response, both in rodents and humans (Avitsur, Powell, Padgett, & Sheridan, 2009; Miller et al., 2008; Powell et al., 2013). In mice, repeated social defeat has been shown to lead to glucocorticoid resistance and an increased inflammatory response (Avitsur et al., 2009; Avitsur, Stark, & Sheridan, 2001; Powell et al., 2013; Stark et al., 2001). In humans, chronic social stress in the form of low socioeconomic status has been shown to be associated with the greater presence of immature pro-inflammatory monocyte transcriptome in peripheral blood mononuclear cells (Powell et al., 2013). Examining fear of terrorism as a form of social stress,

Melamed et al. (2004) also found that fear was positively correlated with elevated C-reactive protein levels in employed Israeli adult women. These data suggest that psychological well-being can significantly modulate physiological function.

Another variable used to study low psychological well-being is social isolation (in animal models) or perceived loneliness (in human studies). In rats, social isolation can increase levels of the adrenocorticotrophic hormone, as well as alter the immune response. For example, Krügel et al (2014) found that single-housed rats, relative to rats housed in groups of four, showed increased levels of adrenocorticotrophic hormone, TNF- α , interleukin-4 (IL-4) and interleukin-10 (IL-10). In turn, in a large study of 5000 ageing adults (50 years or older), Shankar et al. (2011) found that perceived social isolation was positively correlated with high levels of C-reactive protein. Similarly, Jaremka and colleagues (2013) have found that both healthy adults and posttreatment breast-cancer survivors who showed greater perceived loneliness as per the UCLA Loneliness Scale exhibited a stronger immune response following an acute stressor (delivering a five minute speech in front of an audience) than participants who showed lower perceived loneliness. Specifically, the peripheral blood mononuclear cells of the participants who exhibited high perceived loneliness showed greater production of TNF- α and IL-6 when stimulated with lipopolysaccharide in culture, than did the cells of participants who showed lower perceived loneliness (Jaremka et al., 2013). Moreover, Cole et al. (2007) have shown that individuals who experience high levels of loneliness have an up-regulation of genes involved in pro-inflammatory processes, and a down-regulation of genes involved in anti-inflammatory processes, such as the genes

responsible for the glucocorticoid response. The results from the studies reviewed above provide supporting evidence for the proposal that inflammation can be both a cause and a consequence of decreased psychological well-being.

Changes post spinal cord injury relevant to psychological well-being

As seen in the sections above, decreased psychological well-being can be attributed to various processes, three of which are changes in cytokine levels, alteration of the HPA axis, and changes in microglia. Importantly, SCI also affects these three systems. In the following paragraphs, we review how these three systems are impacted following a SCI. We combine the microglia and immune system changes as these two are closely interrelated in SCI.

Spinal cord injury causes changes to microglia and the immune system

Following the injury, microglia, macrophages, and astrocytes are recruited to the site of trauma. Glial cells (microglia, astrocytes, and oligodendrocytes) are vital to neuronal function (Carson, Thrash, & Walter, 2006), and one of their key roles is maintaining homeostasis (Barres, 2008). They regulate the levels of excitatory amino acids, such as glutamate, and the pH of the synaptic interstitial fluid (Carson et al., 2006; Sofroniew & Vinters, 2010). Microglia, specifically, are the macrophages of the central nervous system and serve as resident immune cells (Carson et al., 2006; Hulsebosch, 2002) and they are beneficial in the regulation of homeostasis after SCI. Pathologically, however, they also release pro-inflammatory cytokines and this may adversely affect psychological well-being. Pro-inflammatory cytokine genes and other inflammation-

related genes are up-regulated within hours of injury (Dumont et al., 2001; Yip & Malaspina, 2012). For example, in the hours following injury, the interleukin-6 gene, the *TNF* gene, and the interleukin-1 β gene are up-regulated (Hayashi, Ueyama, Nemoto, Tamaki, & Senba, 2000; Pan et al., 2002). The levels of cytokines such as IL-6, IL-1 β , IL-1 α , and TNF α have also been shown to increase in contused subjects, peaking at approximately 6 hours post SCI, and returning to near basal levels by 24 hours post SCI (Stammers, Liu, & Kwon, 2012; Yang et al., 2005). In addition to this early phase of inflammation, long-term cellular inflammation also occurs post SCI. Using flow cytometry in the injured spinal cord, Beck et al. (2010) demonstrated that starting at 14 days post injury, T cells, microglia and neutrophils increase in the spinal cord, and are detectable up to 180 days post injury (Beck et al., 2010). Thus, SCI results in increased immune activity at the level of the spinal cord both immediately after the injury, and in the days and even weeks following it.

These changes in pro-inflammatory cytokine expression are not restricted to the injury site. Elevated serum levels of pro-inflammatory cytokines have also been found in patients with chronic SCI (Davies, Hayes, & Dekaban, 2007; Hayes et al., 2002), as well as in animal models of SCI (Hasturk et al., 2009). As early as the 1990s, SCI researchers suggested that cytokine dysregulations may be involved in SCI secondary complications such as pressure ulcers (Segal, 1992). Later, studies examined cytokine levels in spinal cord injured patients suffering from pressure ulcers, and found that actually, all spinal cord injured patients, regardless of whether they experienced secondary complications or not, exhibited higher levels of cytokines (IL-6, IL-2R and intracellular adhesion

molecular 1) than healthy controls (Segal, Gonzales, Yousefi, Jamshidipour, & Brunnemann, 1997). Since then, additional studies have examined more closely the changes in cytokine levels post SCI in patients without medical complications. Hayes et al. (2002) found that spinal cord injured patients (n=24) showed increased levels of IL-2 and TNF- α compared to healthy controls (n=26), but no differences for levels of IL-4 or IL-10. Moreover, they found that 57% of the patients showed such increases, suggesting that it is a subset of spinal cord injured patients that shows chronic immune system activation post SCI (Hayes et al., 2002). Another recent cross-sectional study comparing 56 patients with SCI with 35 control participants, confirmed that even asymptomatic patients (no medical complications such as neuropathic pain, pressure ulcers or infections) displayed higher levels of IL-6, TNF- α , and IL-1ra than the non-injured control participants (Davies et al., 2007). These findings suggest that SCI itself, not the secondary complications, have an immune activation effect in the periphery.

Pro-inflammatory signals produced in the periphery can reach the brain via a number of routes: (1) active transport-they can be transported from the peripheral circulation to the brain via specific transporters; (2) leaky blood-brain barrier- at sites of fenestration or as a result of illness; (3) macrophage activation- activation of macrophages and endothelial cells lining the cerebral vasculature; (4) directly from the blood (Roman et al., 2013). To the best of our knowledge, changes in brain cytokines post SCI have not been studied. However, given the ruptured blood-brain barrier resulting from SCI, the various routes through which peripheral cytokines can reach the

central nervous system and the fact that peripheral cytokine levels are altered post SCI, it is likely that changes in these cytokines occur in the brain as well.

Spinal cord injury causes hypothalamic-pituitary-adrenal axis changes

A great deal is known about the effects of synthetic glucocorticoids on SCI, but less is known about how SCI affects endogenous glucocorticoids and the HPA axis. Nonetheless, some clinical and case studies have demonstrated that SCI can alter the HPA axis, both in the acute and the chronic phase of injury (Huang, Wang, Lee, & Lai, 1998; Lee et al., 2002; Lerch, Puga, Bloom, & Popovich, In Press). For example, Huang et al. (1998) assessed functioning of the HPA axis following SCI by comparing plasma levels of corticotrophin-releasing hormone and insulin-induced hypoglycemia in spinal cord injured patients versus healthy controls. Overall, spinal cord injured patients exhibited alterations of the HPA axis, such as decreased adrenocorticotrophic hormone and cortisol release following insulin-induced hypoglycemia and corticotropin-releasing hormone relative to healthy controls, thus indicating a blunting of the HPA axis. Long-term abnormal adrenocorticotropin secretion may cause mild adrenocortical atrophy and, thereby, a reduced cortisol response. Lee et al. (2002) also examined adrenal gland volume post SCI. Using computed tomographic imaging to conduct volumetric measurements of adrenal glands, they compared the adrenal glands of chronic spinal cord injured patients with impaired adrenal reserve, to those of healthy controls. The adrenal glands of the spinal cord injured patients, on average, were found to be larger than those of the healthy controls, even after controlling for patients' height and weight. Adrenal gland volume is known to increase with depression, becoming up to 70% larger

in patients with depression versus healthy individuals (Rubin, Phillips, Sadow, & McCracken, 1995), and it thought to be due to hyperactivity of the pituitary-adrenocortical axis. Given the enlarged adrenal glands observed in chronic spinal cord injured patients, it is possible that such hyperactivity also play a role in depression post SCI.

A few studies have also shown that changes in glucocorticoid expression specifically occur following SCI. Using a rodent model of SCI, Popovich et al. (2001) found that serum corticosterone levels increased within 24 hours following SCI and remained elevated for at least one month post injury. Importantly, sham subjects did not show this elevation (Lucin, Sanders, Jones, Malarkey, & Popovich, 2007; Popovich et al., 2001). Similar results have been found in humans. In a study of 54 spinal cord injured patients, urine cortisol and plasma adrenocorticotropin hormone levels were shown to be increased relative to non-injured patients, two weeks following injury (Cruse et al., 1993). It is thought that these changes in glucocorticoid expression post SCI may affect numerous cellular and physiological processes, such as apoptosis and inflammation (Lerch et al., In Press).

To the best of our knowledge, changes in brain glucocorticoid levels post SCI have not been studied. However, we know that the blood-brain barrier is ruptured as a result of SCI (Sharma, 2005; Whetstone, Hsu, Eisenberg, Werb, & Noble-Haeusslein, 2003), that glucocorticoids can cross the blood-brain barrier (Karssen, Meijer, & de Kloet, 2005), and that the level of urine glucocorticoids increase post SCI (Cruse et al.,

1993). It is, therefore, possible that brain levels of glucocorticoids are increased by SCI. Moreover, given that elevated serum glucocorticoids are maintained at least for a month following SCI, chronic elevation of glucocorticoids may also occur in the brain, which could result in the development of glucocorticoid resistance, sustained inflammation, and eventually, cytokine-induced depression.

Spinal cord injury causes changes in neurotransmitter and neurotrophic systems involved in psychological well-being

SCI also causes alterations in neurotransmitter and neurotrophic systems that have been implicated in psychological well-being. In the 1970s and 80s, several laboratories studied changes in the levels of serotonin, norepinephrine and dopamine post SCI, with contradictory results. Osterholm et al. (1972) assessed the levels of monoamines post SCI in cats, hypothesizing that these neurotransmitters could act as mediators in secondary damage following SCI. Using spectrofluorometric analyses, they observed that spinal cord norepinephrine levels increased within 30 minutes of SCI, began decreasing at 3 hours post, and were below baseline levels 24 hours post. Dopamine displayed an opposite pattern: spinal cord levels of dopamine decreased within 30 minutes, and remained so for 24 hours post SCI. No spinal cord serotonin level changes were observed (Osterholm & Mathews, 1972). However, since then, contradictory results have been reported. For example, Naftchi et al. (1974) found no changes in norepinephrine and increases in dopamine, and Rodriguez et al. (1977) observed increases in dopamine and norepinephrine above the lesion in spinal cord sections collected 7 days after a transection injury, and decreases in the same

neurotransmitters below the lesion. In the 90s, microdialysis studies resolved some of the contradictions of earlier studies by collecting data across time post injury. Levels of norepinephrine and serotonin were recorded in the spinal cord (Liu, Valadez, Sorkin, & McAdoo, 1990). Supporting the original findings of Osterholm et al. (1972), Liu et al. (1990) observed increased norepinephrine shortly after SCI. In addition, they noted substantial increases in serotonin in the 30 to 45 minutes following SCI. Despite the different reports over the years, what these studies highlight is the impact of SCI on neurotransmitter systems at the level of the spinal cord, in the acute phase of injury.

Further, in a unique study, Naftchi et al. (1981) examined post SCI monoamine levels in the heart, adrenal glands, and brainstem of paraplegic rats. To the best of our knowledge, this is the only study having assessed levels of all three monoamines in organs other than the spinal cord following SCI. They reported 30 to 60% decreases in heart norepinephrine and serotonin levels 24 hours post SCI in transected animals relative to shams. They also reported increased norepinephrine and serotonin levels in the brainstem of transected animals 7 days following SCI relative to shams (Naftchi et al., 1981). This study provides strong evidence that SCI can affect neurotransmitter systems in organs beyond the spinal cord itself.

Based on the current data available, it is unclear how these changes in spinal cord monoamine levels post SCI affect psychological well-being, if at all. Most of the changes discussed above appear to be transient- lasting at the most a few days. Longer-term changes in spinal cord monoamines post SCI, as far as we know, have not been

investigated. The results of the study by Naftchi et al. (1981) do suggest that levels of serotonin and norepinephrine increase supraspinally, at least a week post injury. Paradoxically, this would, in theory, be associated with decreased depressive symptoms. It may be that these increases in serotonin are not sufficient to modulate psychological well-being, or that they do not affect brain levels. Brain levels of serotonin post SCI remain unknown. One human study published in 1998, however, looked at serum levels of serotonin pre and post exercise in paraplegic patients. This was done to assess whether chronic spinal cord injured patients experienced serotonergic dysregulations or not. Following exercise, increases in serotonin are expected, and in this sample, chronic spinal cord injured patients exhibited increases in serum serotonin levels post exercise, thus indicating normal serotonergic responses. This suggests to us that changes in serotonin at the level of the spinal cord in the acute phase post SCI likely do not affect peripheral serotonin levels in the long-term.

In addition to altering neurotransmitter concentration at the level of the spinal cord, SCI also results in decreased expression of members of the neurotrophin family. Certain studies report that SCI decreases NGF, BDNF and neurotrophin-3 (NT-3) expression at the level of the spinal cord (Hajebrahimi, Mowla, Movahedin, & Tavallaei, 2008), whereas others have mostly observed decreases in BDNF (Hyun, Lee, Son, & Park, 2009). Decreased BDNF expression has also been shown in the hippocampus of spinally contused rats, from one week to a month post SCI (Fumagalli et al., 2009). Sprague-Dawley male rats were contused, laminectomized only, or intact. At 24 hours or 28 days post injury, they were sacrificed and brain tissue (prefrontal cortex, frontal

cortex, and hippocampus) was collected for RNAase Protection Assays. mRNA levels of BDNF were assessed, as well as mRNA levels of the trophic factor fibroblast growth factor 2 (FGF-2) and growth associated protein 43 (GAP-43), for comparison. Results revealed that although there was an initial (first 24 hours) decrease in BDNF in all brain areas studied, levels returned to normal in all areas except the hippocampus. Moreover, the authors suggested that the fact that levels of FGF-2 and GAP-43 remained unchanged following SCI provided initial evidence that the decreased expression post SCI was selective for BDNF. As discussed earlier, serum and brain levels of BDNF and NGF are decreased in depressed individuals. Although it is not currently known whether these neurotrophic factors cause depression or vice-versa, it is worth considering that decreases in BDNF and NGF levels due to SCI may contribute to the pathophysiology of depression following the injury. Fumagalli et al.'s results provide unique empirical evidence that SCI not only affects the spinal cord physiology, but also the brain. Given the ruptured blood-brain barrier resulting from SCI, and the fact that BDNF changes have been confirmed in the brains of contused rats, it is possible that other changes at the level of the spinal cord also influence the brain.

While SCI is known to cause changes in serotonin, norepinephrine and dopamine, as well as glucocorticoids and cytokines, at the level of the spinal cord, the evidence for supraspinal effects is limited. Future studies must assess changes in the supraspinal levels of the molecules post SCI to delineate possible mechanisms associated with decreased psychological well-being following injury.

Specific aims

Approximately 60% of depressed patients do not respond well to traditional antidepressant medications based on SSRIs (Cattaneo et al., 2013; Krishnadas & Cavanagh, 2012; Mathew & Charney, 2009). As reviewed in the earlier sections, there is increasing evidence suggesting that in a subset of patients with depression, the etiology of the disorder involves dysregulation of the immune system (Maes, 1999; Smith, 1991). This is of particular relevance for spinal cord injured patients, for we know that important immune changes occurs in the spinal cord following SCI. Nonetheless, the effects of SCI on supraspinal levels of cytokines and psychological well-being remain unknown. Our experiments are designed to address this.

We propose that SCI, by activating the immune system, may hinder psychological well-being, potentiating the development of depression and anxiety-like signs post injury. We further suggest that psychological well-being may in turn influence the physical course of recovery by modulating immune system activation in the long-term. We recognize that individual factors (i.e., pain reactivity, resilience to stress) may interact with SCI to cause individual variations in pro-inflammatory cytokine expression that affect psychological well-being. However, identifying these factors is beyond the scope of this proposal. Instead, as an initial step, we aim to test the hypothesis that changes in pro-inflammatory cytokine expression potentiate the development of behavioral changes indicative of depression and anxiety in the rodent SCI model and examine whether these changes in turn affect SCI recovery.

Specifically, the experiments proposed here are designed to determine whether SCI affects psychological well-being and also to explore the reverse relationship; whether psychological well-being affects SCI. In two pilot studies in our lab, we found that two forms of external psychological stress (sound stress and uncontrollable electrical stimulation) did not significantly decrease psychological well-being following SCI. We suspected this to be due to the fact that SCI itself is a strong stressor, and is masking the effect of any other additional external stressor. To investigate this, we have designed the experiments reported in Chapter III and IV. The experiment described in Chapter III assesses the effects of SCI on psychological well-being at the behavioral level. The experiment reported in Chapter IV is designed to evaluate, at the molecular level, the role of the inflammatory response as a function of injury severity in the development of depression and anxiety-like signs post SCI. Lastly, in another pilot study in our lab, we have found that social housing diminishes the negative effects of uncontrollable electrical stimulation on locomotor recovery of function. Consequently, in Chapter V, we conduct an experiment to test the potential beneficial effects of social housing in protecting from decreased psychological well-being post SCI and, as a result, perhaps also promoting physical recovery in our rodent SCI model.

CHAPTER II

GENERAL METHODS

Subjects

Male Sprague-Dawley rats obtained from Harlan (Houston, TX), approximately 90-110 days old (300-350 g) were individually housed in Plexiglas bins [45.7 (length) x 23.5 (width) x 20.3 (height) cm] with food and water continuously available. The rats were maintained on a 12 hour light/dark cycle and all behavioral testing was conducted during the light cycle. Food consumption and subject weights were recorded daily. Following surgery, subjects were manually expressed in the morning (8-9:30 a.m.) and in the evening (6-7:30 p.m.) until they regained full bladder control (which was operationally defined as three consecutive days with an empty bladder at the time of expression), and were checked daily for signs of autophagia and spasticity.

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

An Infinite Horizons (IH) impactor (Precision Systems and Instrumentation) fitted with a 2.5 mm impact probe was used to deliver a contusion injury to the T12 spinal cord. Subjects were anesthetized with 5% isoflurane gas, and once a stable level of anesthesia was reached, the concentration was lowered to a 2-3% maintenance

level. While under stable anesthesia, an area extending approximately 2.5 cm above and below the injury site was shaved and disinfected with iodine. A 3-4 cm incision was made in the skin along the midline of the animal, followed by 1.5 cm incisions on either side of the spinous process at the level of injury. A laminectomy was performed removing the T12 vertebra and exposing the spinal cord. The animal was fixed in the impactor and the probe exerted a force onto the cord, remaining in contact with the cord for one second (dwell time). Depending on the experiment, a force of 110 kDynes for a mild injury, 150 kDynes for a moderate injury, or 200 kDynes for a severe injury was applied to the cord. Immediately after the contusion, the incision was closed with Michel clips and subjects were treated with 100,000 units/kg Pfizerpen (penicillin G potassium), both immediately following surgery and again two days later, to help prevent infection. To compensate for fluid loss, subjects received 3.0 ml of filtered saline at the time of surgery and again two days later. In addition, rats were kept in a recovery room (maintained at 26.6°C) for 24 hours following the injury.

Behavioral tests

Assessment of psychological well-being

Anhedonia

The sucrose preference test was used to assess anhedonia. The test was conducted in the home cage, from 14:00 to 16:00. For testing, one pre-weighed water bottle filled with approximately 250 ml of 2% sucrose solution and one pre-weighed bottle filled with an equal amount of water were placed on either side of the subject's

cage, and left for two hours. The placement of the sucrose and the water solutions (on either the left or right sides) was counterbalanced between subjects and across testing periods. The position of the bottle in the cage (left/right) was also reversed after one hour, to prevent any positional biases from confounding results. At the end of the test period, the change in the weight of each bottle of solution was determined. Sucrose preference (SP) was then calculated using the following formula: $\%SP = [\text{sucrose solution intake (g)} / (\text{sucrose solution intake (g)} + \text{water intake (g)})] \times 100$ (Wang et al., 2009).

Subjects were acclimated to the sucrose preference test in three sessions beginning 13 days prior to surgery. Baseline preferences were collected five days prior to surgery. Sucrose preference was then measured during Test Phase 1 and 2 post injury, on Days 10 and 21 post SCI. A decrease in sucrose preference is a sign of anhedonia, or lack of ability to experience pleasure (Luedtke et al., 2014).

Psychomotor activity and center field activity

The open field test was used to assess psychomotor activity and center field activity. The test was conducted in a black plywood box [100 (length) x 20 (height) x 100 (width) cm], from 8:00 to 11:00. The floor of the box was partitioned into 100 squares [10 (length) x 10 (width) cm] delineated with silver marker. A layer of clear Plexiglas was used to cover the top of the box. The testing room was dark and the open field environment was illuminated from above by a 60 W white light. Subjects were acclimated to the testing room (in their transport boxes) for ten minutes prior to testing.

Following the acclimation period, the subject was placed in the center of the plywood box to begin a five-minute test session. Between each trial, the open-field environment was cleaned with Nolvasan to eliminate any olfactory cues. The test was video recorded from above. Subjects were acclimated to the open field environment during three sessions beginning 13 days prior surgery. Baseline activity levels were collected five days prior to surgery. Open field activity was then assessed during Test Phase 1 and 2, on Days 10 and 21 post injury.

Open field activity was scored *post hoc* from video analyses. The number of squares crossed in the outer squares of the fields were operationalized as having two paws or more in the outside row of squares. The number of squares crossed in the center (28 center squares), as well as time spent in the center squares were also recorded, and center field activity was computed in the following manner: center squares crossed x center time. A decrease in the total number of squares crossed is interpreted as a sign of psychomotor retardation, while a decrease in center field activity is interpreted as a passive anxiety-like sign (Luedtke et al., 2014).

Social exploration

Social exploration was assessed in the same black plywood box used for open field activity. Subjects were placed into the center of the open field and allowed to explore for five minutes. A juvenile rat (<250 g weight), not exposed to any experimental treatment, was then placed into the open field as far from the experimental subject as possible. The experimental subject and the juvenile rat were filmed for 5

minutes. The test apparatus was disinfected with Nolvasan in between trials. Subjects were acclimated to the open field environment as described previously (see *Open field activity*). Baseline social exploration was assessed 5 days prior to surgery. Social exploration was assessed during Test Phase 1 and 2, on Days 10 and 21.

Videos were scored *post hoc*, recording the total time the experimental subject spent interacting with the juvenile rat. Interactions were operationalized as (1) pursuing the juvenile rat (while within three squares of the juvenile rat), and (2) sniffing any part of the juvenile rat's body (Swain & Le, 1998). A total percent time spent in social exploration was obtained by computing the following: (social exploration behavior/300 sec) x 100. Reduced social exploration behavior is a measure of loss of interest or pleasure (Luedtke et al., 2014).

Learned helplessness

The forced swim test was used to assess learned helplessness. Subjects were allowed to acclimate in the testing room for ten minutes. The subject was then placed in a cylinder [15 (diameter) x 40 (height) cm] filled with water ($23 \pm 1^\circ\text{C}$) from which they could not escape. The forced swim test is traditionally conducted using a 10-minute acclimation period and a 10-minute test period 24 hours later. However, Abel and Bilitzke (1990) found that immobility measured during a ten minute acclimation period was highly correlated with immobility measured in the test period conducted 24 hours later. Therefore, in the current study the subjects were filmed for a 10-minute test period

only (without the preceding acclimation to the water). The forced swim test was conducted during Test Phase 2, on Day 23 post injury.

Time spent immobile was scored *post hoc*. Immobility was operationalized as the lack of movement except that required to keep the nose above water (Porsolt, Bertin, & Jalfre, 1977; Slattery & Cryan, 2012)- this included slight tail movement. Immobility is interpreted as a symptom of learned helplessness, and characteristic of depression in rodent subjects (Luedtke et al., 2014).

Food consumption

Food was provided by laboratory members every day. Food weight was recorded before and after injury, daily, at 9 AM. Food consumption was calculated by subtracting the previous day's food weight to the current day's food weight.

Anxiety

The shock probe burying test was used to assess active anxiety. Rats were placed in a plastic box (56.51 cm L x 40.33 cm W x 33.35 cm H) with a small probe (5 cm) placed 2 cm above the bedding level at one far end of the box. The probe was built by wrapping two copper wires around a glass rod, and connecting the extremities to two dragon snaps. The probe was connected to a source of mild electrical stimulation (0.08 mA). As soon as the subject came into contact with the probe (nose or forepaws touch), the electrical stimulation was turned off. The subjects were filmed for ten minutes. The time that the rats spent engaged in burying and freezing behavior was recorded from *post hoc* video analyses. The shock probe burying task was conducted during Test Phase 1

and 2, on Days 5 and 20 post injury. The shock probe test was conducted on Day 20 prior to the depression tests to ensure that behavioral testing stress itself would not affect the rats' behavior in the test. It must be noted that a baseline test of shock probe burying was not conducted. Performance on this test is sensitive to repeated testing as the rats are less likely to touch the probe after having experienced the electrical stimulation produced by the probe. Studies indicate that rats can show retention of the learned aversion to the shock probe up to 20 days after the trial, especially if tested more than once before (De Boer & Koolhaas, 2003). Given the importance of obtaining behavioral data post SCI, and the learning effects of repeated testing, we decided to test subjects early in Test Phase 1, and then once more during Test Phase 2.

The shock probe burying task is widely used as a measure of active anxiety, and has been validated by several groups (De Boer & Koolhaas, 2003; Poling, Cleary, & Monaghan, 1981; Treit, Pinel, & Fibiger, 1981). Greater time spent burying is interpreted as a sign of active anxiety.

Assessment of pain reactivity

Thermal reactivity

The tail-flick test was used to assess thermal reactivity. Subjects were placed in clear restraining tubes and allowed to acclimate to the room and the tubes for ten minutes. The testing room was maintained at 26.5°C. The subjects were then placed on the tail-flick apparatus, with their tail positioned in a 0.5 cm deep groove, cut into an aluminum block, and allowed to acclimate to the apparatus (IITC Inc., Life Science, CA)

for five minutes. Thermal reactivity was then tested by applying the heat source (light) onto the rat's tail (3.8 cm from the tip of the tail). Withdrawal latency from the light source was recorded. If a subject failed to respond, the test trial was automatically terminated after 8 s of heat exposure, to prevent tissue damage. Three tests occurred at 2-minute intervals, and the last two test tail-flick latencies were recorded, and averaged. Baseline tail-flick scores were obtained 4 days prior to the surgery. The tail-flick test was subsequently conducted during Test Phase 1 and 2, on Days 11 and day 22 post injury.

Mechanical reactivity

The tactile test was used to assess mechanical reactivity. Subjects were placed in clear restraining tubes and allowed to acclimate to the room and the tubes for 15 minutes. Mechanical reactivity was assessed using Von Frey filaments (Semmes-Weinstein Anesthesiometer, Stoelting Co., Chicago, IL). Von Frey filaments were applied every 2s in sequence to the plantar surface of the rats' hindpaw (the L5 plantar dermatome, between the footpads). The stimuli were presented until subjects exhibited a paw withdrawal/motor (spinal) and vocalization (supraspinal) response. The intensity of the stimuli that produced the responses was reported using the formula provided by Semmes-Weinstein: $\text{Intensity} = \log_{10}(10,000 * \text{g force})$. If one or both responses were not observed, testing was terminated at an intensity of 6.65. Each subject was tested twice on each foot in a counterbalanced ABBA order.

In the girdle test, mechanical reactivity is assessed at the level of injury (Christensen & Hulsebosch, 1997). The girdle zone for allodynic responding was drawn as a map grid on the rats with an indelible marker (44 squares). A von Frey hair with bending force of 204.14 mN (26 g force) was applied to each point on this grid, and vocalization responses were recorded and mapped onto a grid map of that animal. The von Frey stimulus was applied first to the left of the animal's spinal column, in a rostral to caudal direction (11 squares per column, 2 columns), and then likewise to the right. The total number of vocalizations was recorded.

Both the tactile and the girdle tests were conducted at baseline (five days prior to surgery), and during Test Phase 1 and 2, on Days 11 and day 22 post surgery.

Assessment of recovery and general health

Locomotor recovery

The recovery of hindlimb stepping was scored using the Basso, Beattie and Bresnahan (BBB) scale (Basso, Beattie, & Bresnahan, 1995). This 21-point scale is used as an index of hindlimb functioning after a spinal injury. Using this scale, no movement of the hindlimbs (ankle, knee or hip) is designated a score of 0, and intermediate milestones include slight movement of one joint (1), extensive movement of all three joints (7), occasional weight supported stepping in the absence of coordination (10), and consistent weight supported stepping with consistent front limb- hind limb coordination (14). Higher scores reflect consistent limb co-ordination and improved fine motor skill.

The locomotor capacity (BBB) of subjects was observed for 5 minutes in an open field test area (99 cm diameter, 23 cm deep) and scored by a trained observer blind to the experimental groups on days 1-7, 9, 11, 13, 15, 18, and 21 post injury, from 9 am to noon. Care was taken to ensure that all observers' scoring behavior had high intra- and inter-observer reliability (all r 's > 0.90). BBB scores were then transformed, as described in Ferguson et al. (2004), to help assure that the data were amendable to parametric analyses.

Tissue collection

Serum

Twenty-four hours prior to blood collection, the rats' hindlimbs were shaven with an electric trimmer to expose the saphenous vein. The next day, topical lidocaine was applied to the shaven leg. After waiting for six minutes (to give Lidocaine the time to act), each rat was inserted into a clean sock, with an opening for its nose, to breathe comfortably. A compression point at the base of the leg was made to make the saphenous vein bulge out (similar to using a tourniquet). The vein was punctured using a 20G needle and the blood was collected with a blood collection tube with a clot activator (MCB 16440 with Serum/Clotting activator, Braintree Scientific). When enough blood had been collected, a clean compress was held on the puncture site to stop the bleeding. A 2.5 ml (s.c.) of saline was administered to the rats to prevent dehydration. Care was taken to perform this procedure in two minutes or less. Blood was collected at baseline (2 days prior to surgery), on Day 1 post surgery, and during Test Phases 1 and 2, on

Days 10 and 24 post surgery, two hours prior to the beginning of the dark cycle. Saphenous veins punctured were alternated on each day of collection (i.e., left, right, left, right).

The collected blood was left at room temperature for 30 minutes to allow clotting. The samples were then centrifuged at 12 000 RPM for 15 minutes, at 4 °C. The top clear layer (serum) of each sample was extracted with a pipette and stored in a -20 °C freezer.

Fresh tissue collection

Brain tissue

Subjects were anesthetized with beuthanasia (.02 ml administered i.p.) and after verification of a deep level of anesthesia (absence of pedal and corneal reflex), an incision above the skull was made. The nasal bone and the cranial bone were cut to extract the brain. The brain was placed immediately on a glass dish on ice, and the hippocampi were extracted and flash frozen in liquid nitrogen, in less than 3 minutes (Spijker, 2011), for the subsequent extraction of total protein (detailed in the Chapter IV).

Spinal cord tissue and thymus

A 1 cm section of the spinal cord, centered over the injury site was removed and frozen in liquid nitrogen for the subsequent extraction of total protein (detailed in the Chapter IV). In addition, the thymus was extracted and weighed as an index of psychological stress. All tissue was collected on Day 25 post surgery.

Molecular analyses

Alpha-2 ELISA

Alpha-2 macroglobulin serum levels were assessed using an ELISA kit (ab157730; Abcam, Cambridge, MA; sensitivity 2.2277 ng/mL minimum calculated detectable dose) according to the manufacturer's instructions. Samples were read at 450 nm using a microplate reader (Wallac Victor2 1420 Multilabel Counter, PerkinElmer, Waltham, MA). A 5-parameter logistic regression was used to derive the equation for the standard curve (optical density by alpha-2 macroglobulin ng/mL). The alpha-2 macroglobulin level (ng/mL) for all samples was obtained by entering the mean optical density minus the Blank optical density in the equation for the standard curve, and solving for alpha-2 macroglobulin levels. All alpha-2 macroglobulin levels were multiplied by the appropriate dilution factor.

Cytokine/chemokine multiplex

Serum and hippocampus homogenate levels of 27 cytokine/chemokines were assessed using a magnetic bead panel Milliplex MAP Kit (RECYMAG65K27PMX; EMD Millipore Corporation, Billerica, MA). The analytes assessed with this panel were G-SCF, Eotaxin, GM-CSF, IL-1 α , Leptin, MIP-1 α , IL-4, IL-1 β , IL-2, IL-6, EGF, IL-13, IL-10, IL-12p70, IFN- γ , IL-5, IL-17A, IL-18, MCP-1, IP-10, GRO/KC/CINC-1, VEGF, Fractalkine, LIX, MIP-2, TNF- α , and RANTES. Spinal cord homogenate levels of 20 cytokine/chemokines were assessed using a magnetic bead panel Bio-Plex Pro Rat Cytokine 23-Plex Assay (L80-01V11S5; Bio-Rad Laboratories, Hercules, CA). This

panel assessed the same analytes as the Milliplex MAP Kit, except that Eotaxin, Leptin, MIP-1 α , EGF, IP-10, Fractalkine, and MIP-2 were not included. Both kits followed the same basic protocols and used the same reagents. All plates were read using a mini-PROTEAN system (Bio-rad), and Bio-plex Array reader with High Throughput Fluidics system (Biorad).

Experimental schedule

The experimental timeline used for the injury severity (Chapter III) and group housing (Chapter IV) experiments is shown in Figure 1. Baseline measures for depression were collected in the week prior to injury. Following the contusion the tests were repeated on Days 10-11 and 20-21. The forced swim test was also conducted on the day immediately prior to injury and repeated on Day 23 post injury. As a measure of active anxiety, the shock probe test was conducted on Day 5 and 20 post injury. Recovery of locomotor function was assessed throughout the recovery period with tests of sensory function conducted on Day 11 and 22 post injury (see Figure 2).

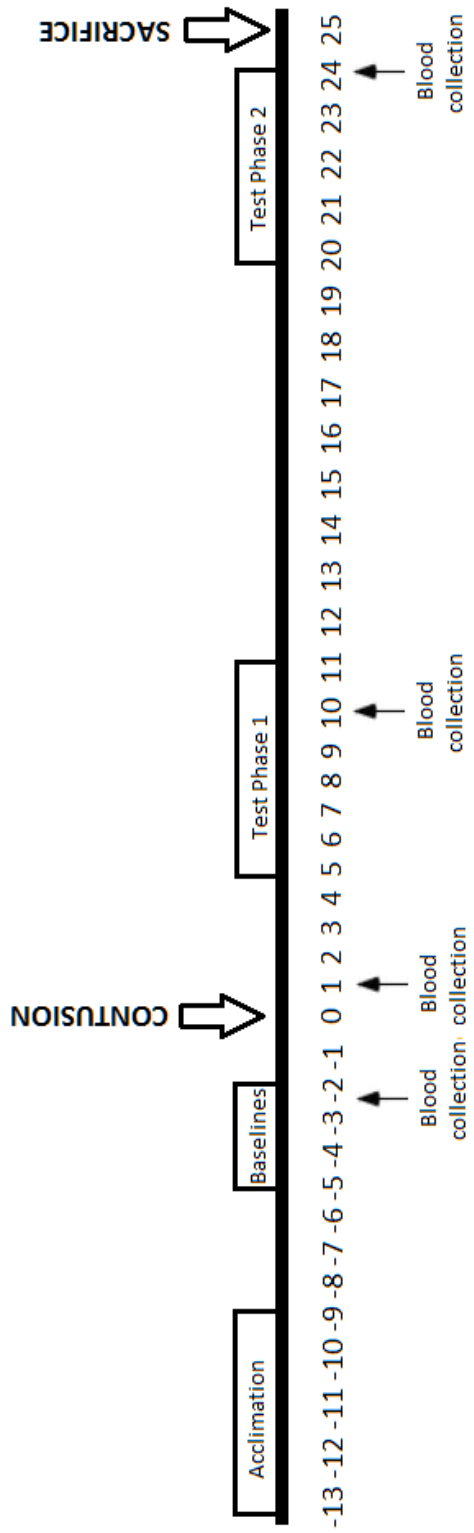


Figure 2 - Experimental schedule.

Statistical analyses

Identifying psychological well-being groups

Principal components analysis

The principal components analysis is a variable reduction technique that transforms a group of observations from a related set of variables into a (typically) smaller set of linear uncorrelated variables. These uncorrelated variables are referred to as principal components. In both experiments (Injury Severity and Stress, and Group Housing), subjects' scores on each of the behavioral measures (sucrose preference, forced swim, open field psychomotor activity, center field activity, social exploration, and shock probe burying) were averaged across Test Phase 1 and 2 and then subjected to a principal components analysis using orthogonal Varimax rotation. The scores were averaged so that the equation derived in subsequent analyses would characterize behavioral signs that persisted into Test Phase 2 as well as have strong predictive value for Test Phase 1, or the early phase of SCI. Factors with loadings of ≥ 0.32 on more than one component (complex structure) were removed (Tabachnick & Fidell, 2007) and the analysis was repeated until no complex structure factors remained.

Hierarchical cluster analysis

Hierarchical cluster analysis is a statistical procedure used to separate a sample into clusters that the experimenter can operationally define. In both the Injury Severity and Stress experiment, and the Group Housing experiment, a hierarchical cluster analysis was performed using the measures with moderate-strong loadings on the

components retained in the PCA. Specifically, average scores (derived from both Test Phase 1 and 2), on each of the retained behavioral tests, were first standardized by z scores. Then a hierarchical cluster analysis was performed using Ward's method and applying squared Euclidean distance as the distance measure. The number of appropriate clusters based on the behavioral measures was obtained by looking for a break in the agglomeration coefficient change and by observing the dendrogram, which illustrates the distance between linked clusters. It must be noted that the cluster sizes need not be even. The cluster analysis was repeated using the same parameters but restricting it to a single solution of three clusters. A new variable, cluster membership, was generated for all subjects.

The three clusters' performance on all measures retained by the principal component analysis was compared across the recovery period (Test Phase 1 and 2) using repeated measure analyses of variance (ANOVAs). Baseline scores were used as a covariate when significant ($p < 0.05$). Based on the pattern of behaviors exhibited by each cluster, the subject cohorts were labeled as exhibiting anxiety and depression like signs, depression like signs or as being healthy.

CHAPTER III

CHARACTERIZING PSYCHOLOGICAL WELL-BEING AS A FUNCTION OF INJURY SEVERITY AT THE BEHAVIORAL LEVEL

Introduction

This experiment characterized depression and anxiety-like signs as a function of spinal cord injury (SCI) severity to determine whether post SCI inflammation contributes to decreased psychological well-being in a rodent model of SCI. Despite the high prevalence of depression and anxiety after SCI and their impact on the post-SCI experience, there has only been one empirical study of psychological well-being in an animal model of SCI (see Luedtke et al. 2014). Luedtke et al. (2014) demonstrated that following SCI, approximately 35% of spinally contused rats show a depression-like profile. This profile includes decreased psychomotor activity (open field test), decreased interest in social interactions (social exploration test), increased learned helplessness (forced swim test), and increased anhedonia (sucrose preference test). Moreover, and critically for a SCI model of depression, Luedtke et al. (2014) provided solid evidence that the depression-like signs in these subjects were not simply due to reduced motor activity following SCI. Indeed, there were no significant differences in terms of locomotor recovery of function and tissue sparing between depressed and non-depressed subjects. This study also showed that some of the signs of depression could be reversed by SSRIs, but not all. Specifically, learned helplessness on the forced swim test was reversed by administration of fluoxetine. This further suggested that the depression-like

signs observed post SCI may not be due, at least not solely, to a serotonin imbalance. Instead, the paper proposed that inflammation may precipitate the development of depression after SCI.

There is a growing body of literature pointing to a role of chronic peripheral and central inflammation in psychiatric illnesses such as major depressive disorders, anxiety, and even schizophrenia (Anisman, 2009; Black & Berman, 1999; Dantzer et al., 2008; Maes, 1999; Muller, 2013; Pace & Miller, 2009; Rosenblat et al., 2014; Uddin et al., 2011; Wager & Gianaros, 2014; Walker et al., 2014; Zunszain et al., 2012). For example, endotoxins can induce transient depressive-like symptoms in both humans and rats (De La Garza, 2005; Makino et al., 2000; Reichenberg et al., 2001) and immunotherapy can induce depression symptoms in cancer patients (Dantzer et al., 2008). Similarly, in a mouse model of premature aging, high levels of anxiety have been found to be associated with chronic inflammation (Vida et al., 2014). Activation of the innate immune system particularly increases in brain IL-1 β levels, increase anxiety-like behaviors at the open-field test and the elevated plus maze test (Chiu et al., 2014). These findings highlight the association between inflammation and symptoms of depression and anxiety.

SCI is characterized by inflammation and activation of the immune system. Following the injury, microglia, macrophages, and astrocytes are recruited to the site of trauma. Pro-inflammatory cytokine genes and other inflammation-related genes are up-regulated within hours of injury (Dumont et al., 2001; Yip & Malaspina, 2012), and

some for weeks following the injury (Jokic, Yip, Michael-Titus, Priestley, & Malaspina, 2010; Malaspina, Jokic, Huang, & Priestley, 2008). For example, in the hours following injury, the interleukin-6 gene (*IL-6*), the *TNF* gene, and the interleukin-1 β gene (*IL-1 β*) are up-regulated (Hayashi et al., 2000; Pan et al., 2002). In addition, as spinal cord injury severity increases, pro-inflammatory cytokines are further up-regulated (Yang et al., 2005). In a rodent study of SCI, inflammation post SCI was compared in mild and severe SCI subjects (10 g weight drop from 3 and 12 cm to T12, respectively). Immunocytochemical, RT-PCR and Western Blot assays on spinal cord tissue collected one to six hours post SCI indicated that IL-1 β , IL-6 and TNF- α production is increased in neurons and microglia of the spinal cord following SCI, and that this production is significantly greater following a severe injury rather than a mild injury (Yang et al., 2005). Therefore, immune system activation triggered by the SCI may influence psychological well-being, and may do so incrementally as injury severity increases.

The current experiment aimed to extend our previous study (Luedtke et al. 2014) assessing behaviors not only characteristic of depression but also of anxiety in the rodent contusion model. Further, given that inflammation at the level of the spinal cord is known to increase with severity of injury, we hypothesized that if inflammation at the level of the spinal cord directly results in decreased psychological well-being, the incidence of depression and anxiety should increase as a function of injury severity.

Methods

Experimental design

Subjects (N=47) received a mild, moderate, or severe contusion (see General Methods, Surgery, for detailed procedures) or were intact controls (n=12/group). For all subjects, psychological well-being was assessed at baseline with a comprehensive battery of tests as well as during Test Phase 1 (days 5 and 10) and 2 (days 20-21) post injury (See Figure 2). Intact subjects were tested at ages compatible with injured subjects. The procedures for each of the tests are described in detail in the General Methods section (see Assessment of Psychological Well-being). Food consumption was also collected daily, as an index of appetite deviation which is associated with depression in the clinical population.

Locomotor function was assessed throughout the 21-day recovery period with the BBB scale (Basso et al., 1995), as described in the general methods. Tests of sensory function (girdle, tactile and thermal reactivity), were also conducted during Test Phase 1 (day 11) and Test Phase 2 (day 22). Blood was collected in each test phase (pre-injury, days 2, 10, and 24). Finally, at the end of the recovery assessment period, subjects received a lethal injection of beuthanasia (100 mg/kg, i.p.) and the injured spinal cord (1 cm section centered on the lesion) and brain tissue (hippocampus) were collected. Thymus weight was also recorded as a physiological index of stress.

ELISAs

Alpha-2 macroglobulin levels were assayed on Days 1, 10 and 24 with ELISAs on serum (Abcam 2013, kit ab157730). Dilution tests were run to determine the appropriate dilution rate for all serum samples. For intact subjects, the dilution rate was determined to be 300 x, whereas for all contused subject samples, the dilution rate was determined to be 3000 x. Assays were run following the manufacturer's protocol. The best fit equation was determined to be a 5-parameter logistic regression. The concentration readings were multiplied by the appropriate dilution factors (300 for intact subject samples, and 3000 for all contused subject samples).

Statistical analyses

We first used a series of statistical analyses to determine the psychological well-being of the subjects in the experiment. Detailed statistical steps are found in the General Methods chapter (Chapter 2). First, a principal component analysis was used in order to determine which behavioral measures were correlated with each other. This allowed us to select the behavioral measures providing the most informative and non-repetitive information. Next, we ran a hierarchical cluster analysis on the behavioral measures retained by the principal component analysis in order to identify the subject clusters in our sample. The hierarchical cluster analysis identified subjects as exhibiting depression, anxiety/depression, or as being healthy. After identifying the psychological well-being groups, we ran post-cluster repeated measure analyses of variance (ANOVAs) on the behavioral measures retained by the principal component analysis in order to confirm the hierarchical cluster group identifications. Following confirmation

of the hierarchical cluster groups, we conducted one-way repeated measure ANOVAs on all test variables, to look for main effects of injury severity and psychological well-being on measures of depression, anxiety, pain, recovery of function and physiological markers.

Results

Principal component analyses: assessment of test validity for identifying psychological well-being

The principal component analysis produced two components, which cumulatively explained 73.62% of the variance between subjects. The first component contained the sucrose preference test and the shock probe burying test. Open field activity and social exploration loaded on the second component (see Table 1). Both components had Eigenvalues greater than 1, and explained a significant proportion of the variance between subjects. They were retained for subsequent analyses.

Behavioral test	Components	
	1	2
Sucrose preference	.178	.817
Social exploration	.872	-.150
Open field activity	.872	.214
Shock probe burying	.120	-.802

Table 1 - Components of principal component analyses in Injury Severity experiment. Component loadings are shown for the behavioral tests retained. To be retained, behavioral tests had to load with $>.03$ on one component only.

Hierarchical cluster analyses: classification of psychological well-being

Average scores (derived from both Test Phases 1 and 2), for each of the behavioral tests retained in the principal component analysis (sucrose preference, shock probe burying, open field and social exploration), were used in the hierarchical cluster analysis. The dendrogram produced by this analysis showed that subjects separated into three psychological well-being clusters, with 15 subjects in Hierarchical Cluster (HC) Group 1, 12 subjects in HC group 2, and 20 subjects in HC group 3 (see Figure 3).

To identify the psychological well-being characteristics of each cluster group, the three groups were compared on each of the behavioral measures retained in the principal component analysis, across Test Phase 1 and 2. Based on their performance on these measures, subjects in HC group 1 were labeled as exhibiting anxiety and depression like signs (or ANX/DEP), subjects in HC group 2 as exhibiting depression-like signs (or DEP) and subjects in HC group 3 as being healthy (or HEALTH). Baseline differences were significant for the social exploration test and the open field activity test, [$F(2,44)=5.82$, $p=.006$ and $F(2,44)=12.88$, $p=.000$, respectively]. For these two variables, repeated measures analyses of covariances were conducted. It is important to note that including the baseline as a covariate does not correct fully for the differences pre-contusion on these two variables; however, it was not possible to counterbalance by psychological well-being pre contusion, since groups were determined post injury. All post-hocs were conducted with multiple comparison Sidak tests.

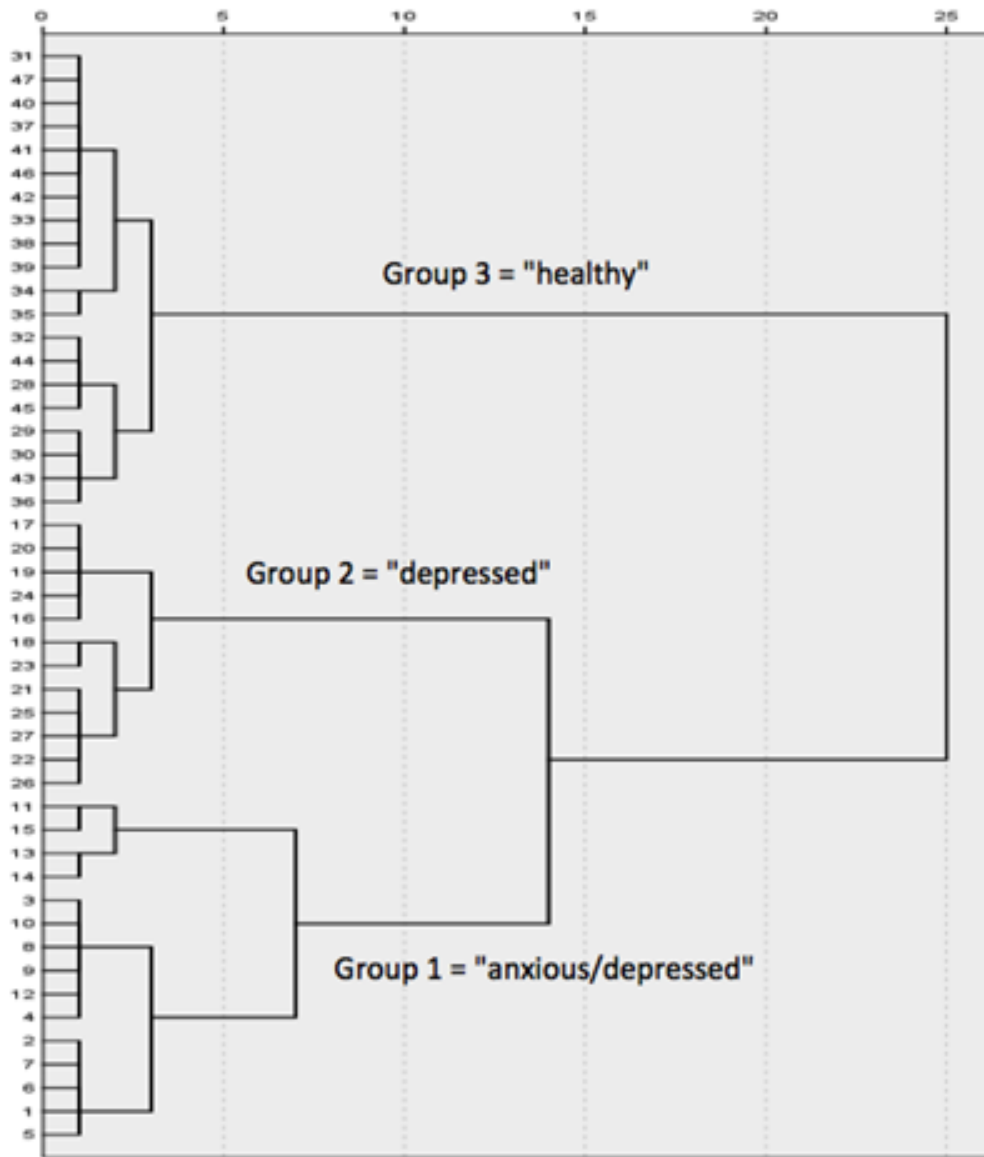


Figure 3- Hierarchical cluster dendrogram, Injury Severity and Stress experiment. The dendrogram illustrates the results of the hierarchical cluster analysis, which separated the sample into three main clusters (Group 1 (n=15), Group 2 (n=12), Group 3 (n=20)), based on the subjects' average performance on the behavioral tests retained by the principal component analyses. The numbers on the y-axis represent the subjects.

Post hierarchical cluster comparisons

Repeated measure ANOVAs revealed a main effect of psychological well-being (HC group) for the sucrose preference test [$F(2,44)=8.39, p=.001$], the open field activity test [$F(2,43)=20.95, p=.000$], the social exploration test [$F(2,43)=7.67, p=.001$], and the shock probe burying test [$F(2,40)=41.32, p=.000$]. For sucrose preference, subsequent ANOVAs (on Days 10 and 21 separately) confirmed that the HC groups differed at both time points [$F(2,44)=8.67, p=.001, F(2,44)=3.89, p=.028$, for Day 10 and 21 respectively]. *Post hoc* analyses revealed that both on Day 10 and 21, the ANX/DEP group showed significantly lower sucrose preference than the HEALTH group, $p= .05$, Figure 4A and Figure 4B. For open field activity, separate ANOVAs on Days 10 and 21 also confirmed main effects of psychological well-being at both time points [$F(2,43)=14.93, p=.000, F(2,43)=11.65, p=.000$.] *Post-hoc* analyses revealed that on Day 10, the DEP group showed decreased activity in an open field relative to the ANX/DEP and HEALTH group, $ps < .05$ (Figure 4C), and at Day 21, both the ANX/DEP and the DEP group showed decreased activity relative to the HEALTH group, $ps \leq .05$, Figure 4D. On the social exploration test, subsequent ANOVAs on Days 10 and 21 revealed that the HC groups also differed significantly on social exploration at Day 10, $F(2,43)=14.27, p=.000$.

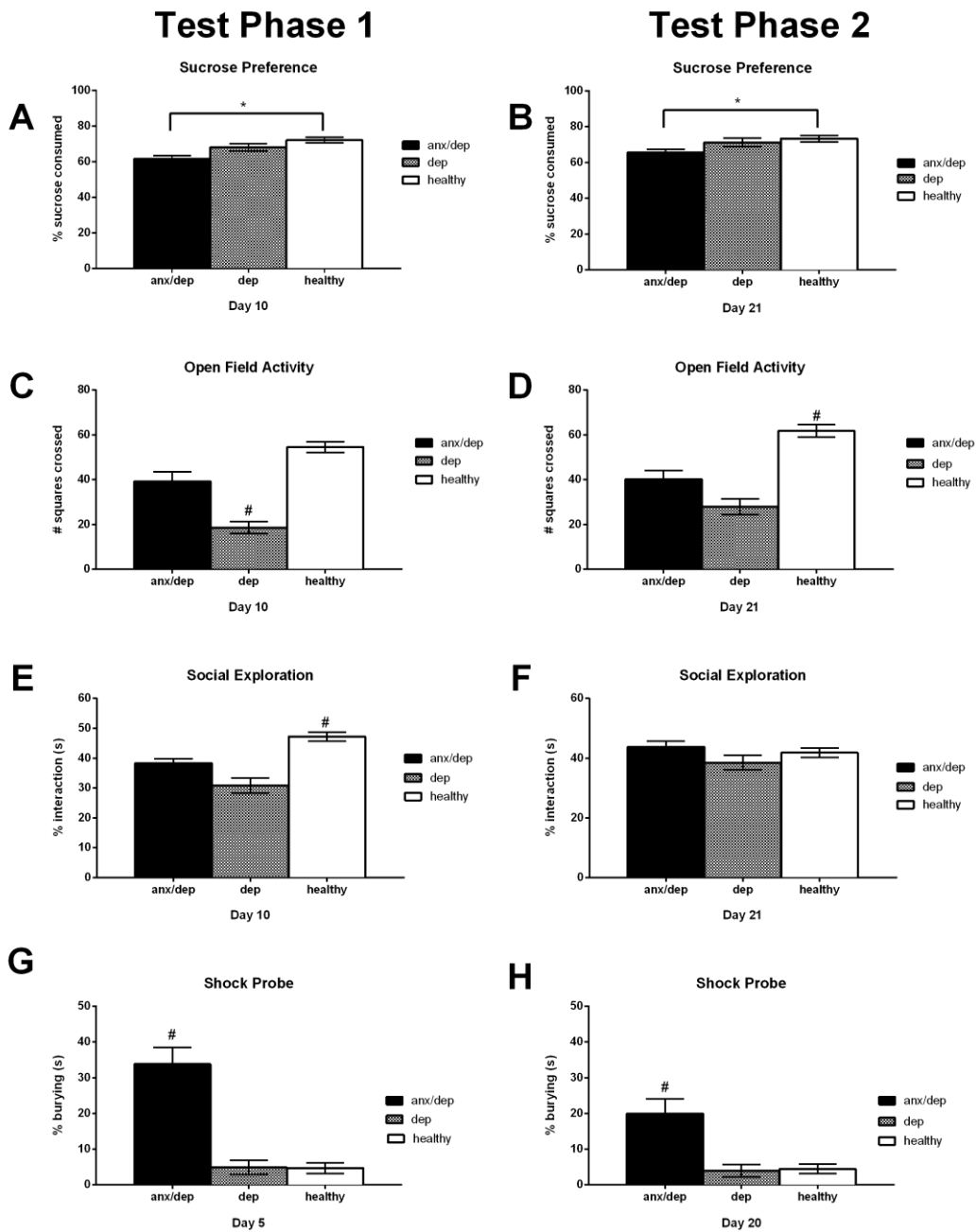


Figure 4- Post hierarchical cluster differences. The average (\pm standard error of the mean) performance of the ANX/DEP, DEP, and HEALTH group are shown on each task. ANX/DEP subjects showed decreased sucrose preference relative to the HEALTH group on Days 10 and 21 (A and B), as well as increased shock probe burying relative to the DEP and HEALTH groups on Days 5 and 20 (G and H). The DEP group showed decreased open field activity relative to the ANX/DEP and HEALTH group on Day 10 (C) and relative to the HEALTH group on Day 21 (D). The DEP group also showed decreased social exploration on Day 10 (E) relative to the HEALTH group. * $p < .05$, # significant from all others.

Post-hoc analyses revealed that on Day 10, both the ANX/DEP and the DEP group showed significantly lower social exploration time than the HEALTH group, $ps \leq .05$, Figure 4E. Finally, on the shock probe burying test, separate ANOVAs on Days 5 and 20 data confirmed that the HC groups differed significantly on shock probe burying at both time points [$F(2,43)=28.66, p=.000, F(2,44)=11.12, p=.000$]. *Post-hoc* analyses revealed that the ANX/DEP group showed significantly higher percent time burying at the shock probe burying test than both the DEP and HEALTH groups, at both Days 5 and 20, $ps \leq .05$, Figure 4G and Figure 4H.

Group membership

As shown in Figure 5, examining group membership by psychological well-being and injury severity, we found that most intact subjects were classified as healthy, whereas most contused subjects were classified either as depressed or anxious/depressed. Although it did appear that the number of depressed subjects increased with injury severity and that the number of anxious/depressed subjects decreased with injury severity, there was no clear linear relationship overall with increased injury severity and number of subjects classified as either depressed or anxious/depressed.

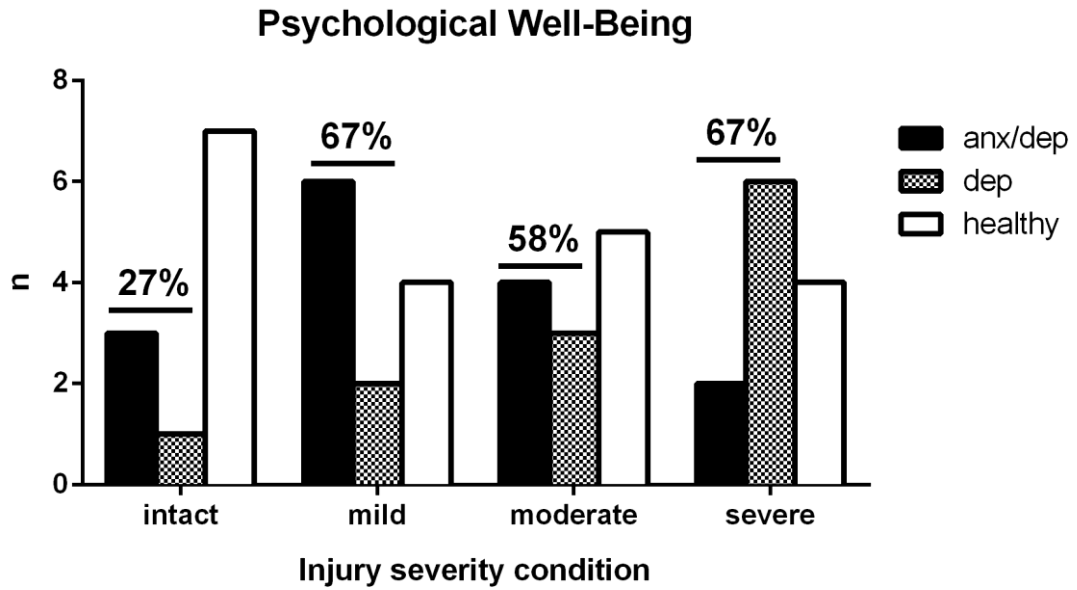


Figure 5- Psychological well-being membership by injury severity. This figure shows group membership by injury severity, and indicates the percentage of subjects identified as either anxious/depressed or depressed within each injury severity level.

Psychological well-being, pain, recovery of function and physiological markers following spinal cord injury

First, to verify the effect of injury severity itself, one-way repeated measure ANOVAs were conducted by injury severity on all measures of depression- and anxiety-like behavior, as well as pain, recovery of function, and physiological markers, followed by multiple comparison Sidak *post-hocs* tests to identify group differences on specific test days. Then, to determine differences among psychological well-being groups on measures other than those retained by the principal component analyses and used for hierarchical clustering, one-way repeated measure ANOVAs were conducted by psychological well-being on measures of anxiety (center activity) and depression (forced swim) not used for hierarchical clustering, as well as on measures of pain, recovery of function, and physiological markers. These comparisons across time were followed by one-way ANOVAs for each independent variable separately (injury severity and psychological well-being). Subsequently, multiple comparison Sidak *post-hocs* tests were conducted to identify group differences on specific test days. For each variable, the injury severity effect is reported first, followed by the psychological well-being effect. Note that for sucrose preference, open field activity, social exploration, and shock probe burying, psychological well-being effects are not reported here, as these were already reported in the post-hierarchical cluster ANOVAs used to characterize the hierarchical cluster subgroups. Finally, correlations were conducted to investigate associations between pain reactivity (tactile tests, tail-flick test, and girdle test) and behavioral measures (open field activity, social exploration, sucrose preference, forced

swim, shock probe burying, and center activity), as well as pain reactivity and peripheral inflammation (serum alpha-2 macroglobulin levels). Correlations are reported only where found significant. All graphs show psychological well-being groups (anxious/depressed, depressed, and healthy) subdivided by injury severity groups (intact, mild, moderate, and severe) for consistency.

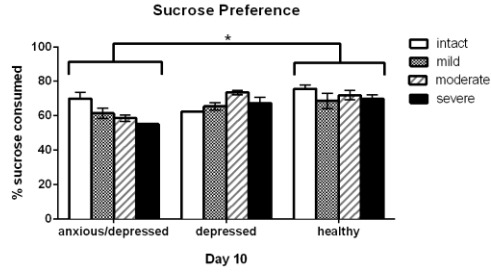
Depression measures

While main effects of sucrose preference, open field activity, and social exploration were found by psychological well-being (Figure 6), as per the post hierarchical cluster repeated measure analyses run in the previous section, repeated measure ANOVAs found no main effect of injury severity on sucrose preference across time [$F(3,43)=1.67, p=.188$], open field activity across time, [$F(3,42)=2.12, p=.112$], social exploration across time [$F(3,42)=1.55, p=.215$], or forced swim on Day 24 [$F(3,41)=.175, p=.913$]. Finally, repeated measure ANOVAs did not significant differences among psychological well-being groups on the forced swim test, $F(2,42)=2.016, p=.146$ (Figure 6G).

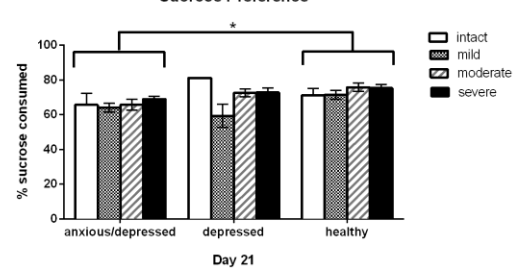
Figure 6- Depression measures by psychological well-being and injury severity. The average (\pm standard error of the mean) performance of the ANX/DEP, DEP, and HEALTH group, separated by injury severity level, are shown on all depression measures. No main effects of injury severity were observed. Main effects of psychological wellbeing were found. The ANX/DEP group showed decreased sucrose preference relative to the HEALTH group (A and B). They also showed decreased open field activity and social exploration relative to the HEALTH group (C and D). The DEP group showed decreased open field activity relative to the ANX/DEP (Day 10) and HEALTH group (Day 10 and 21), (C and D). The DEP group showed decreased social exploration relative to both the ANX/DEP and the HEALTH group on Day 10 (E). No main effects of injury severity were found. $*p < .05$

Test Phase 1

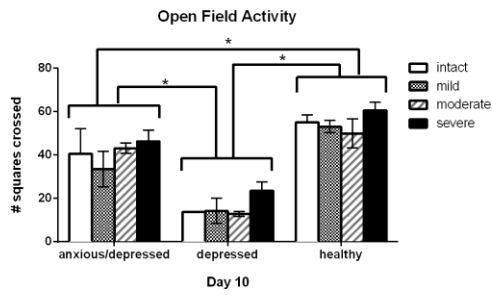
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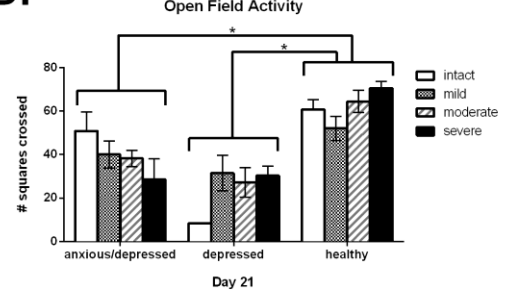
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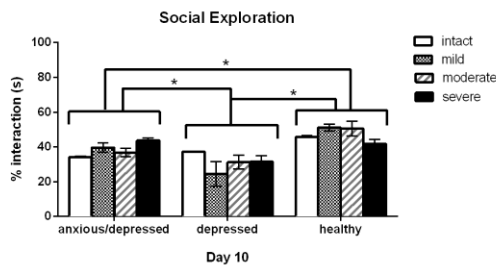
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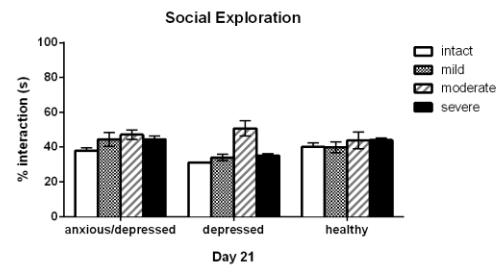
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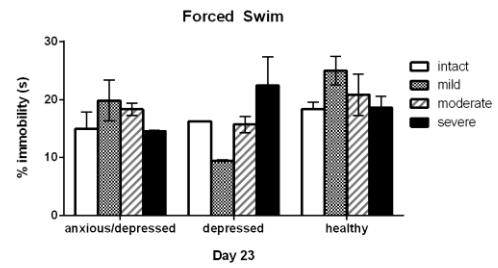
E.



F.



G.



Anxiety measures

Shock probe burying. While main effects of shock probe burying was found by psychological well-being (Figure 7), as per the post hierarchical cluster repeated measure analyses run in the previous section, repeated measure ANOVAs found no main effect of injury severity on shock probe burying across time, $F(3,39)=1.37, p=.267$.

Center activity. First, a repeated measures ANOVA revealed a main effect of injury severity for center activity in the open field, $F(3,43)=3.27, p=.030$. Subsequent ANOVAs on Days 10 and 21 separately confirmed a main effect of injury severity on Day 21, $F(3,43)=3.08, p=.037$. *Post-hoc* Sidak comparisons by injury severity indicated that on Day 21, the severe injury subjects showed a trend for greater center activity than the mild and moderate injury groups, $p=.101$ and $.057$ respectively, Figure 7D. Second, a repeated measure ANOVA by psychological well-being revealed a main effect of psychological well-being for center activity, $F(2,44)=4.19, p=.021$. Subsequent ANOVAs on Days 10 and 21 separately confirmed a main effect of injury severity on Day 21, $F(2,43)=5.63, p=.007$. *Post-hoc* Sidak comparisons indicated that on Day 21, both the ANX/DEP and DEP groups showed reduced center activity relative to the HEALTH group, $ps \leq .05$, Figure 7D.

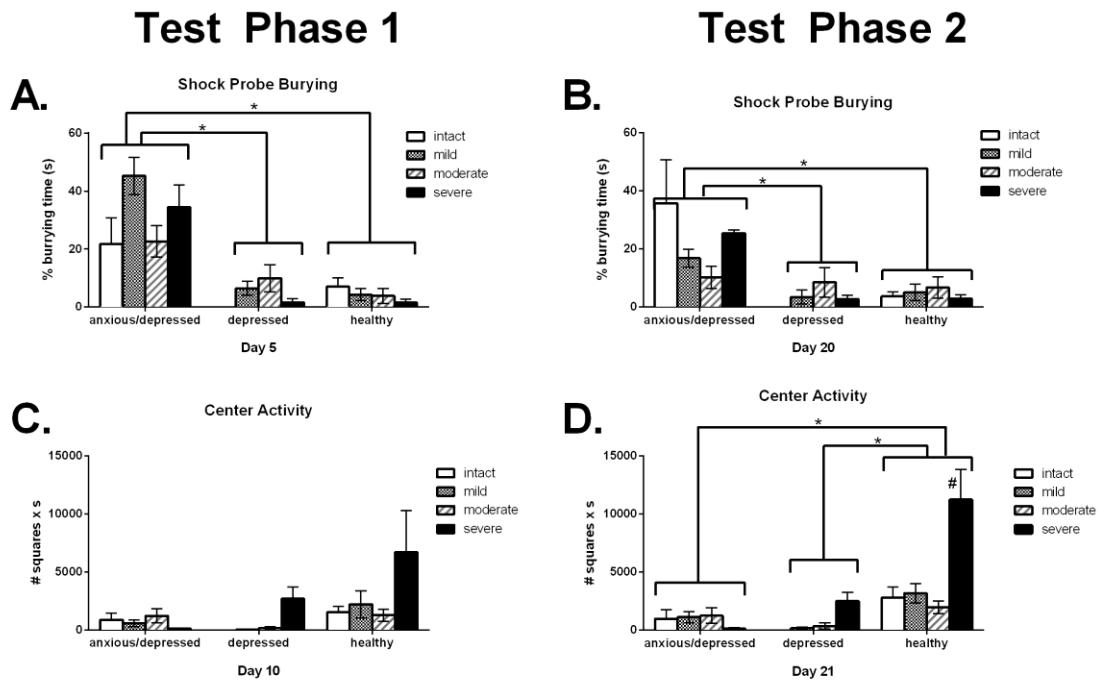


Figure 7- Anxiety measures by psychological well-being and injury severity. The average (\pm standard error of the mean) performance of the ANX/DEP, DEP, and HEALTH group, separated by injury severity level, are shown on all anxiety measures. The severe injury group showed greater center activity than all other injury severity groups on Day 21 (D). The ANX/DEP group showed increased shock probe burying relative to the DEP and HEALTH groups on Days 5 and 20 (A and B). On Day 21, the ANX/DEP and DEP groups showed decreased center activity relative to the HEALTH group (D). * $p < .05$, # significant from all others.

Pain measures

Tail-flick. A repeated measures ANOVA revealed no main effect of injury severity nor psychological well-being, [$F(3,43)=1.413, p=.252, F(2,44)=.676, p=.514$, respectively.]. There were no baseline differences across groups. However, we did find a positive correlation between withdrawal latency at the tail-flick test on Day 11 and number of squares crossed in an open field on Day 10, $r = .288, p < .049$.

Tactile test- paw withdrawal threshold. First, a repeated measure ANOVA revealed a main effect of injury severity for the motor tactile test (paw withdrawal threshold), [$F(3,43)=8.55, p=.000$]. *Post-hoc* Sidak comparisons by injury severity confirmed that across the recovery period, the intact group showed a significantly higher paw withdrawal threshold than all other groups, $ps \leq .05$. Second, a repeated measure ANOVA revealed a trend for differences among psychological well-being groups for the motor tactile test (paw withdrawal threshold), [$F(2,44)=2.758, p=.074$]. Subsequent ANOVAs on Days 11 and 22 separately confirmed a main effect of psychological well-being on Day 22, $F(2,44)=3.451, p=.041$. *Post-hoc* Sidak comparisons by psychological well-being indicated that on Day 22, the ANX/DEP group showed a significantly lower paw withdrawal threshold than the HEALTH group, $p < .05$, Figure 8.

Tactile test- vocalization threshold. First, a repeated measure ANOVA found no main effect of injury severity for the vocal tactile test (vocalization threshold), $F(3,43)=.985, p=.409$. Second, a repeated measure ANOVA revealed a main effect of psychological well-being for the vocal tactile test, $F(2,44)=8.75, p=.001$. Subsequent ANOVAs,

comparing vocalization thresholds on Days 11 and 22 separately, confirmed a main effect of psychological well-being on test Day 22, $F(2,44)=7.024$, $p=.002$, but not on Day 11. *Post-hoc* Sidak comparisons by psychological well-being indicated that on Day 22, the DEP group and the ANX/DEP group showed a lower vocalization threshold than the HEALTH group, $ps < .05$, Figure 8B. Finally, vocalization thresholds on Day 22 were negatively correlated with percent time spent burying on the shock probe burying test on Day 20, $r = -.30$, $p < .04$.

Girdle test. A repeated measures ANOVA revealed a main effect of injury severity for the girdle test, $F(3,43)=2.957$, $p=.043$. Subsequent ANOVAs on Days 11 and 22 separately confirmed a main effect of injury severity on Day 22, $F(3,43)=2.892$, $p=.046$. *Post-hoc* comparisons indicated that on Day 11, the intact group showed a trend towards a lower number of vocalizations at the girdle test than the severe group, $p=.052$. Second, repeated measure ANOVAs did not find a main effect of psychological well-being for the girdle test, $F(2,44)=.190$, $p=.828$. No differences were found at baseline either. However, the number of vocalizations on the girdle test on Day 11 were negatively correlated with sucrose preference on Day 10, $r = -.322$, $p < .027$.

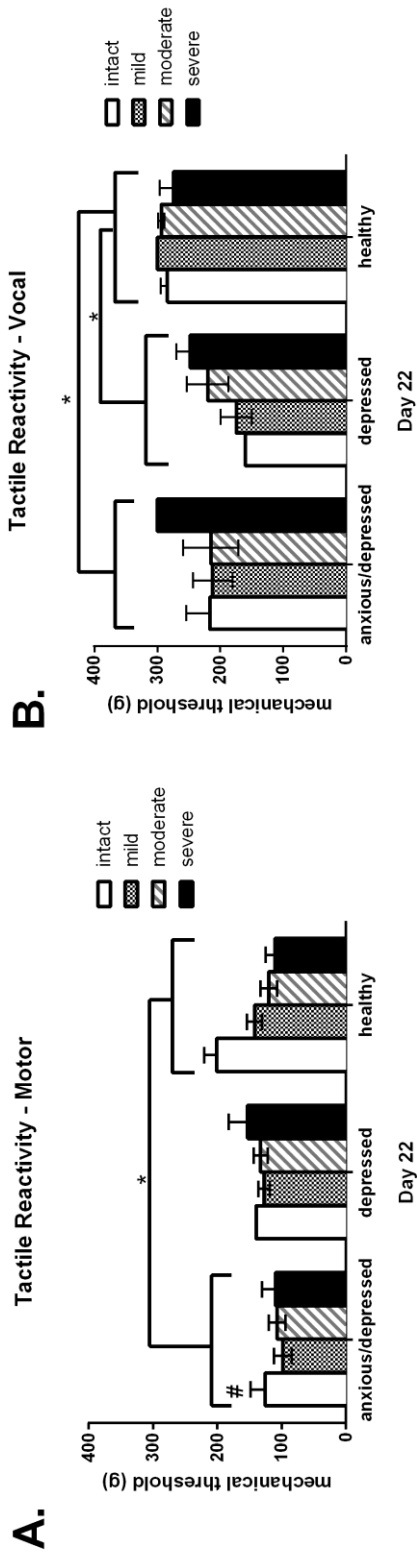


Figure 8- Pain measures by psychological well-being and injury severity. The average (\pm standard error of the mean) performance of the ANX/DEP, DEP, and HEALTH group, separated by injury severity level, are shown on the tactile tests. The intact group showed a higher paw withdrawal threshold than all other groups (A). The ANX/DEP group showed a lower paw withdrawal threshold than the HEALTH group (A). Both the ANX/DEP and the DEP group showed a lower vocalization threshold than the HEALTH group (B). The intact group showed a significantly higher paw withdrawal threshold than all other groups (shown in the ANX/DEP group only in A). * $p < 0.05$, # significant from all others.

Recovery measures

Locomotor recovery of function. First, a repeated measures ANOVA revealed a main effect of injury severity, $F(3,35)=55.15, p=.000$. *Post-hoc* Sidak comparisons by injury severity confirmed that all four groups differed significantly from each other across time, $ps \leq .05$, Figure 9. Second, repeated measure ANOVAs found no main effect of psychological well-being, $F(2,44)=.555, p=.578$, Figure 10.

Weight gain. First, a repeated measures ANOVA revealed a main effect of injury severity on weight gain, $F(3,35)=7.003, p=.001$. *Post-hoc* Sidak comparisons by injury severity confirmed that across time, the severe injury group gained significantly less weight post contusion injury than all the other groups, $ps \leq .05$, Figure 11A and Figure 11B. Second, a repeated measure ANOVA found no main effect of psychological well-being, $F(2,44)=1.809, p=.176$.

Food consumption. First, a repeated measures ANOVA revealed a main effects of injury severity on food consumption across time [$F(3,43)= 3.92, p = .015$]. *Post-hoc* Sidak comparisons found that the severe injury group showed significantly lower weight gain relative to the intact and moderate injury groups across time, $ps \leq .05$. Second, a repeated measure ANOVA did not find a main effect of psychological well-being on food consumption, $F(2,44)= 2.161, p = .127$.

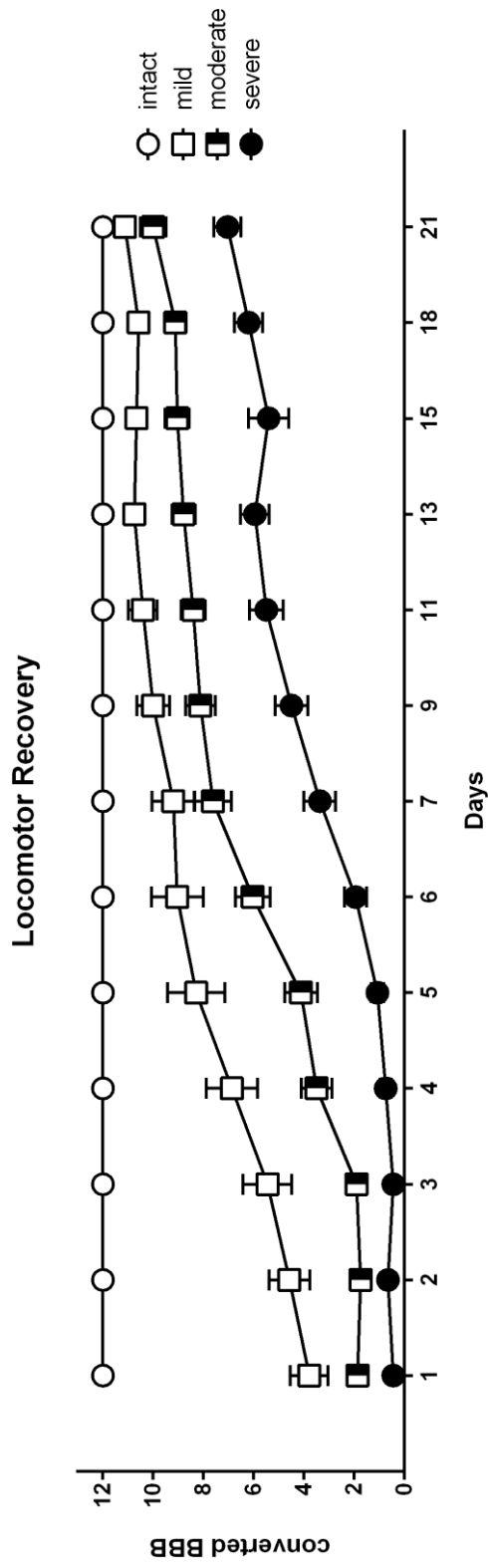


Figure 9- Locomotor recovery of function by injury severity. The average (\pm standard error of the mean) performance of the intact, mild, moderate and severe injury groups are shown on the converted BBB test in the 21 days post injury. All groups differed significantly from each other (significance not shown on graphs).

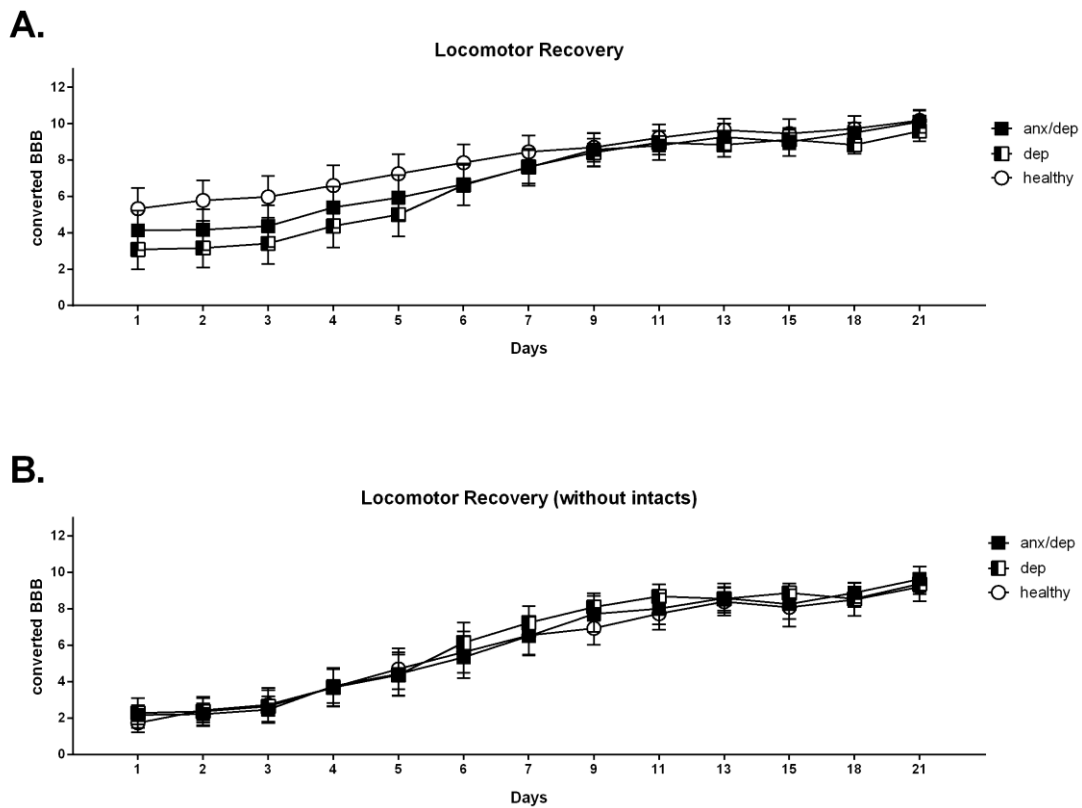


Figure 10 - Locomotor recovery of function by psychological well-being. The average (\pm standard error of the mean) performance of the ANX/DEP, DEP, and HEALTH groups are shown on the converted BBB test in the 21 days post injury. Results with intact subjects included (A) and removed (B) are shown, for clarity. No significant differences were found among the groups.

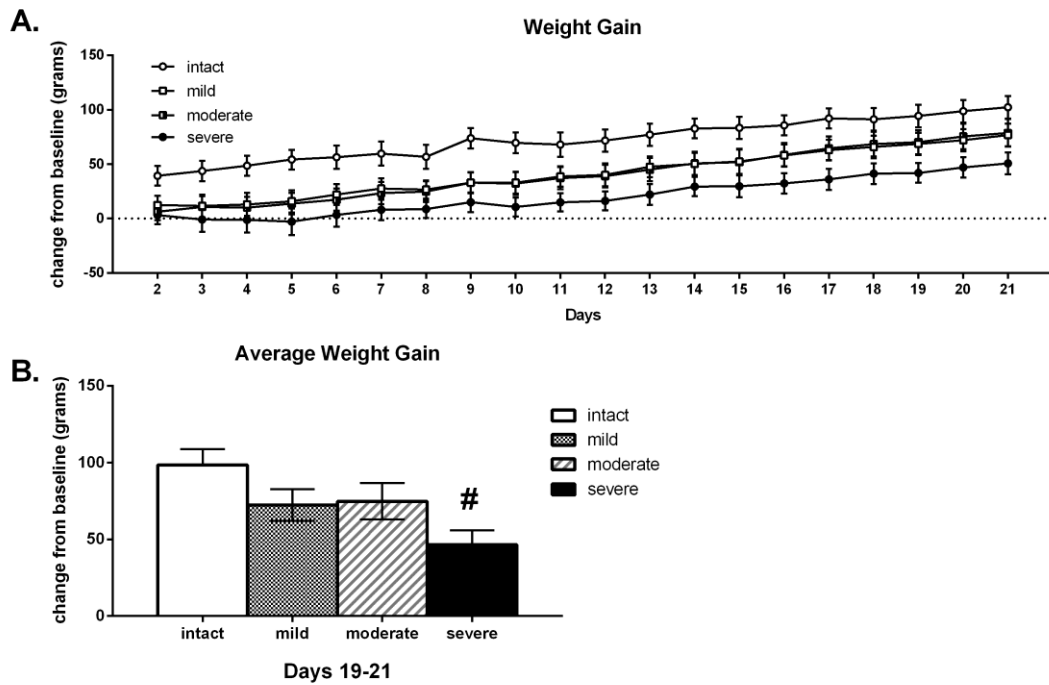


Figure 11- Weight gain by injury severity. The average (\pm standard error of the mean) weight gain of the intact, mild, moderate and severe injury groups in the 21 days post injury are shown (A). The severe injury group showed a significantly lower average weight gain than all other groups (B). * $p < .05$, # significant from all others.

Physiological measures

Thymus. First, no main effect of injury was found. Second, an ANOVA revealed a main effect of psychological well-being on thymus weight, with or without intact subjects included in the analysis [$F(2,44) = 5.09, p = .010, F(2,33) = 5.161, p = .011$, respectively]. *Post-hoc* Sidak comparisons found that thymus weight was significantly decreased in the DEP group relative to the HEALTH group, $p < .05$, Figure 12.

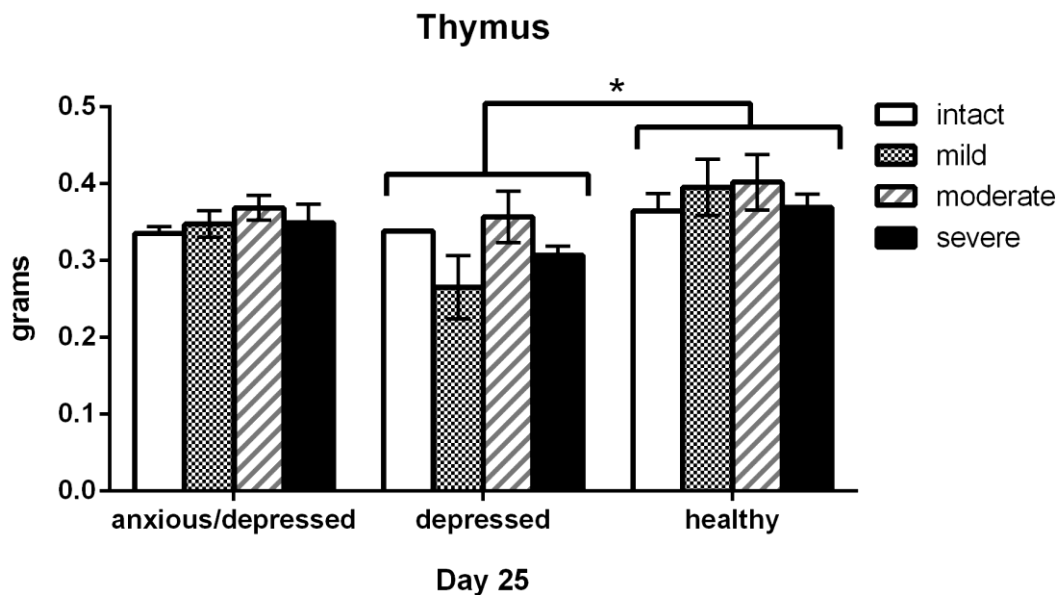


Figure 12- Thymus weight by psychological well-being and injury severity. The average (\pm standard error of the mean) thymus weights are shown by psychological well-being and injury severity groups. No main effect of injury severity was found. The DEP group showed decreased thymus weight relative to the HEALTH group, $* p < .05$.

Alpha-2 macroglobulin. It was expected that levels of alpha-2 macroglobulin would be extremely low in intact subjects. Therefore, they were excluded from analyses, and instead, all alpha-2 macroglobulin levels were normalized to intact animals by dividing each data point for a given day of collection by the intact subjects' average level of alpha-2 macroglobulin for that same day. Furthermore, because alpha-2 macroglobulin is an acute phase protein, we expected group differences to be evident shortly after the spinal cord injury only. Therefore, we conducted ANOVAs on days 1, 10 and 24 separately. First, we found no main effect of injury severity for alpha-2 macroglobulin on any day. Second, we found a trend towards a main effect of psychological well-being at Day 1 post injury only, $F(2,31)= 3.007, p = .064$. *Post-hoc* analyses revealed a trend for both the ANX/DEP and DEP groups to show increased levels of serum alpha-2 macroglobulin on Day 1 relative to the HEALTH group (normalized to intact animals), $p = .16$ and $.09$ respectively, Figure 13A and Figure 13B.

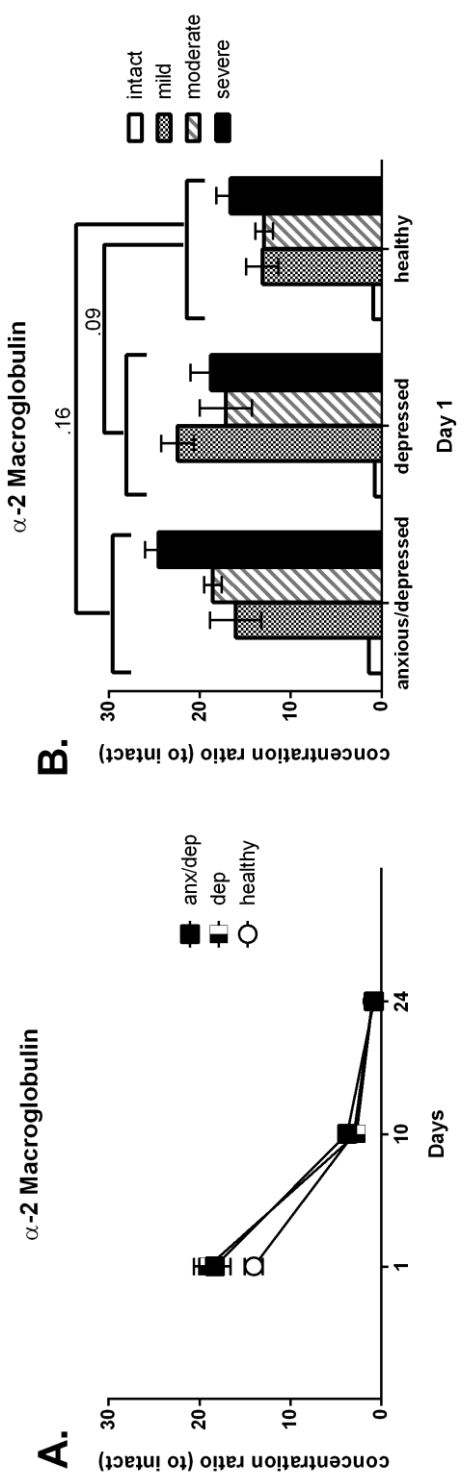


Figure 13- Alpha-2 macroglobulin levels in serum. The average (\pm standard error of the mean) alpha-2 macroglobulin levels normalized to intact are shown by psychological well-being and injury severity groups. No main effect of injury severity was observed among the contused groups. A trend for both the ANX/DEP and DEP groups to show increased levels of serum alpha-2 macroglobulin on Day 1 relative to the HEALTH group was found (A and B), $p = .16$ and $.09$ respectively.

Discussion

These data support the hypothesis that the inflammation due to SCI decreases psychological well-being. Using principal components and cluster analyses, based on behavioral measures of depression and anxiety, we determined that 25.5% of rodents exhibited a depression-like profile (30.5% of contused subjects), consistent with results of an earlier depression study (Luedtke et al., 2014). Another 31.9% of rodents exhibited an anxiety- and depression-like profile (33.3% of contused subjects). This is a novel assessment of psychological well-being in the rodent SCI model. Subjects were identified as exhibiting an anxiety-like profile based on performance on an active anxiety test, the shock probe burying task, and the categorization was confirmed by group differences on a passive anxiety test, center time and activity in the open field task. Depressed subjects showed psychomotor reduction (decreased squares crossed in an open field), decreased interest in social interactions, and passive anxiety (reduced center activity in an open field). Anxious/depressed subjects in turn showed anhedonia (decreased sucrose preference), psychomotor reduction, decreased interest in social interactions, and clear active and passive anxiety (shock probe burying and center activity in an open field, respectively). Notably, behavioral signs of depression and anxiety persisted in both the acute and chronic phases of injury, long after sickness behavior would have resolved.

We observed a large proportion of subjects with a “non-healthy” profile (either falling into the depressed or anxious/depressed categories). In Luedtke et al. (2014), 34.6% of subjects showed a “non-healthy” profile, which was formed by the depressed

cluster (9 subjects out of 26). Conversely, in the present study of 47 subjects, 57.4% of subjects showed a non-healthy profile, which comprised both a depressed cluster (n=12), and an anxious/depressed cluster (n=15). This increased proportion of non-healthy subjects may be attributed to the rise of an anxiety-like profile. Indeed, if we exclude the anxious/depressed cluster, and only consider the depressed cluster versus the healthy cluster, as was done in Luedtke et al. (2014), we find that 37.5% of subjects showed a “non-healthy” profile, a figure similar to that of Luedtke et al. (2014). The increase in anxiety-like signs in this experiment could be explained by one important difference between the two studies. In Luedtke et al. (2014), no blood draws were performed, whereas in the present experiment, blood draws were performed prior to the contusion injury, and on days 1, 10 and 24 post injury. We suspect that the repeated blood draws, in combination with the invasive contusion surgery, may have acted as a stressor to increase anxiety-like behavior. However, these blood draws were necessary in this study, to assess peripheral inflammation across days post SCI.

At a physiological level, the depression-like profile and the anxiety/depression like profile were both associated with increased expression of an acute phase protein, alpha-2 macroglobulin, which is commonly used as an index of peripheral inflammation in the rat. It is also known that thymus weight decreases with depression (Leonard & Song, 1996; Song & Leonard, 2000). Consistent with this, we also found that at time of sacrifice, the thymus weight of depressed subjects was significantly smaller than that of the healthy subjects. Elevated serum levels of pro-inflammatory cytokines have been found in patients with chronic SCI (Davies et al., 2007; Hayes et al., 2002), as well as in

animal models of SCI (Hasturk et al., 2009). As expected, spinally contused subjects overall exhibited increased peripheral inflammation relative to intact subjects.

Given that spinal cord inflammation is known to increase with severity of injury in the acute phase of injury, we initially expected psychological well-being to decrease as a function of injury severity. However, there were no significant differences in alpha-2 macroglobulin levels between the mild, moderate, and severe injury groups, and psychological well-being also did not decrease as a function of injury severity. Indeed, on most behavioral tasks (sucrose preference, open field activity, social exploration, forced swim, and shock probe burying), there was no main effect of injury severity. A main effect of injury severity was only observed for activity in the center of an open field. However, it was the severe injury group that displayed the least anxiety-like behavior (i.e., greater center activity on Day 21 post injury (see Figure 7)). These results, in addition to the fact that we found no differences in locomotor recovery of function between psychological well-being groups, support the initial findings of Luedtke et al. (2014), demonstrating that symptoms of depression and anxiety in the rodent model of SCI cannot be attributed simply to reduced motor function affecting performance in the behavioral tasks used to assess psychological well-being.

The effects of pain are more difficult to ascertain. We found that the anxious/depressed group showed a lower paw withdrawal threshold than the healthy group on the tactile test, and that both the anxious/depressed and the depressed group showed a lower threshold for vocalization than the healthy group at the tactile test.

Although we found some correlations between pain reactivity (tail-flick test, girdle test, and tactile vocal) and performance on certain behavioral tasks to assess depression- or anxiety-like behavior (open field activity, sucrose preference, and shock probe burying, respectively), these were not systematic. They appeared on one day only (tail-flick and girdle on Day 11, tactile on Day 22), and affected only one behavioral measure each. It is possible that increased pain sensitivity reduces motivation to perform in the behavioral tasks, therefore leading to apparent depression-like behavior that in reality is simply reduced motivation. However, our experimental design does not allow us to systematically address this concern. We can only surmise that the lack of a generalized effect across tasks refutes this explanation. An experiment in which non-steroidal anti-inflammatory drugs are administered before behavioral testing would be useful to answer this question in the future. Based on our results, we suggest that decreased pain thresholds and decreased psychological well-being may share a common underlying factor- inflammation. Rather than pain or decreased motor function producing behavior that resembles depression, we propose that inflammation is the proximate cause of decreased psychological wellbeing (and pain) after SCI.

This experiment is the first SCI study to examine in combination, psychological well-being, pain reactivity, and inflammation following a spinal cord contusion injury in a rodent model. We provide a first set of evidence indicating that following a spinal cord contusion injury, a subgroup of rats exhibit depression- and anxiety-like signs, which are accompanied by increased peripheral inflammation and decreased pain thresholds. These results support the hypothesis that SCI impacts the immune system

which then contributes to decreased psychological well-being following SCI. In the next chapter we assess a tableau of cytokine and chemokine expression peripherally (serum) and centrally (brain and spinal cord) post SCI, and investigate how they might be associated with depression. A better understanding of the role of SCI-triggered inflammation in decreased psychological well-being at a molecular level could eventually lead to better targeted treatments for people with SCI suffering from depression.

CHAPTER IV

**MOLECULAR CHANGES ASSOCIATED WITH PSYCHOLOGICAL WELL-
BEING FOLLOWING SPINAL CORD INJURY**

Introduction

This experiment aimed to investigate the relationship between inflammation post spinal cord injury (SCI) and psychological well-being, as well as pain reactivity. In Chapter III, we found that a subgroup of spinally contused rodents exhibited depression- and anxiety-like signs post SCI, as well as increased serum alpha-2 macroglobulin levels relative to subjects with a healthy psychological well-being. Our results are in line with a growing literature suggesting that at least sub-types of mood disorders are characterized by increased activation of the immune system (Smith, 1991; Maes, 1999).

According to the cytokine hypothesis, pro-inflammatory cytokines can be directly involved in inducing depression. Cytokines such as IL-6 and TNF- α can assist in the breakdown of 5-serotonin into 5-hydroxyindoleacetic acid (5-HIAA). In addition, pro-inflammatory cytokines can increase the activity of indolamine 2,3-dioxygenase (IDO) (Maes et al., 2011), which catalyses the conversion of tryptophan, the precursor for serotonin, into kynurenine. As a result, levels of tryptophan available to produce serotonin are reduced (Capuron et al., 2003; Capuron et al., 2001). Cytokines can also alter the synthesis of catecholamines by triggering the synthesis of precursors for

dopamine, norepinephrine and epinephrine (Sperner-Unterweger et al., 2014). These mechanisms are thought to contribute to a cytokine-mediated form of depression.

It is well known that in the hours and weeks following SCI, pro-inflammatory cytokine genes and other inflammation-related genes are up-regulated in the spinal cord (Dumont et al., 2001; Jokic et al., 2010; Malaspina et al., 2008; Yip & Malaspina, 2012). In addition, increased serum levels of pro-inflammatory cytokines have been observed in patients with chronic SCI (Davies et al., 2007; Hayes et al., 2002), as well as in animal models of SCI (Hasturk et al., 2009). Given the psychoneuroimmunological evidence suggesting an association between immune activation and depression, it is possible that increases in pro-inflammatory cytokines following SCI contribute to decreased psychological well-being.

To the best of our knowledge, changes in brain cytokines post SCI have not been studied. However, we know that the blood-brain barrier is ruptured as a result of SCI. We also know that peripheral cytokines can reach the central nervous system via various routes, such as active transport, leaky sites, macrophage activation, or through the blood (Roman et al., 2013). Furthermore, there are some indications that peripheral cytokine levels are altered post SCI. Therefore, it is possible that changes in cytokines expression at the level of the spinal cord following injury impact peripheral and supraspinal levels of cytokines, and subsequently play a role in decreased psychological well-being post SCI.

In this study, we hypothesized that the increased incidence of depression and anxiety-like signs observed in contused subjects in Chapter III would be associated with increased peripheral and central levels of pro-inflammatory cytokines. Building on data collected in Chapter III, in the present chapter, we used Multiplex assays to identify changes in cytokine and chemokine levels both peripherally (serum) and centrally (brain and spinal cord).

Methods

The tissue collected from the subjects in the Injury Severity and Stress experiment (Chapter III) was analyzed. This included serum, hippocampi and spinal cords from subjects in the mild, moderate, or severe contusion (see General Methods, *Surgery*, for detailed procedures) conditions or in the intact control condition (n=12/group), at time points corresponding to baseline and days 1, 10 and 24 (serum) or 25 (hippocampi and spinal cord) post contusion injury. The hippocampi were chosen for these brain analyses because pre-clinical and clinical studies of depression consistently find that depressed subjects show decreased hippocampal volume (Duman et al., 1999; MacQueen et al., 2003; Paradise et al., 2012), increased microglial density (Stockmeier et al., 2004) and increased glucocorticoid levels (Bremner et al., 2000).

Protein extraction

The lysis buffer was made as described in Bake, Selvamani, Cherry, & Sohrabji, 2014 and Jezierski & Sohrabji, 2001: 0.5M Tris (pH 7.4), 5M NaCL, 50% glycerol to stabilize protein, 100mM EGTA (pH 8) to inhibit metalloproteases and cysteine

proteases, 10mM Na-orthovanadate (pH 10) as a phosphatase inhibitor, 1mM ZnCl₂ as a phosphatase inhibitor, 0.5M NaF as a phosphatase inhibitor, reconstituted aprotinin as a phosphatase inhibitor, reconstituted leupeptin to inhibit serine and cysteine proteases, 10% triton X-100 to solubilize membranes and 200mM PMSF with DMSO added at the time of extraction to inhibit serine proteases.

The lysate was obtained by homogenizing the hippocampi and spinal cord in 600 μ L lysis buffer with a pestle on ice. The samples were next centrifuged at 18 000 rpm for 30 minutes at 4 °C, and the supernatant was collected and stored at -20 °C.

Total protein

A BCA Protein Assay Kit was used to assay the total protein concentration in hippocampal and spinal cord supernatant (Pierce, Rockford, IL). Standards from 0 to 200 were made from the Albumin provided in the kit and MQ water (See Table 2).

Samples were prepared by mixing 5 μ L of tissue (spinal and hippocampal) homogenate in lysis buffer and 45 μ L of MQ water in 1.7 mL plastic tubes. The protein assay reagent was prepared in a 50:1 ratio for A:B, and 1mL was added to all standards and all prepared samples. All tubes (standards and samples) were incubated in a water bath for one hour at 37 °C. Then, 200 μ L of each standard and sample were loaded in duplicate onto a 96-well microplate. Absorbance was measured at 562nm on a spectrophotometer (Biomate 3, Thermo Electron Corporation, Waltham, MA). Concentrations were obtained in μ g protein/ μ L.

Standards ($\mu\text{g/mL}$)	Albumin (μL)	MQ Water (μL)
0	0	50
5	1.25	48.75
10	2.5	47.5
15	3.75	46.25
20	5	45
25	6.25	43.75
30	7.5	42.5
35	8.75	41.25
40	10	40
60	15	35
80	20	30
100	25	25
150	37.5	12.5
200	50	0

Table 2- Preparing standard curve (albumin).

Multiplex

Serum and hippocampal levels of 27 cytokine/chemokines were assessed on Days 1 and 25 using a magnetic bead panel Milliplex MAP Kit (Milliplex MAP kit, RECYTMAG-65k). The cytokines and chemokines assessed are listed in Table 3, Table 4 and Table 5. Although we recognize the limitations in identifying cytokines and chemokines as either pro- or anti-inflammatory given the complex dual roles of most, we have organized the analytes according to their most general roles in inflammation. Therefore, Table 3 displays the “pro-inflammatory” cytokines and chemokines, and Table 4 lists the “anti-inflammatory” cytokines and chemokines. Finally, we have added Table 5 to list the cytokines and chemokines for which there is not a clear pro versus anti-inflammatory role defined.

Spinal cord homogenate levels of 20 cytokine/chemokines were assessed using a magnetic bead panel Bio-Plex Pro Rat Cytokine 23-Plex Assay (L80-01V11S5; Bio-Rad Laboratories, Hercules, CA). This panel assessed the same analytes as the Milliplex MAP Kit, except that Eotaxin, Leptin, MIP-1 α , EGF, IP-10, Fractalkine, and MIP-2 were not included. Both kits followed used the same reagents and followed equivalent protocols.

Analyte	Function	References
Eotaxin	Activator and chemoattractor. Also referred to as CCL11.	(Menzies-Gow et al., 2002)
GM-CSF (Granulocyte-macrophage colony-stimulating factor)	Stimulates stem cells granulocyte production (neutrophils, eosinophils, and basophils) and monocytes.	(Shi et al., 2006)
IL-1 α (interleukin-1 α)	Activates a set of immune system response processes shortly after infection.	(Dinarelli, 2009)
MIP1- α (Macrophage inflammatory protein-1 α)	Involved in recruiting and activating leukocytes.	(Cook, 1996)
IL-1 β (interleukin-1 β)	Activates microglia and immune cells (cytokines)	(Audet & Anisman, 2013)
IL-2 (interleukin-2)	Involved in the differentiation of T cells.	(Audet & Anisman, 2013)
IL-6 (interleukin-6)	Stimulates immune response.	(Audet & Anisman, 2013)
IL-12p70 (IL-12 phosphorylated 70 kDa)	Involved in differentiating T cells to Th1 cells. Stimulates production of TNF- α and IFN- γ .	(Muller-Berghaus et al., 2004)
IFN- γ (interferon- γ)	Activates microglia, natural killer cells and macrophages	(Audet & Anisman, 2013)
IL-5 (interleukin-5)	Stimulates B cell growth. Involved in eosinophil activation.	(Kouro & Takatsu, 2009)
IL-17a (interleukin-17a)	Involved in delayed-type reactions. Increases chemokine production for the recruitment of monocytes and neutrophils to inflammation site.	(Jin & Dong, 2013)
IL-18 (interleukin-18)	Stimulates T cell and natural killer cell maturation, and stimulates IFN- γ production.	(Audet & Anisman, 2013)

Table 3- Pro-inflammatory cytokines and chemokines assessed with Multiplex assay.

Analyte	Function	References
MCP-1 (monocyte chemotactic protein-1)	Recruits monocytes, memory T cells and dendritic cells to inflammation site. Also referred to as chemokine C-C motif ligand 2 (CCL2).	(Deshmane, Kremlev, Amini, & Sawaya, 2009)
IP-10 (interferon gamma-induced protein 10)	Chemoattractant for activated T cells. Involved in recruiting T cells to sites of inflammation. Also referred to as C-X-C motif chemokine 10 (CXCL10).	(Dufour et al., 2002)
GRO-KC (growth-related oncogene/keratinocyte derived chemokine)	Chemoattractant for neutrophils. Also referred to as CXCL1.	(Roy, Richard, Dumas, & Vallieres, 2012)
VEGF (Vascular endothelial growth factor)	Critical in angiogenesis. Can increase IFN- γ and decrease IL-10 in T-cells, therefore increasing pro-inflammatory T-cell differentiation.	(Mor, Quintana, & Cohen, 2004)
LIX (lipopolysaccharide-induced CXC chemokine)	Involved in cell migration and activation of neutrophils. Also referred to as CXCL5.	(Choong, Yong, Tan, Luo, & Lodish, 2004)
MIP-2 (macrophage inflammatory protein 2)	Chemoattractant for neutrophils. Also referred to as CXCL2.	(Driscoll, 1994)
TNF- α (Tumor necrosis factor- α)	Activates microglia and immune cells.	(Audet & Anisman, 2013)
RANTES (regulated on activation, normal T cell expressed and secreted)	Chemoattractant for T cells, eosinophils, and basophils. Also referred to as Chemokine (C-C motif) ligand 5 (CCL5).	(Kapp, Zeck-Kapp, Czech, & Schopf, 1994)

Table 3- Continued.

Analyte	Function	References
IL-4 (interleukin IL-4)	Involved in a shift from Th1 to Th2. Decreases production of macrophages, IFN-gamma, and dendritic cell IL-12.	(Audet & Anisman, 2013)
EGF (epidermal growth factor)	Stimulates cell growth, proliferation and differentiation.	(Menard et al., 2012)
IL-13 (interleukin IL-13)	Inhibits cytokine production by B cells and monocytes. Induces protein-degrading enzyme matrix metalloproteinases (MPPs).	(Wynn, 2003)
IL-10 (interleukin IL-10)	Involved in shift from Th1 to Th2. Reduces immune response activation.	(Audet & Anisman, 2013)

Table 4- Anti-inflammatory cytokines and chemokines assessed with Multiplex assay.

Analyte	Function	References
G-CSF (Granulocyte colony-stimulating factor)	Neurogenesis, neuroplasticity. Counters apoptosis. Can have both pro- and anti-inflammatory effects.	(Boneberg & Hartung, 2002; Lawlor et al., 2004)
Leptin	Marker of inflammation. Regulates energy consumption. Related to appetite and hunger, metabolism, and behavior.	(Fantuzzi & Faggioni, 2000)
Fractalkine	Anti-apoptotic. Involved in chemotaxis and leukocyte adhesion, as well as the survival of various cells during inflammation. Also referred to as CX3CL1.	(White & Greaves, 2012)

Table 5- Unclassified cytokines and chemokines assessed with Multiplex assay.

The assays were run according to the manufacturer's instructions, including for dilution recommendation. In summary, a 96-well microplate was first coated with 200 μL assay buffer/well. The plate was left on a plate shaker for 10 minutes at room temperature, and the contents were decanted. Next, 25 μL of standards and controls were added to the appropriate wells (in duplicates), and 25 μL of assay buffer was added to the background wells and the sample wells. Following this, 25 μL of appropriate matrix solution was added to the background, standards, and control wells. For serum samples, the serum matrix provided with the kit was used as matrix. For the tissue supernatant, lysis buffer (with necessary PMSF) was used as matrix. Next, 25 μL of samples was added to the sample wells. Serum samples were diluted 1:2 in assay buffer, whereas tissue supernatant was not diluted. Then, the premixed beads were sonicated thoroughly and 25 μL of the mixed beads were added to all wells in the dark, after which the plate was sealed, wrapped in foil, and incubated at room temperature (20 to 25 $^{\circ}\text{C}$) for two hours on a plate shaker. Next, the wells were decanted and washed two times using a hand-held magnet, 25 μL of detection antibodies was added to each well, and the plate was sealed, wrapped in foil, and incubated for one hour at room temperature on a plate shaker. After this second incubation, 25 μL of Streptavidin-Phycoerythrin was added to each well, and the plate was incubated as described above for 30 minutes. Finally, wells were decanted, washed twice, and 125 μL of Sheath Fluid was added to all wells. The beads were resuspended for five minutes on a plate shaker before running the plate using a Bio-Plex suspension array system (Bio-Rad Laboratories, CA) which was calibrated on the day of use according to the manufacturer's instructions. The program

was set for 100 μ L/well, 50 beads per bead set. The median fluorescent intensity of all 27 analytes was obtained for all standards and samples, and analyte concentration in samples (in pg/mL) was calculated by deriving a 5-parameter logistic standard curve for each analyte separately.

For serum samples, the calculated concentration for all analytes was multiplied by the dilution factor 2. For protein supernatant samples, the calculated concentration for all analytes was normalized to the total protein content, obtained from the BCA Protein Assay.

Results

The results are reported for serum, hippocampi, and spinal cord separately. First, 4 (injury severity groups) x 3 (hierarchical cluster groups) ANOVAs were conducted on Day 1 and Day 24 (for serum) and Day 25 (for tissue) data separately, to identify main effects of injury severity and psychological well-being as well as interaction effects. If main effects of injury severity were ruled out in the 4 x 3 ANOVAs, one-way ANOVAs were conducted to examine the effects of psychological well-being specifically. For spinal cord tissue, however, one-way ANOVAs were also conducted by injury severity groups alone, since differences by injury severity were expected in the spinal cord based on our hypothesis.

Serum

We assessed the levels of 27 cytokine/chemokines in serum on Day 1 and Day 24 post SCI. Day 10 levels could not be assessed for technical reasons beyond our control,

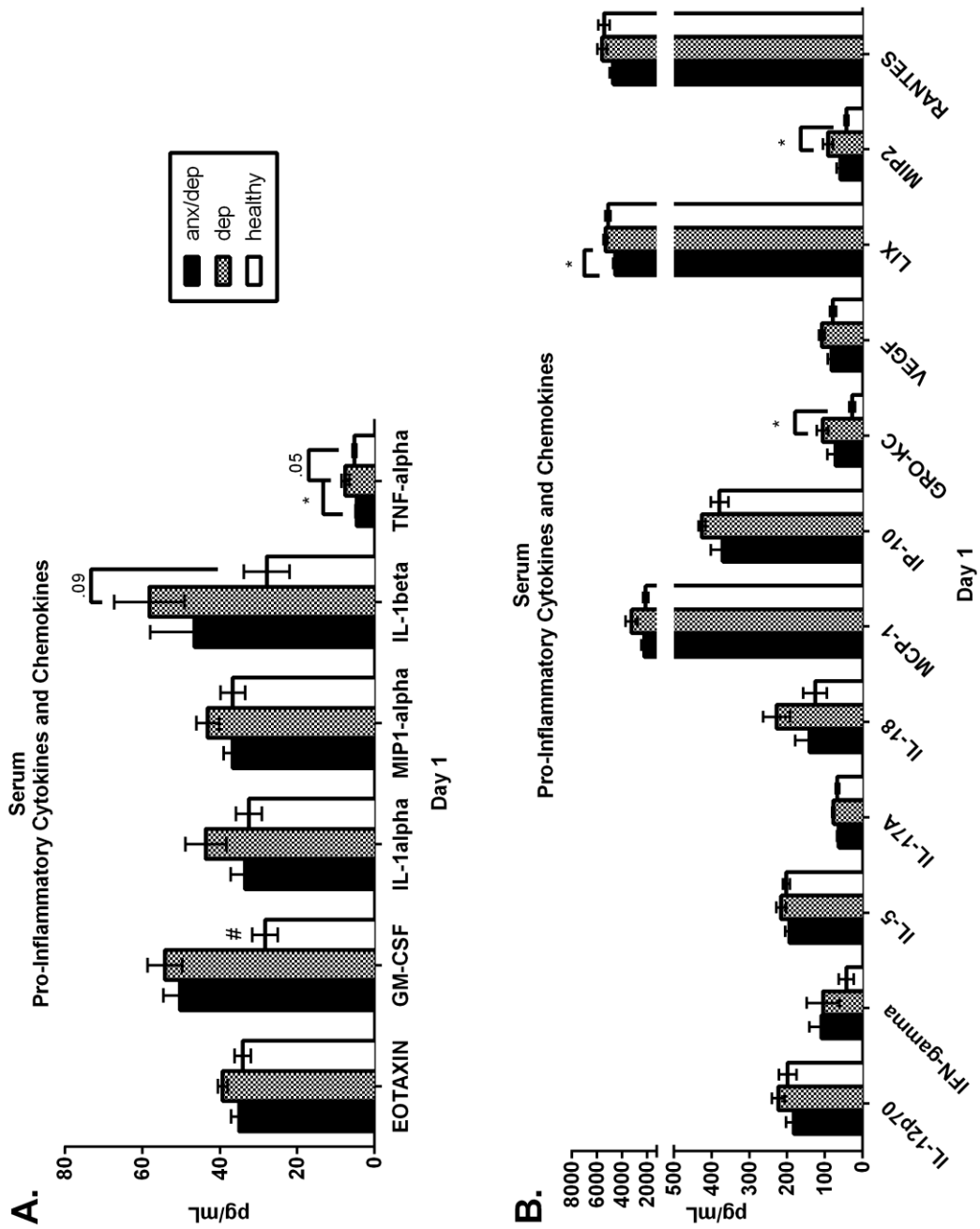
which resulted in the loss of those samples. On Day 1 post SCI, 4 x 3 ANOVAs ruled out a main effect of injury severity for all the analytes except MCP-1, for which a main effect of injury severity was observed [$F(3,19)=5.65$, $p=.006$]. *Post-hoc* Sidak comparisons indicated that the intact group exhibited a significantly lower level of MCP-1 than the moderate and severe injury groups, $ps \leq .05$. For all other analytes, one-way ANOVAs by psychological well-being were conducted, and these were followed by *post-hoc* Sidak comparisons.

One-way ANOVAs revealed a main effect of psychological well-being for GM-CSF [$F(2,27)=11.923$, $p=.000$], GRO-KC [$F(2,27)=5.832$, $p=.008$], MIP-2 [$F(2,27)=5.66$, $p=.009$], TNF α [$F(2,27)=4.889$, $p=.015$], IL-1 β [$F(2,27)=2.727$, $p=.083$], IL-10 [$F(2,27)=4.098$, $p=.028$], Fractalkine [$F(2,27)=3.537$, $p=.043$], and LIX [$F(2,27)=3.731$, $p=.037$]. *Post-hoc* Sidak comparisons revealed that the depressed and anxious/depressed groups showed higher levels of GM-CSF than the healthy group, $ps \leq .05$. Furthermore, the depressed group exhibited levels of GRO-KC and MIP-2 that were higher than those of the healthy group, $ps \leq .05$. The levels of TNF- α , IL-1 β and Fractalkine in the depressed group also showed a trend towards levels higher than those of the healthy group, ($p=.054$, $p=.093$ and $p=.06$, respectively). Moreover, the depressed group showed levels of TNF- α and LIX that were higher than those of the anxious/depressed group, $ps \leq .05$. Furthermore, IL-10 and Fractalkine in the depressed group also showed a trend towards levels greater than those of the anxious/depressed group, ($p=.07$ and $p=.08$, respectively). Finally, the depressed group showed higher levels of IL-10 than the healthy group ($p<.05$) and a trend towards greater levels of

Fractalkine than the healthy group ($p=.06$). The Day 1 differences in pro-inflammatory cytokines by psychological well-being are shown in Figure 14, and anti-inflammatory and unclassified cytokines in Figure 15. Figure 16 shows the significant differences that still hold when the intact subjects are removed from analyses. Non-significant results obtained by injury severity for Day 1 serum are reported in Figure 17 and Figure 18.

On Day 24 post SCI, 4 x 3 ANOVAs ruled out a main effect of injury severity for all the analytes. One-way ANOVAs revealed a main effect of psychological well-being for GM-CSF [$F(2,33)=5.372$, $p=.010$], IL-1 β [$F(2,33)=4.561$, $p=.018$], Rantes [$F(2,33)=4.042$, $p=.027$], and IL-17A [$F(2,32)=3.321$, $p=.049$]. *Post-hoc* Sidak comparisons revealed that the depressed group showed higher levels of GM-CSF and IL-1 β than the anxious/depressed and the healthy group, $ps \leq .05$, Figure 19A. Furthermore, both the depressed group and the anxious/depressed group showed lower levels of Rantes than the healthy group, $ps \leq .05$, Figure 19B. Finally, the anxious/depressed group showed lower levels of IL-17A than the healthy group, $p<.05$, Figure 19B. Although no significant differences were found in anti-inflammatory cytokines, a trend is observed for IL-13, with the depressed group showing increased levels relative to both the anxious/depressed and the healthy group (Figure 20). Figure 21 shows the significant differences that still hold when intact subjects are removed from analyses. Finally, non-significant results obtained by injury severity on Day 24 serum are reported in Figure 22 and Figure 23.

Figure 14- Day 1 serum pro-inflammatory analytes by psychological well-being. The average (\pm standard error of the mean) concentration of pro-inflammatory cytokines and chemokines in serum are shown for the ANX/DEP, DEP and HEALTH groups. Analytes are separated into graphs A and B for ease of reading. IL-6 and IL-2 median fluorescent intensities were out of range for all subjects; therefore, pg/mL concentrations were not obtained. * $p < .05$, # significant from all others.



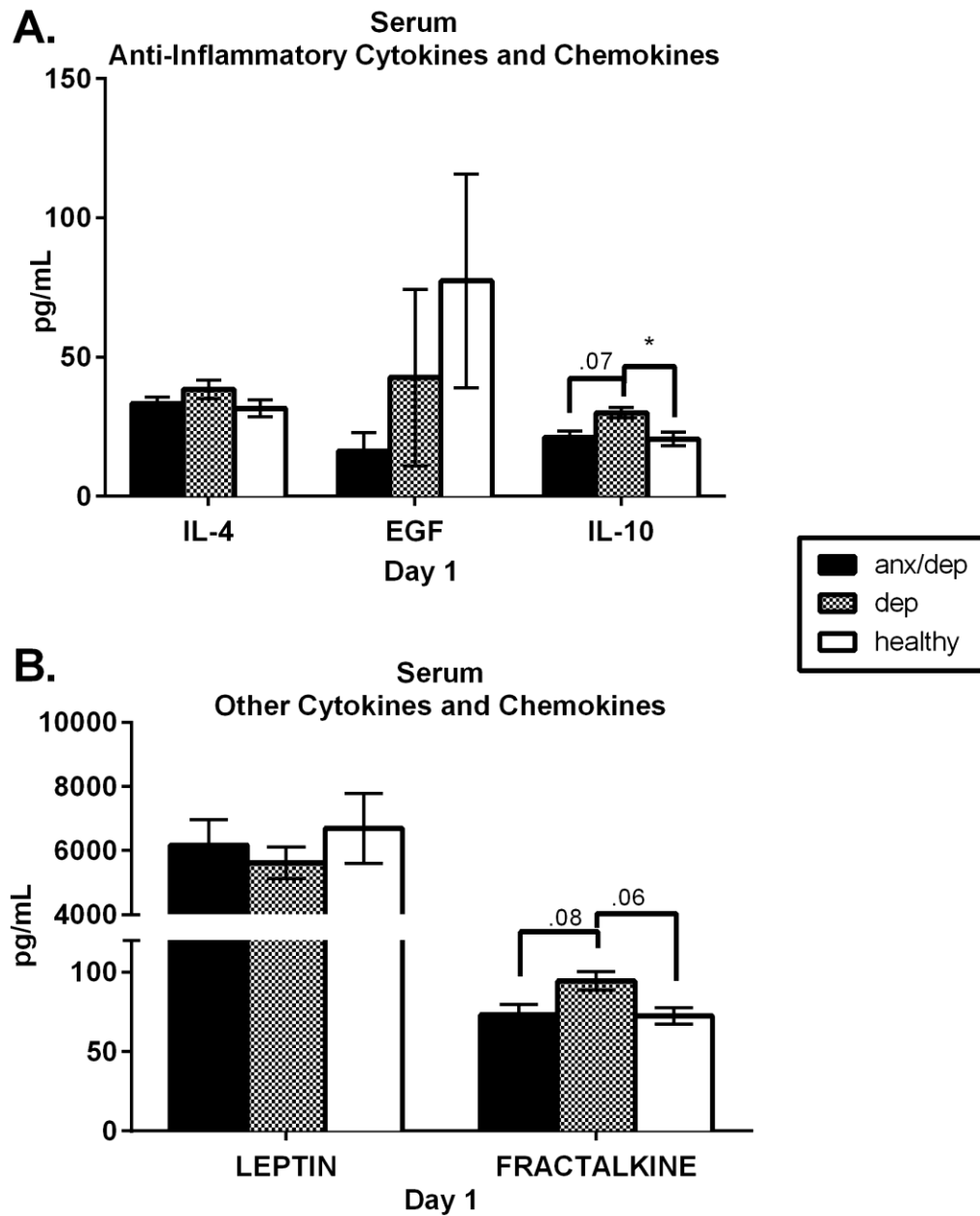


Figure 15- Day 1 serum anti-inflammatory and unclassified analytes by psychological well-being. The average (\pm standard error of the mean) concentration of anti-inflammatory (A) and unclassified (B) cytokines and chemokines in serum are shown for the ANX/DEP, DEP and HEALTH groups. IL-13 and G-CSF median fluorescent intensities were out of range for all subjects; therefore, pg/mL concentrations were not obtained. * $p < .05$.

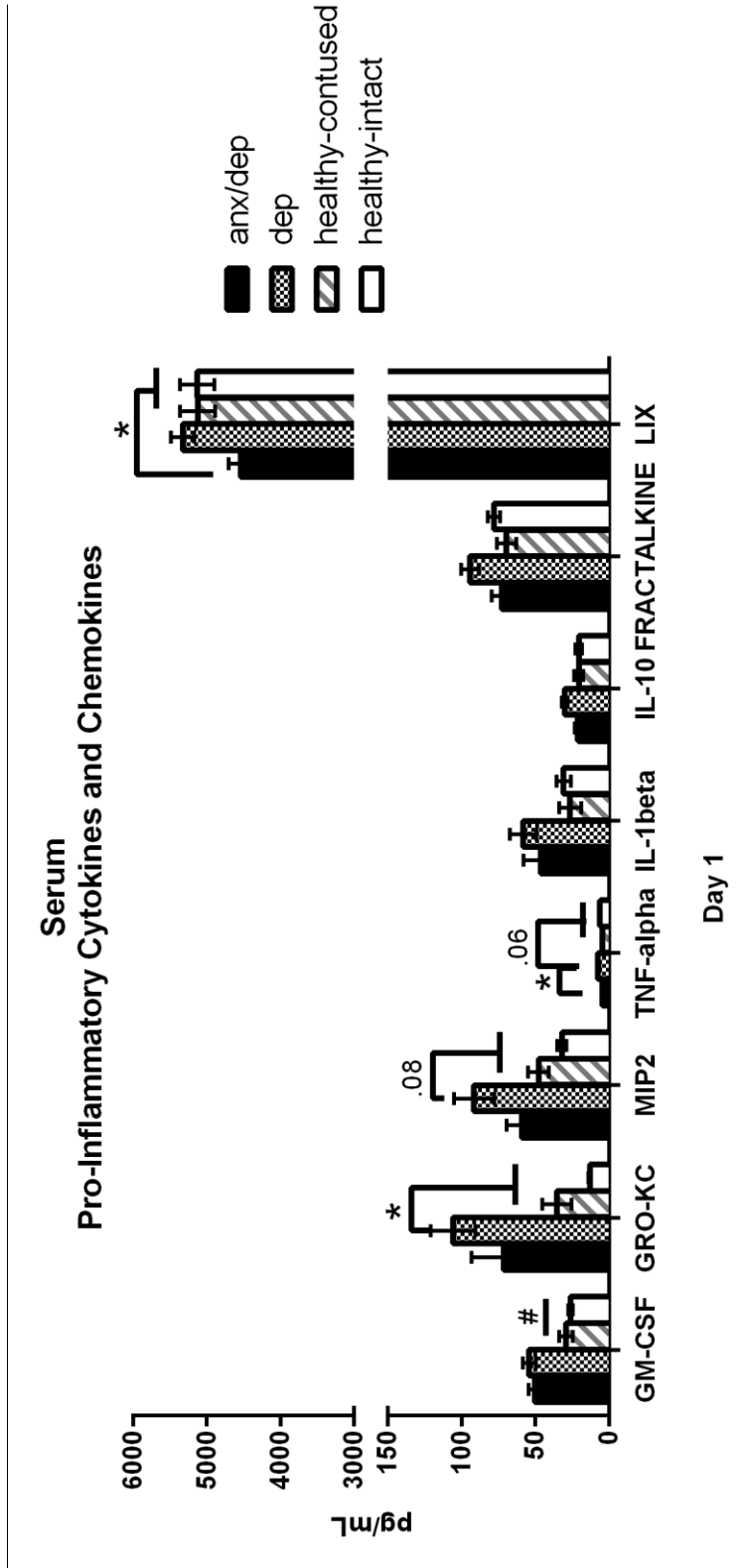
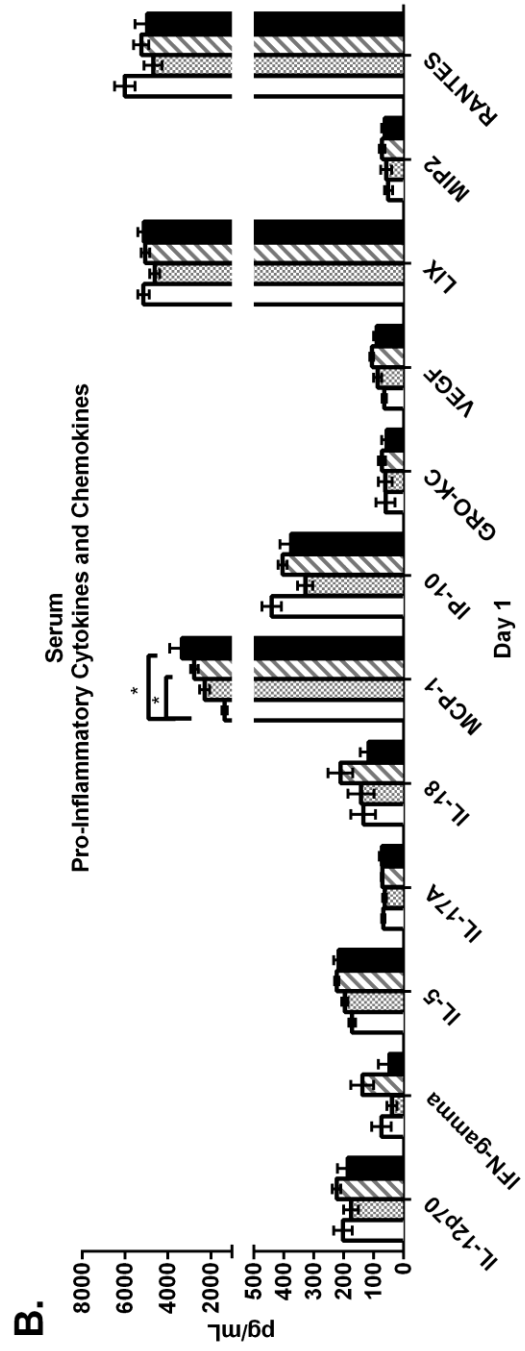
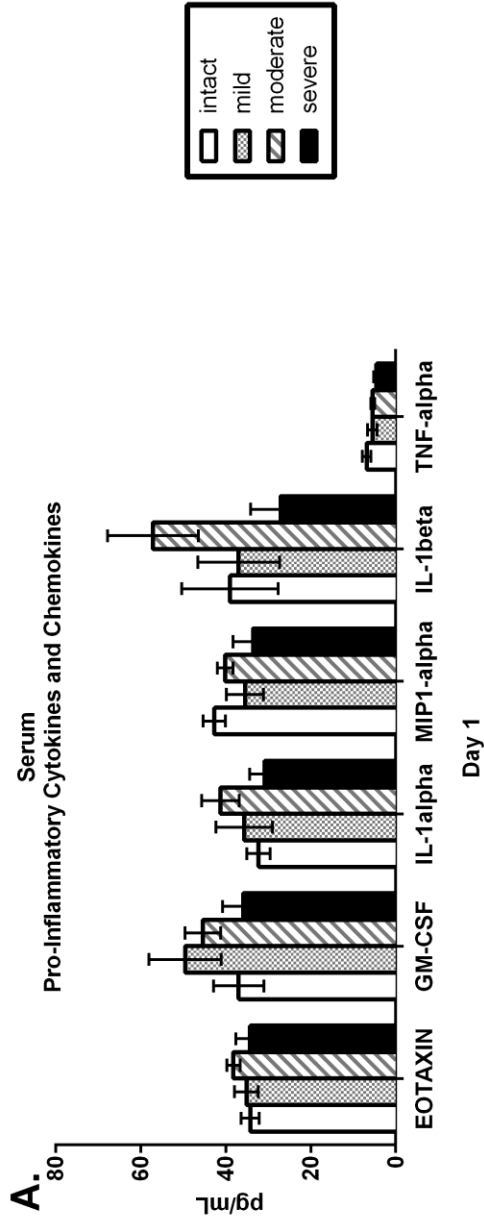


Figure 16- Day 1 significant serum differences, reexamined with healthy intact separated. The average (\pm standard error of the mean) concentration of analytes in serum found to differ by psychological well-being are shown here again. The graph depicts the original data (intacts not removed), with the HEALTH group separated into healthy contused subjects and healthy intact subjects to verify possible HEALTH group differences due to intact subjects. The statistical differences reported here are based on post-hoc Sidak comparisons between the ANX/DEP, DEP and HEALTH groups, with intacts removed. As the graphs indicate, even with intacts removed, the ANX/DEP and DEP groups showed increases in both pro-inflammatory cytokines and chemokines. * $p < .05$, # significant from all others.

Figure 17- Day 1 serum pro-inflammatory analytes by injury severity. The average (\pm standard error of the mean) concentration of pro-inflammatory cytokines and chemokines in serum are shown for the intact, mild, moderate and severe injury groups. Analytes are separated into graphs A and B for ease of reading. IL-6 and IL-2 median fluorescent intensities were out of range for all subjects; therefore, pg/mL concentrations were not obtained. * $p < .05$.



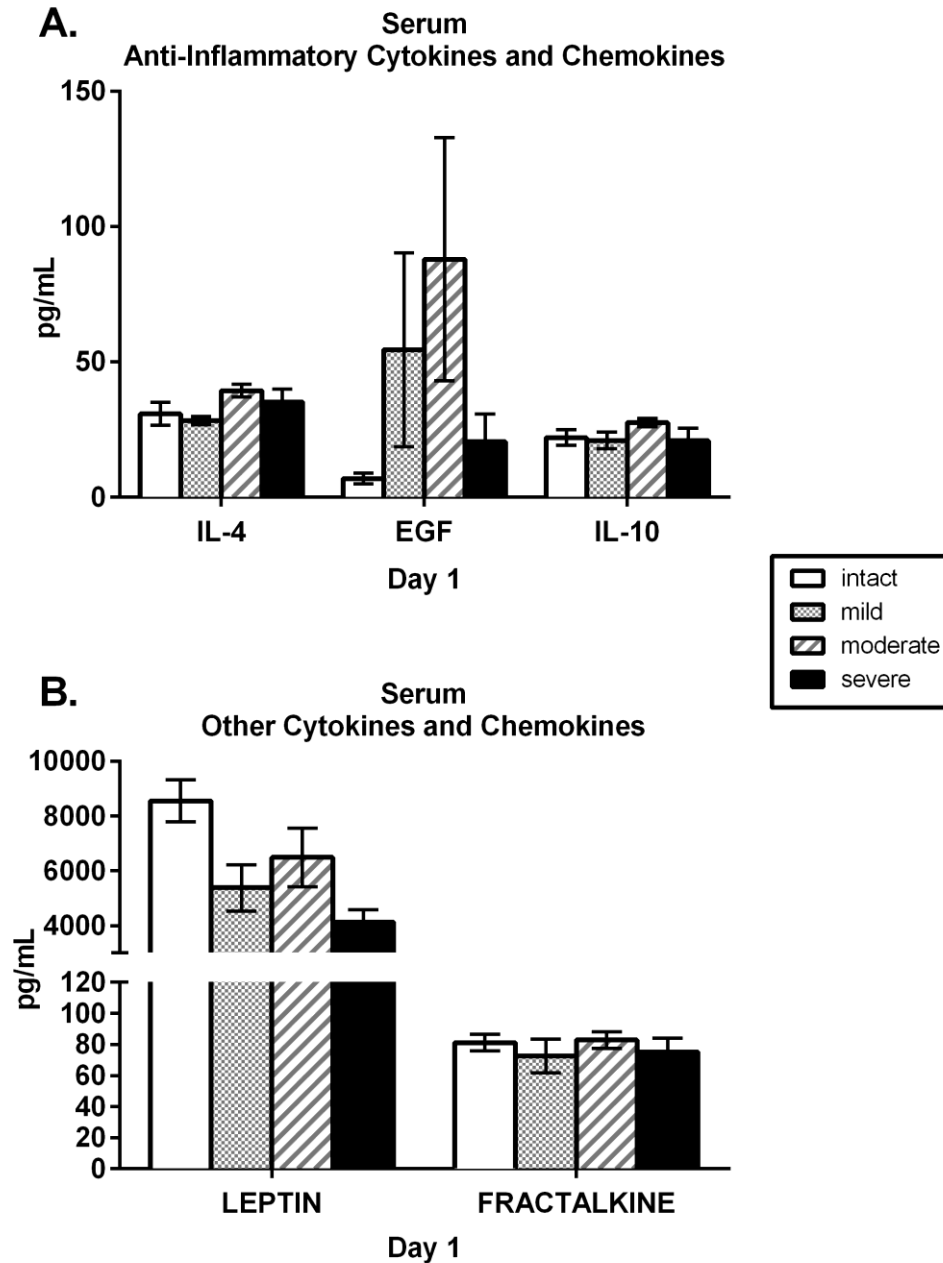
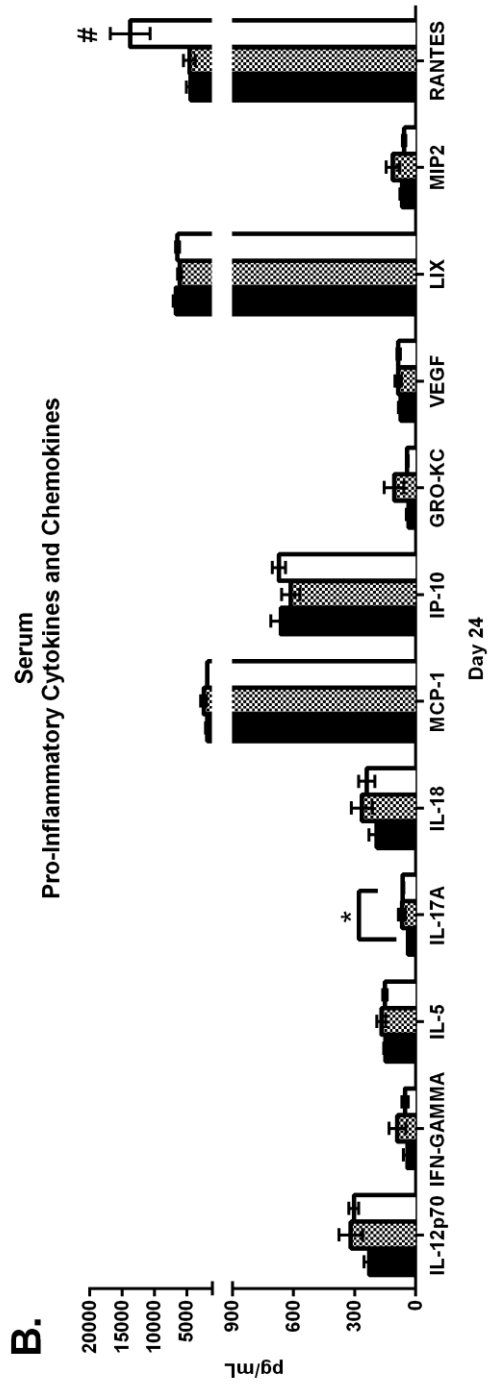
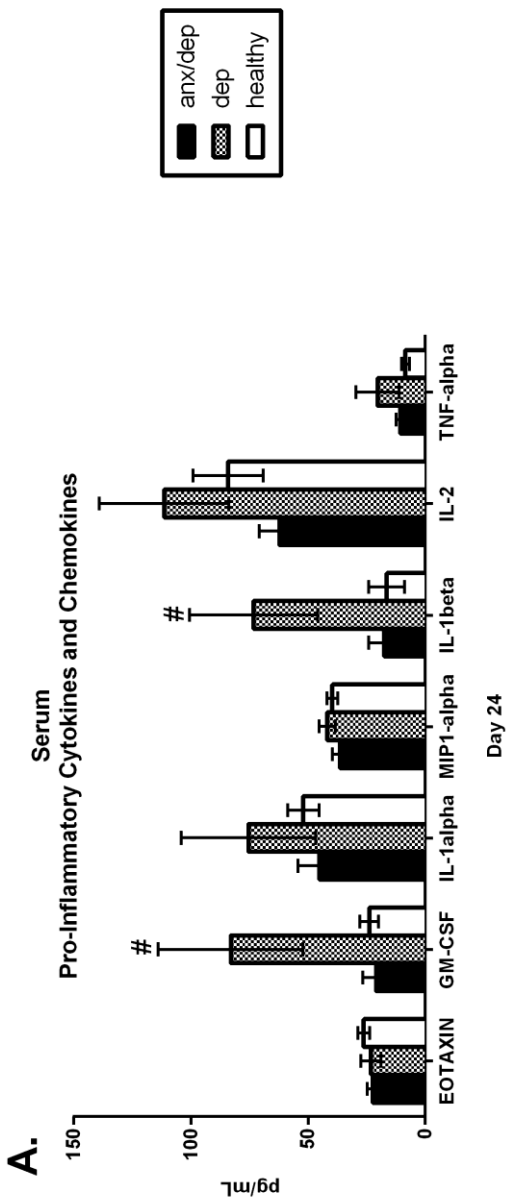


Figure 18- Day 1 serum anti-inflammatory and unclassified analytes by injury severity. The average (\pm standard error of the mean) concentration of anti-inflammatory (A) and unclassified (B) cytokines and chemokines in serum are shown for the intact, mild, moderate and severe injury groups. IL-13 and G-CSF median fluorescent intensities were out of range for all subjects; therefore, pg/mL concentrations were not obtained.

Figure 19- Day 24 serum pro-inflammatory analytes by psychological well-being. The average (\pm standard error of the mean) concentration of pro-inflammatory cytokines and chemokines in serum are shown for the ANX/DEP, DEP and HEALTH groups. Analytes are separated into graphs A and B for ease of reading. IL-6 median fluorescent intensities were out of range for all subjects; therefore, pg/mL concentrations were not obtained. * $p < .05$, # significant from all others.



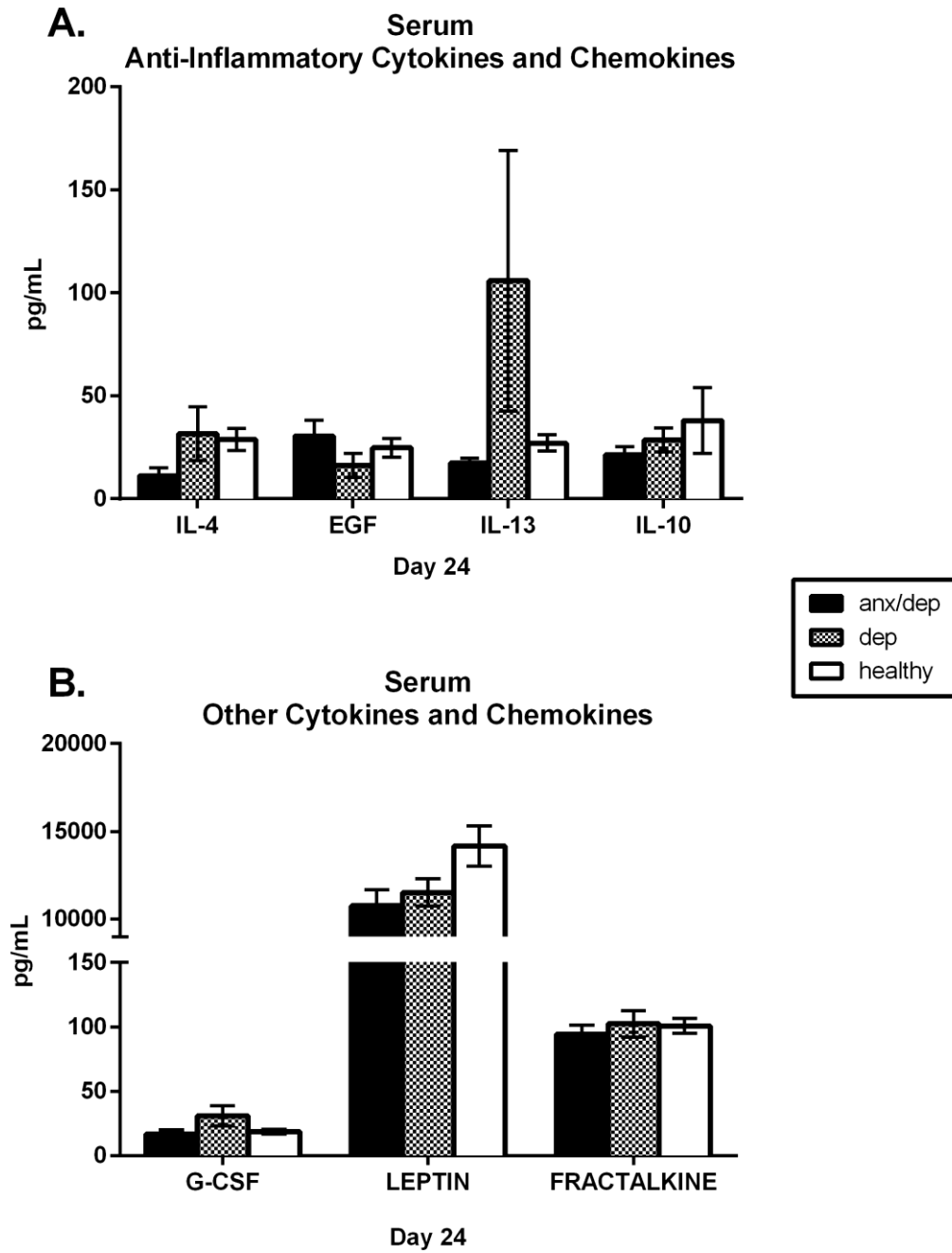


Figure 20- Day 24 serum anti-inflammatory and unclassified analytes by psychological well-being. The average (\pm standard error of the mean) concentration of anti-inflammatory (A) and unclassified (B) cytokines and chemokines in serum are shown for the ANX/DEP, DEP and HEALTH groups.

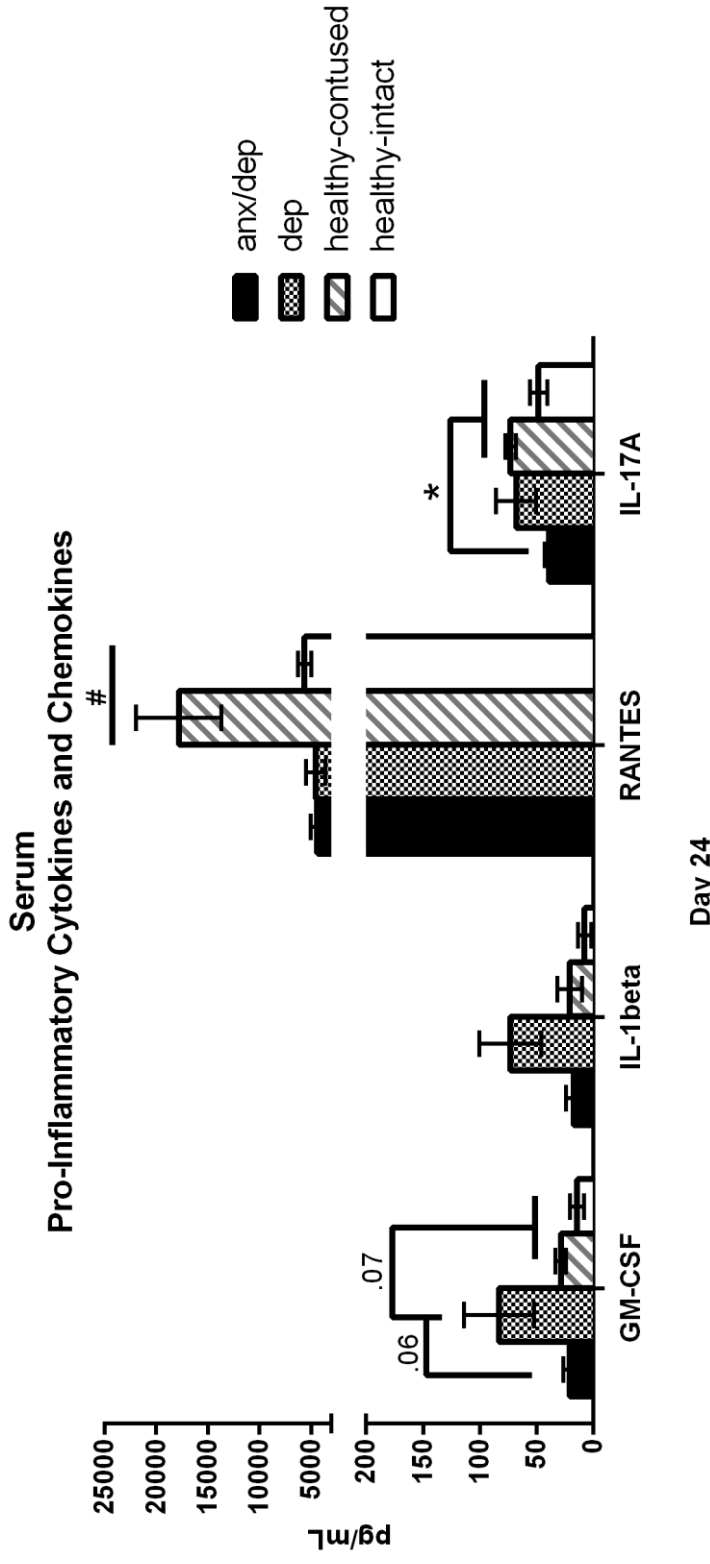
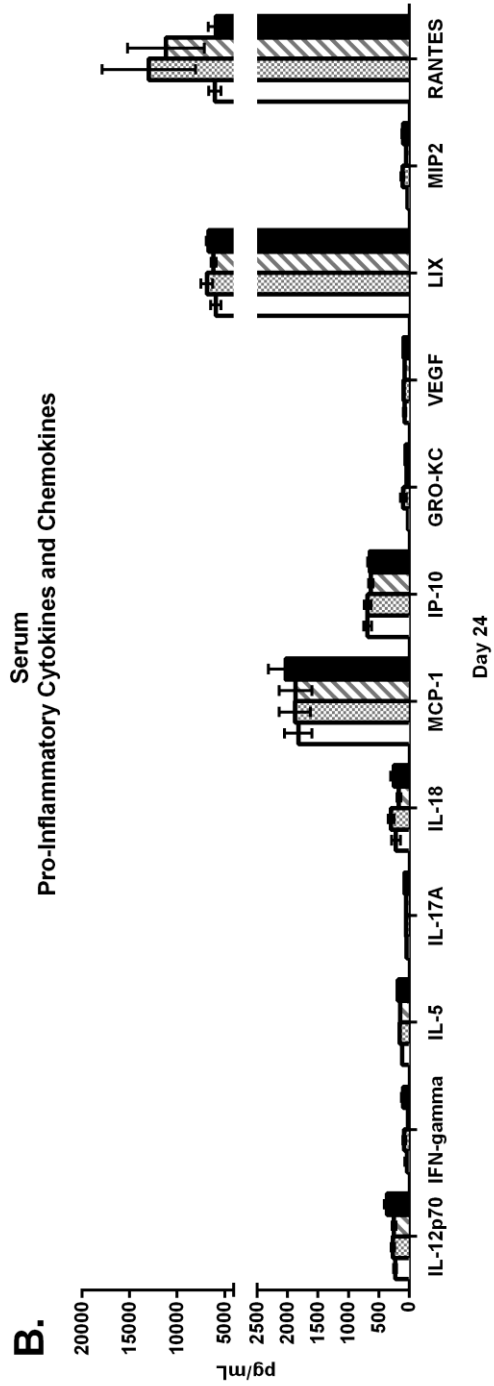
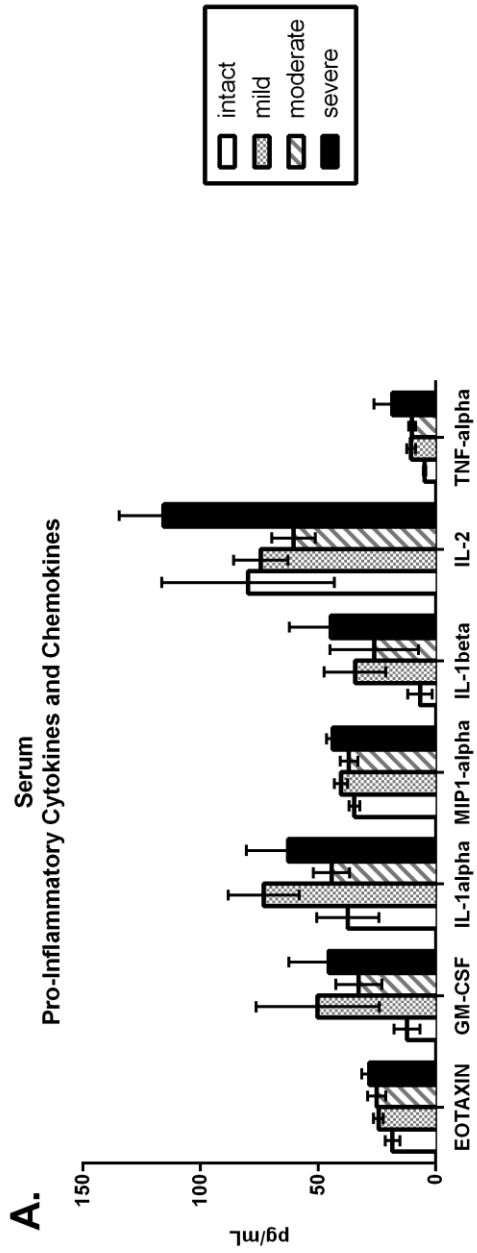


Figure 21- Day 24 significant serum differences, reexamined with healthy intact separated. The average (\pm standard error of the mean) concentration of analytes in serum found to differ by psychological well-being are shown here again, The graph depicts the original data (intacts not removed), with the HEALTH group separated into healthy contused subjects and healthy intact subjects to verify possible HEALTH group differences due to intact subjects. The statistical differences reported here are based on post-hoc Sidak comparisons between the ANX/DEP, DEP and HEALTH groups, with intacts removed. As the graphs indicate, even with intacts removed, the ANX/DEP and DEP groups showed increases in both pro-inflammatory cytokines and chemokines. * $p < .05$, # significant from all others.

Figure 22- Day 24 serum pro-inflammatory analytes by injury severity. The average (\pm standard error of the mean) concentrations of pro-inflammatory cytokines and chemokines in serum are shown for the intact, mild, moderate and severe injury groups. Analytes are separated into graphs A and B for ease of reading. IL-6 median fluorescent intensities were out of range for all subjects; therefore, pg/mL concentrations were not obtained.



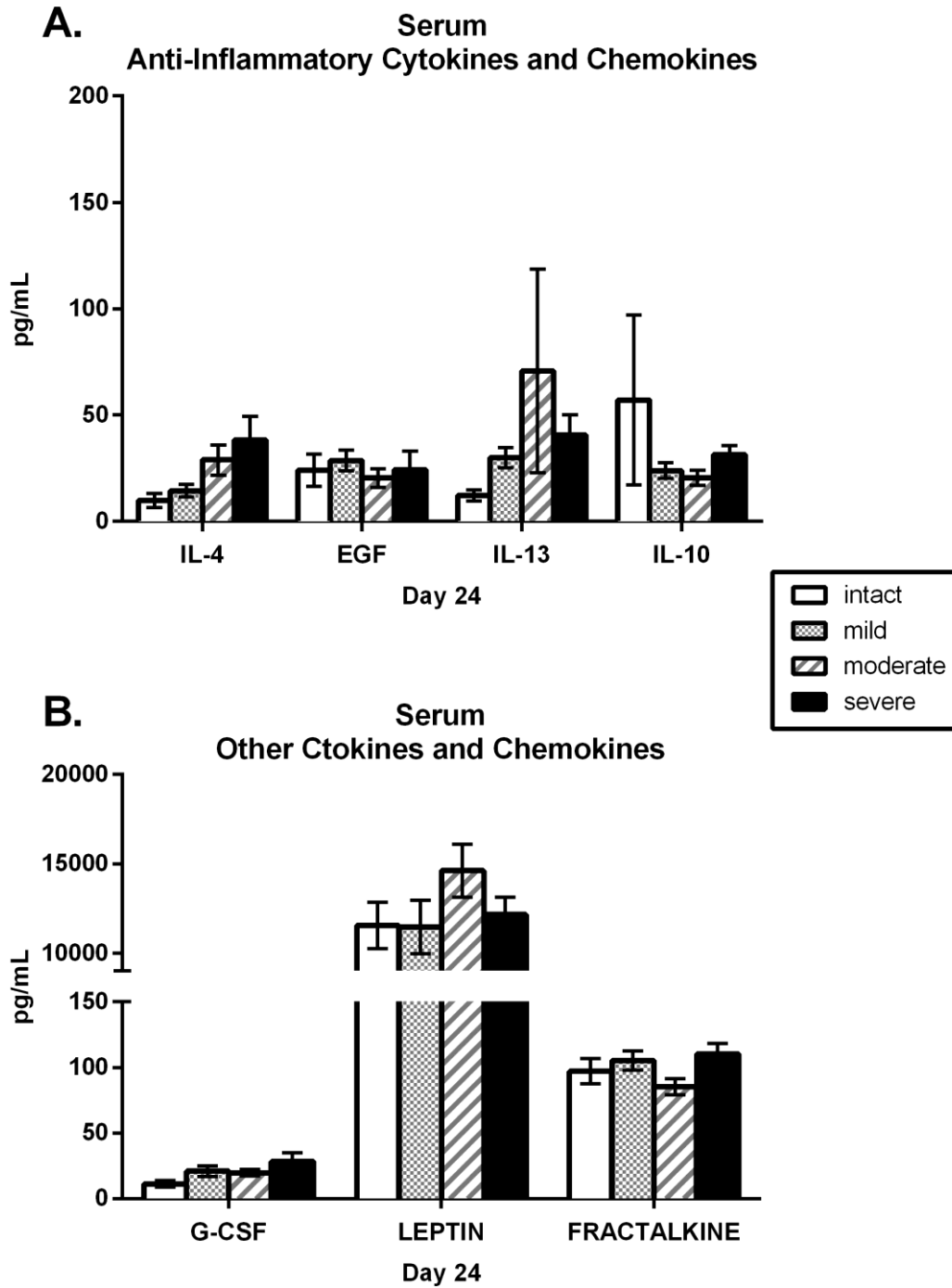


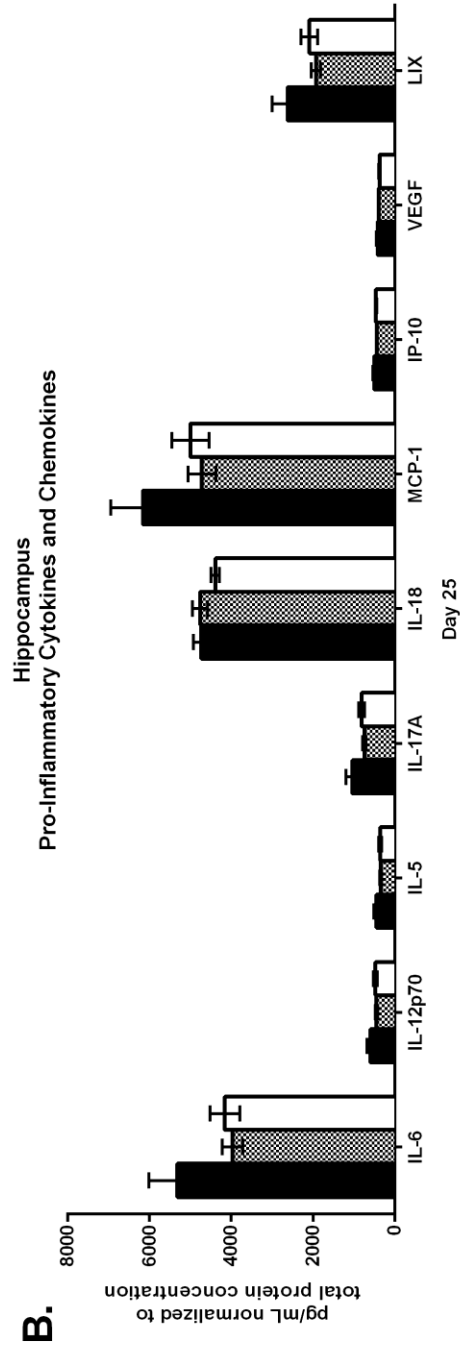
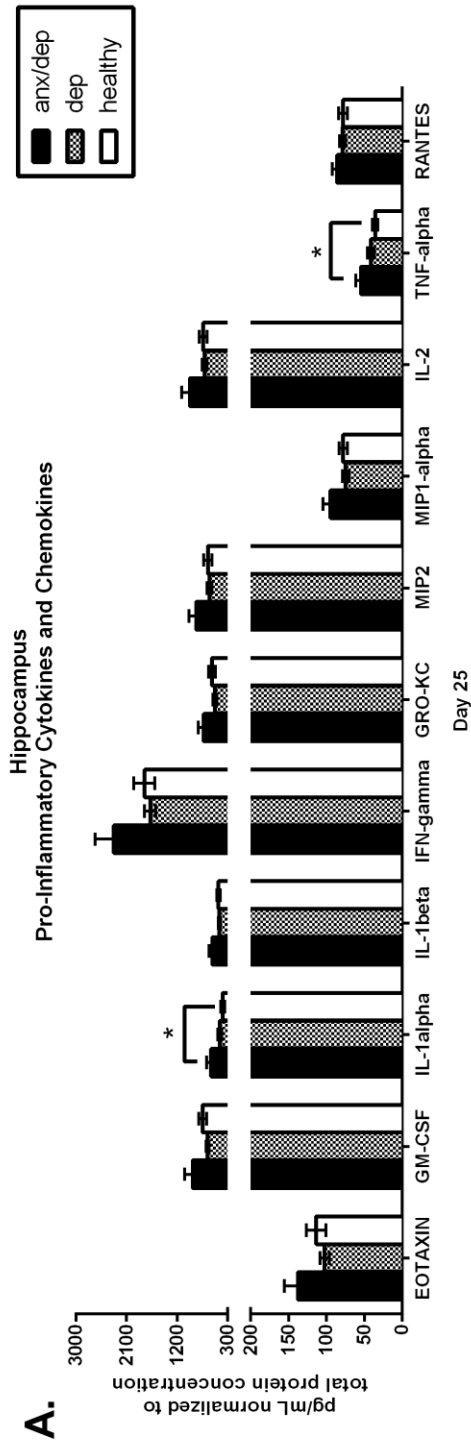
Figure 23 - Day 24 serum anti-inflammatory and unclassified analytes by injury severity. The average (\pm standard error of the mean) concentrations of anti-inflammatory (A) and unclassified (B) cytokines and chemokines in serum are shown for the intact, mild, moderate and severe injury groups.

Hippocampus

We assessed the levels of 27 cytokine/chemokines in the hippocampi protein supernatant (left and right combined). 4 x 3 ANOVAs ruled out a main effect of injury severity for all the analytes. One-way ANOVAs revealed a main effect of psychological well-being for two pro-inflammatory cytokines, Il-1 α [$F(2,32)=4.463, p=.020$] and TNF- α [$F(2,32)=3.537, p=.041$].

Post-hoc Sidak comparisons revealed that the anxious/depressed group showed higher levels of Il-1 α and TNF- α than the healthy group, $ps \leq .05$, Figure 24A. No significant differences were found for anti-inflammatory cytokines and chemokines (Figure 25). Figure 26 shows the significant differences that still hold when the intact subjects are removed from analyses. The non-significant results obtained by injury severity are reported in Figure 27 and Figure 28.

Figure 24- Hippocampus pro-inflammatory analytes by psychological well-being. The average (\pm standard error of the mean) concentration of pro-inflammatory cytokines and chemokines in lysate (normalized to total protein concentration) are shown for the ANX/DEP, DEP and HEALTH groups. Analytes are separated into graphs A and B for ease of reading. * $p < .05$.



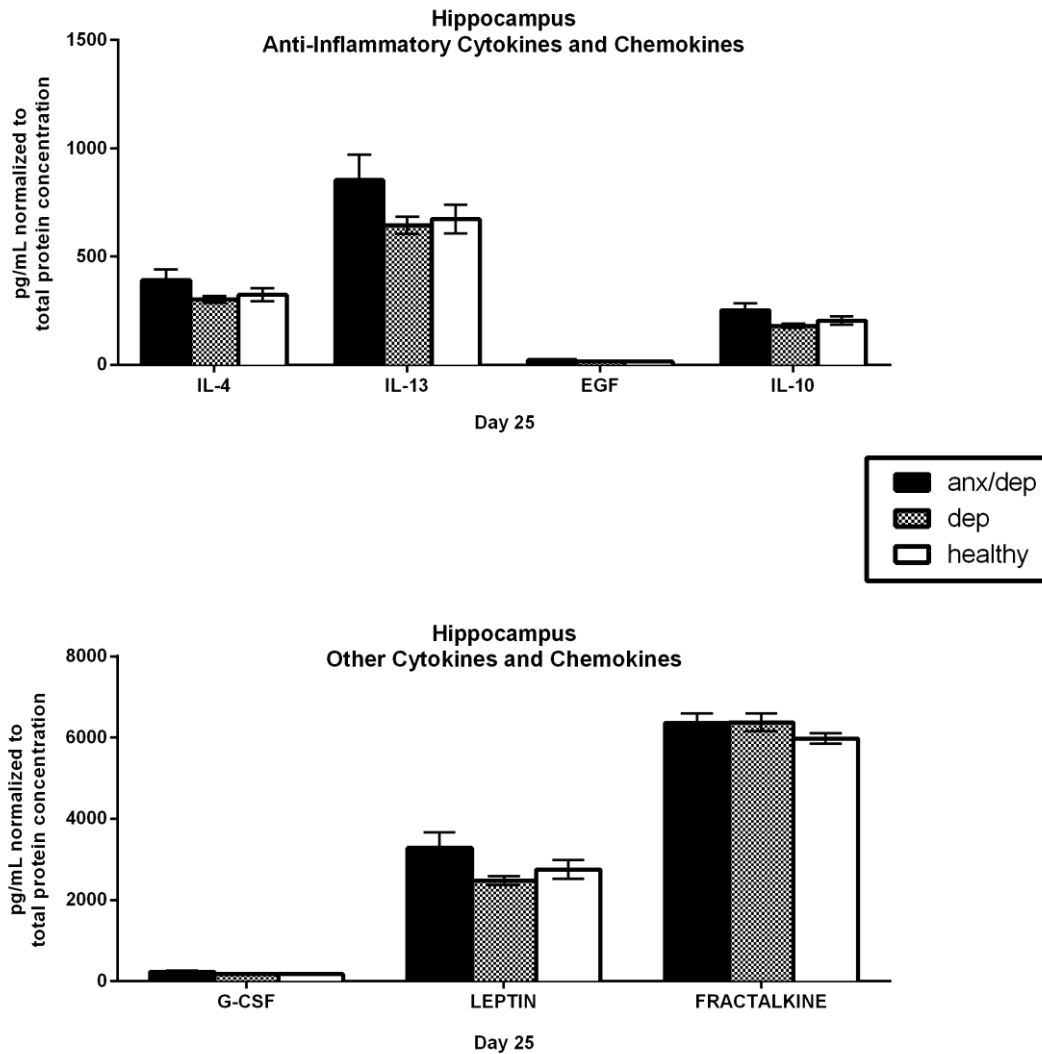


Figure 25- Hippocampus anti-inflammatory and unclassified analytes by psychological well-being. The average (\pm standard error of the mean) concentration of anti-inflammatory (A) and unclassified (B) cytokines and chemokines in lysate (normalized to total protein concentration) are shown for the ANX/DEP, DEP and HEALTH groups.

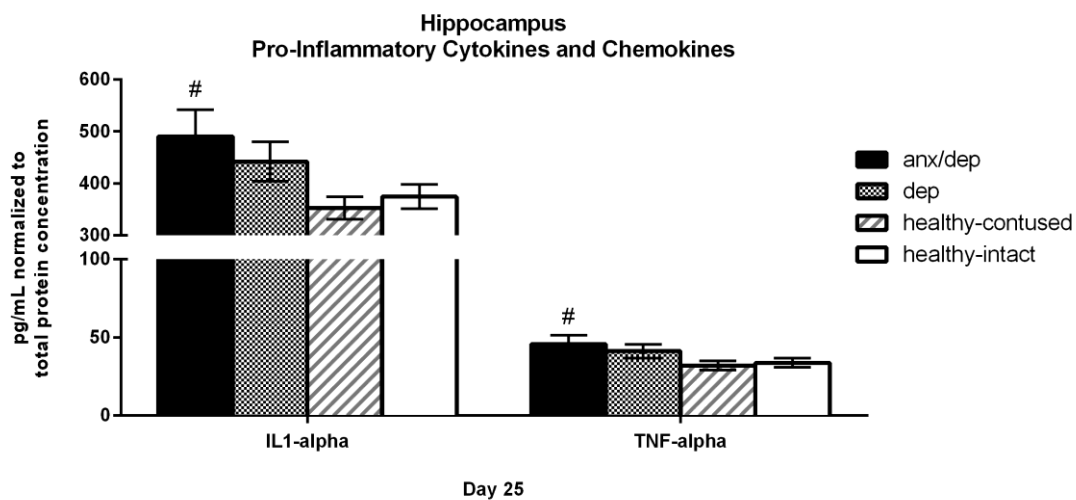
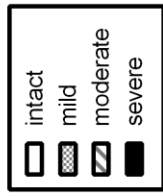
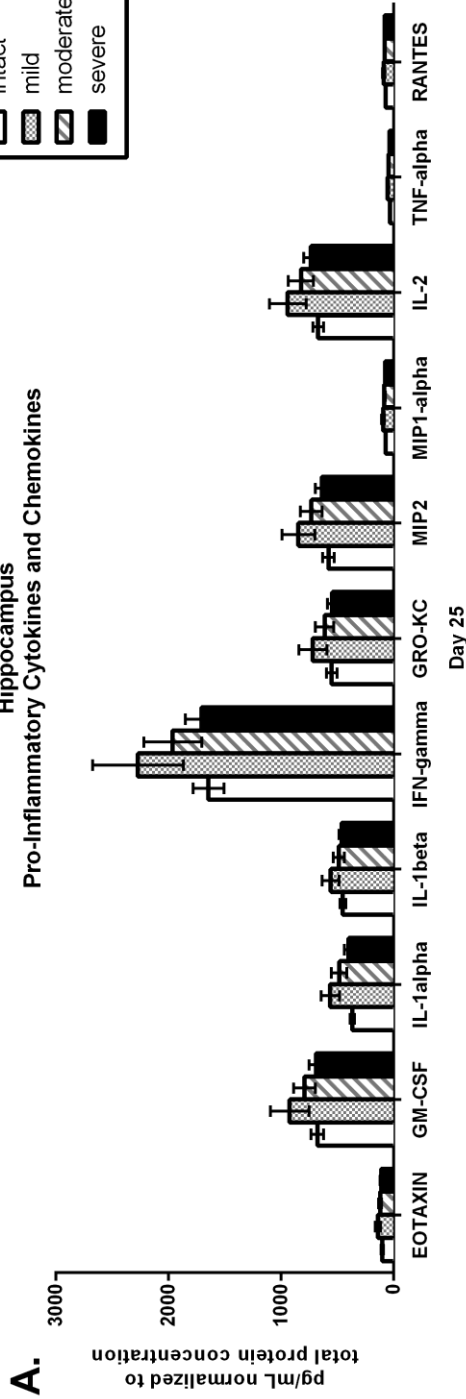


Figure 26- Significant hippocampal differences, reexamined with healthy intact separated. The average (\pm standard error of the mean) concentration of analytes in lysate (normalized to total protein concentration) found to differ by psychological well-being are shown here again. The graph depicts the original data (intacts not removed), with the HEALTH group separated into healthy contused subjects and healthy intact subjects to verify possible HEALTH group differences due to intact subjects. The statistical differences reported here are based on post-hoc Sidak comparisons between the ANX/DEP, DEP and HEALTH groups, with intacts removed. As the graphs indicate, even with intacts removed, the ANX/DEP group shows increases in both pro-inflammatory cytokines and chemokines. * $p < .05$, # significant from all others.

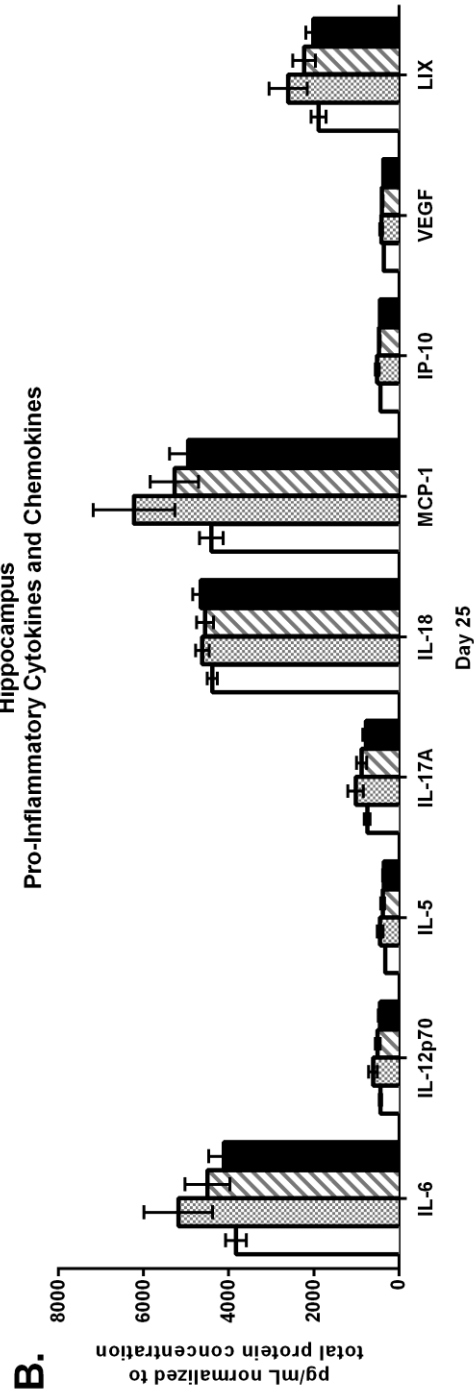
Figure 27- Hippocampus pro-inflammatory analytes by injury severity. The average (\pm standard error of the mean) concentrations of pro-inflammatory cytokines and chemokines in lysate (normalized to total protein concentration) are shown for the intact, mild, moderate and severe injury groups. Analytes are separated into graphs A and B for ease of reading.



Hippocampus
Pro-Inflammatory Cytokines and Chemokines



Hippocampus
Pro-Inflammatory Cytokines and Chemokines



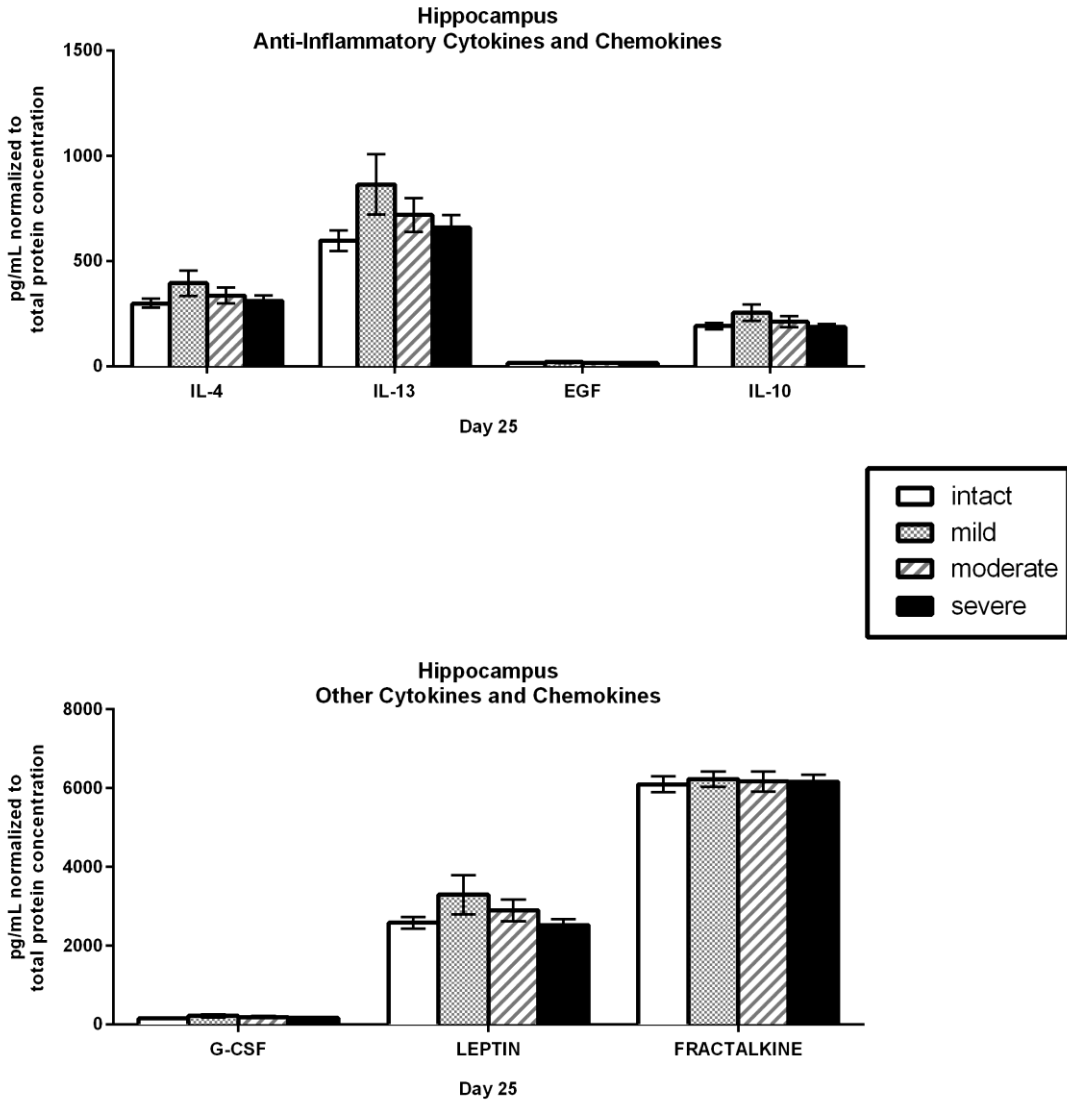


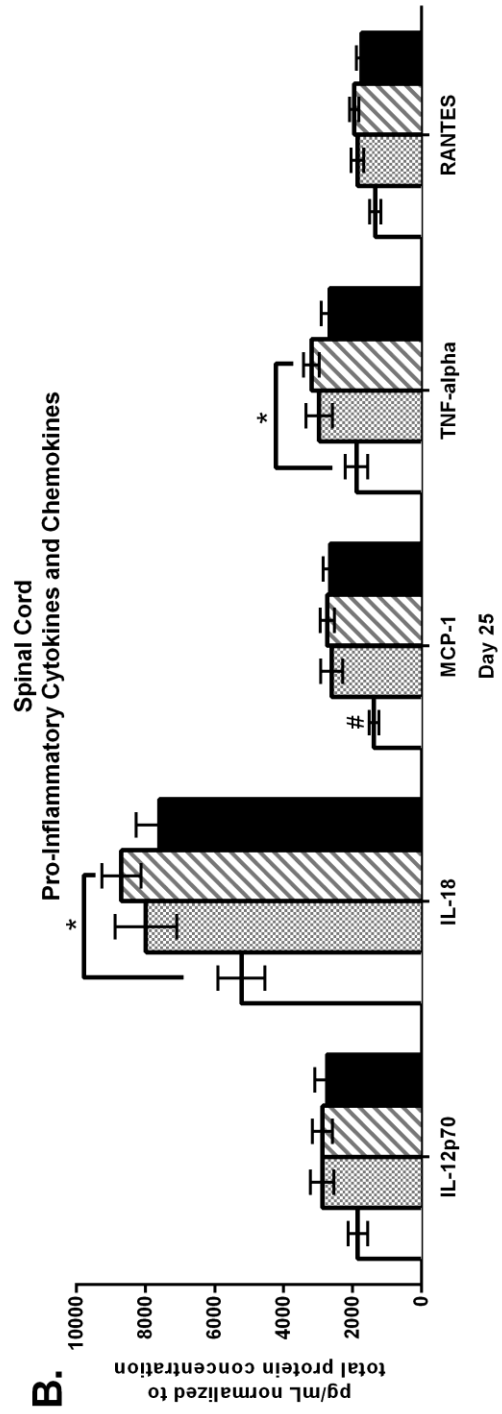
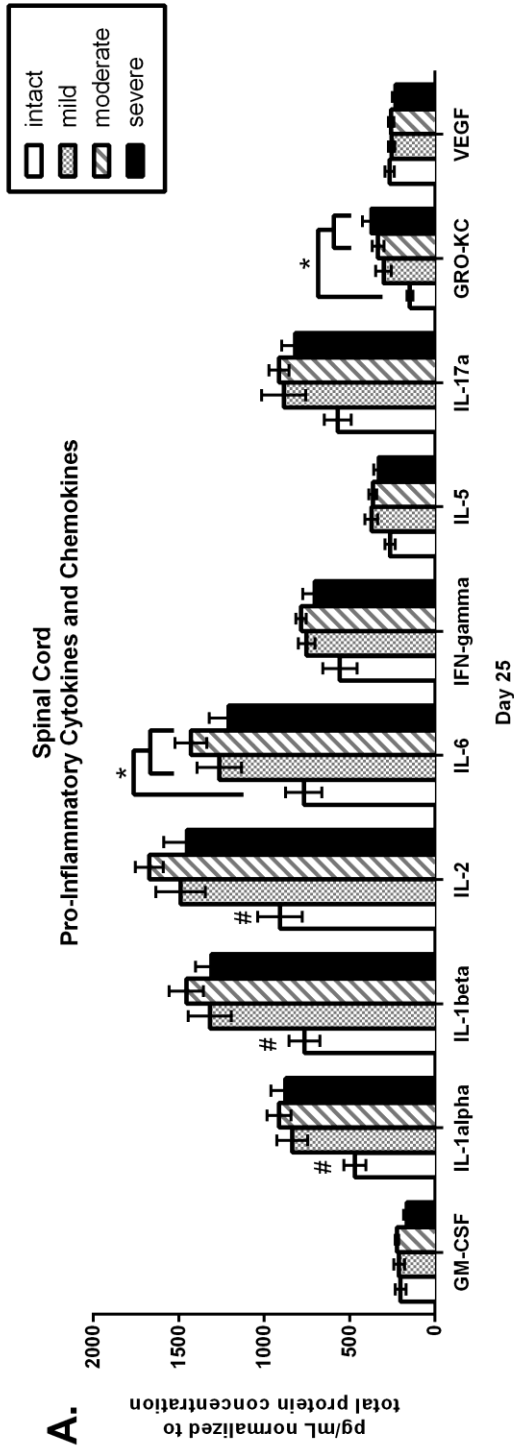
Figure 28 – Hippocampus anti-inflammatory and unclassified analytes by injury severity. The average (\pm standard error of the mean) concentrations of anti-inflammatory (A) and unclassified (B) cytokines and chemokines in lysate (normalized to total protein concentration) are shown for the intact, mild, moderate and severe injury groups.

Spinal cord

We assessed the levels of 20 cytokine/chemokines in the spinal cord protein supernatant. 4 x 3 ANOVAs ruled out a main effect of injury severity for all the analytes except IL-1 β , $F(3,26)=3.026$, $p=.047$. However, one-way ANOVAs by injury severity revealed a main effect for IL-1 α [$F(3,32)=4.803$, $p=.007$], IL-1 β [$F(3,33)=6.361$, $p=.002$], IL-2 [$F(3,33)=5.456$, $p=.004$], IL-6 [$F(3,33)=4.931$, $p=.006$], IL-18 [$F(3,33)=3.349$, $p=.028$], GRO-KC [$F(3,33)=4.452$, $p=.010$], MCP-1 [$F(3,33)=5.636$, $p=.003$], and TNF- α [$F(3,33)=2.838$, $p=.053$]. *Post-hoc* Sidak comparisons revealed that the mild, moderate, and severe injury groups showed higher levels of IL-1 α , IL-1 β , IL-2 (Figure 29A), and MCP-1 (Figure 29B) than the intact group, $ps \leq .05$. The mild and moderate injury groups showed higher levels of IL-6 than the intact group, $ps \leq .05$ (Figure 29A). The moderate and severe injury groups showed higher levels of GRO-KC than the intact group, $ps \leq .05$ (Figure 29A). Finally, the moderate injury group showed higher levels of IL-18 and TNF- α than the intact group, $ps \leq .05$ (Figure 29B). No main effects of injury severity were observed for the anti-inflammatory or the unclassified cytokines/chemokines (Figure 30).

One-way ANOVAs by psychological well-being revealed a main effect for IL-18 [$F(2,34)=3.426$, $p=.044$], IFN- γ [$F(2,34)=3.516$, $p=.041$], MCP-1 [$F(2,34)=3.849$, $p=.031$], and TNF- α [$F(2,34)=4.496$, $p=.019$]. *Post-hoc* Sidak comparisons revealed that the anxious/depressed group showed higher levels of IL-18, MCP-1, TNF- α , IFN- γ than the healthy group, ($p=.041$, $p=.066$, $p=.015$ and $p=.056$ respectively).

Figure 29- Spinal cord pro-inflammatory analytes by injury severity. The average (\pm standard error of the mean) concentrations of pro-inflammatory cytokines and chemokines in lysate (normalized to total protein concentration) are shown for the intact, mild, moderate and severe injury groups. Analytes are separated into graphs A and B for ease of reading. * $p < .05$, # significant from all others.



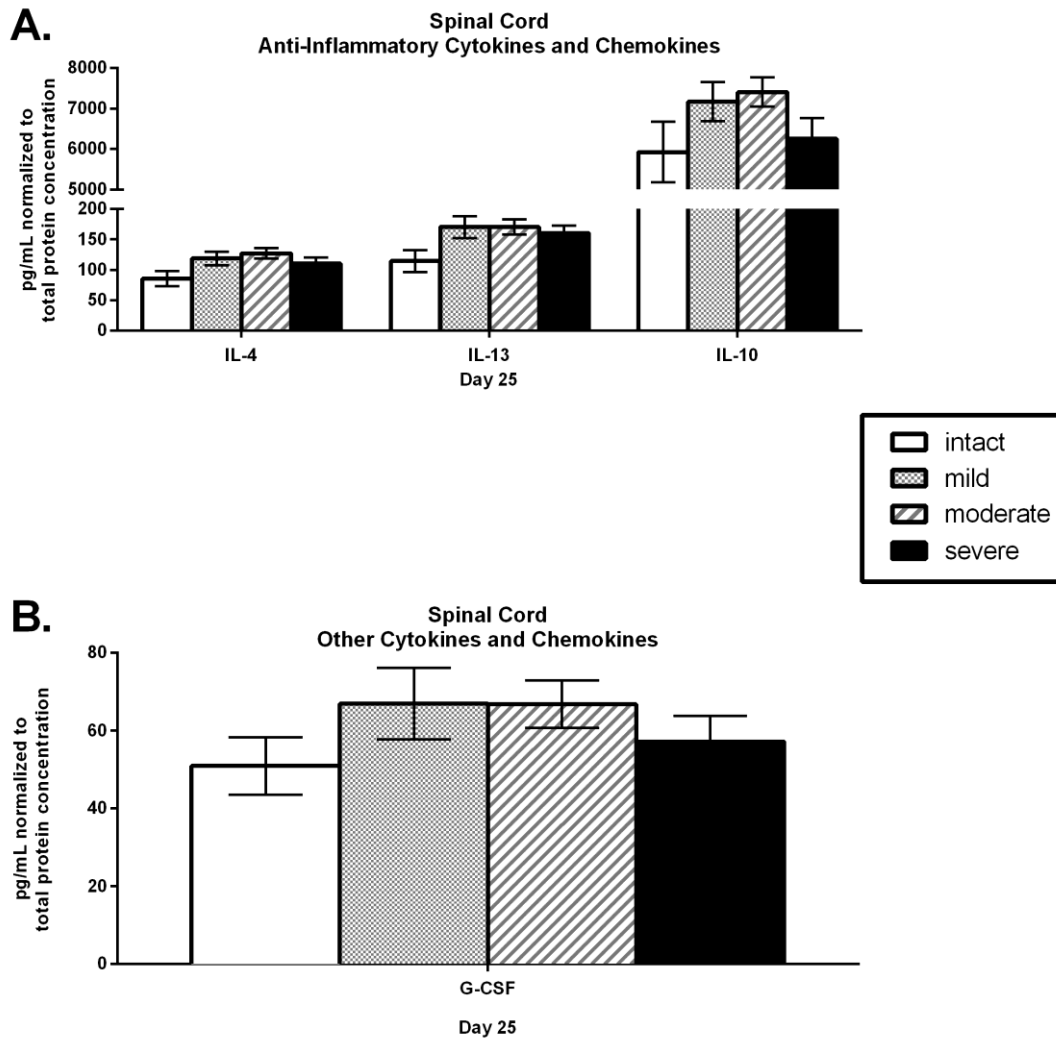


Figure 30- Spinal cord anti-inflammatory and unclassified analytes by injury severity. The average (\pm standard error of the mean) concentrations of anti-inflammatory (A) and unclassified (B) cytokines and chemokines in lysate (normalized to total protein concentration) are shown for the intact, mild, moderate and severe injury groups.

Figure 31 shows the significant differences by psychological well-being that still hold when the intact subjects are removed from analyses. Figure 32 shows the differences by psychological well-being for all pro-inflammatory cytokines, including intact. .No main effect of psychological well-being was observed for the anti-inflammatory or unclassified cytokines/chemokines (data reported in Figure 33).

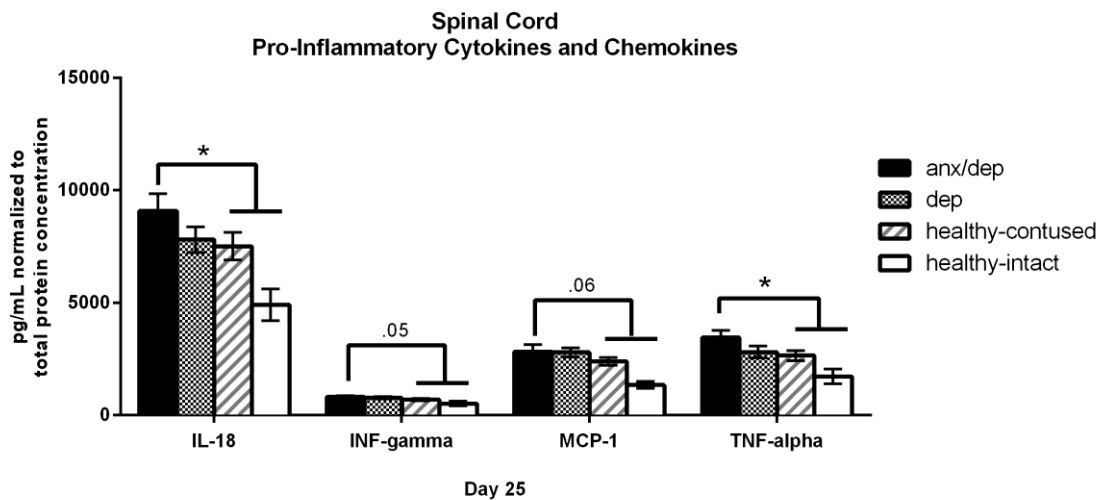
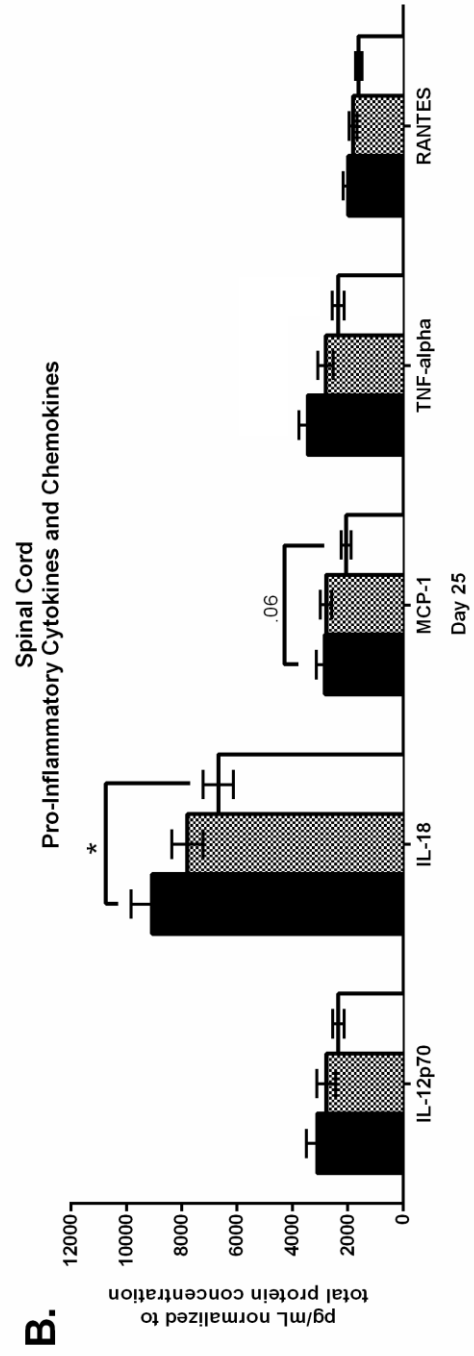
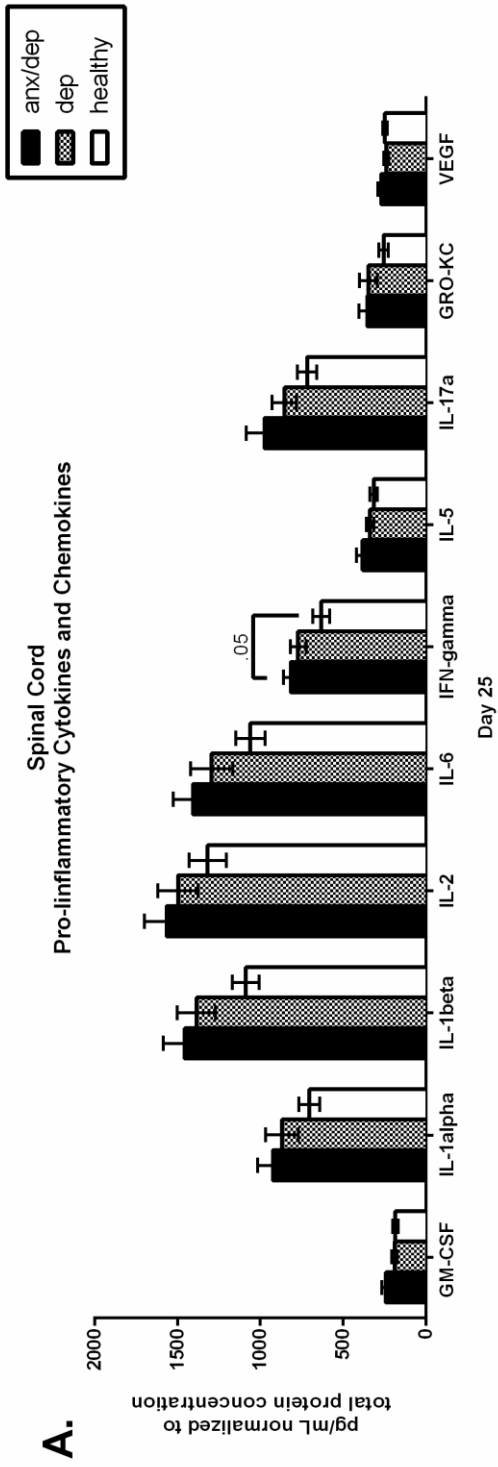


Figure 31- Significant spinal cord differences, reexamined with healthy intact separated. The average (\pm standard error of the mean) concentration of analytes in lysate (normalized to total protein concentration) found to differ by psychological well-being are shown here again, The graph depicts the original data (intacts not removed), with the HEALTH group separated into healthy contused subjects and healthy intact subjects to verify possible HEALTH group differences due to intact subjects. The statistical differences reported here are based on post-hoc Sidak comparisons between the ANX/DEP, DEP and HEALTH groups, with intacts removed. As the graphs indicate, even with intacts removed, the ANX/DEP group shows increases in both pro-inflammatory cytokines and chemokines. * $p < .05$.

Figure 32- Spinal cord pro-inflammatory analytes by psychological well-being. The average (\pm standard error of the mean) concentration of pro-inflammatory cytokines and chemokines in lysate (normalized to total protein concentration) are shown for the ANX/DEP, DEP and HEALTH groups. Analytes are separated into graphs A and B for ease of reading. * $p < .05$.



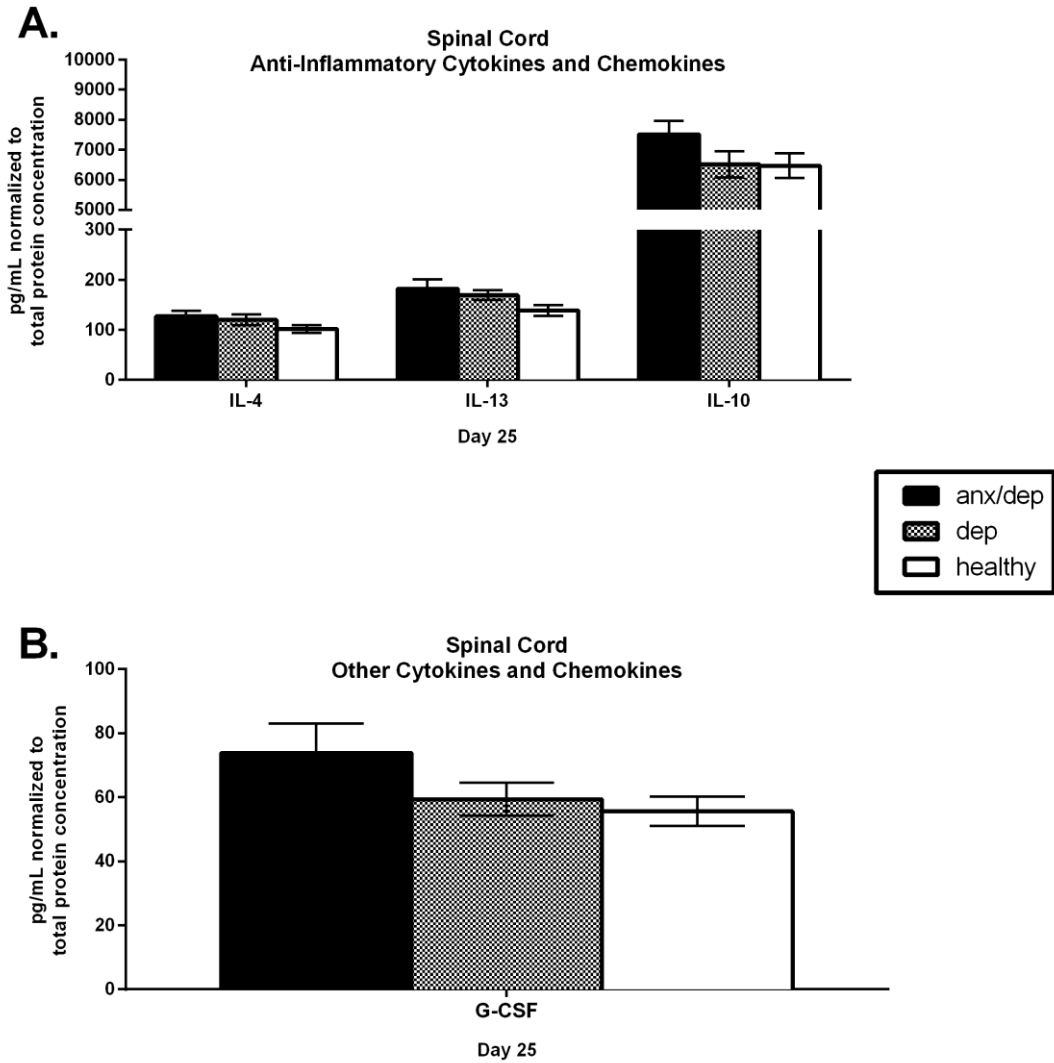


Figure 33- Spinal cord anti-inflammatory and unclassified analytes by psychological well-being. The average (\pm standard error of the mean) concentration of anti-inflammatory (A) and unclassified (B) cytokines and chemokines in lysate (normalized to total protein concentration) are shown for the ANX/DEP, DEP and HEALTH groups.

Discussion

In this chapter, we assessed a tableau of cytokine and chemokine expression peripherally (serum) and centrally (brain and spinal cord) post SCI, to determine whether inflammation is associated with depression- and anxiety-like signs post SCI. Numerous human and rodent studies have found inflammation to be involved in depression (Audet & Anisman, 2013; Dantzer et al., 2008; Maes, 1999; Maes et al., 1997; van West & Maes, 1999; Walker et al., 2014), especially after a central nervous system injury, such as a traumatic brain injury (Juengst, Kumar, Failla, Goyal, & Wagner, 2014). Our results provide similar confirmatory evidence for a role of inflammation in depression following SCI. Overall, the data support the hypothesis that the increased incidence of depression and anxiety-like signs observed in contused subjects in Chapter III is associated with increased levels of pro-inflammatory cytokines in the spinal cord, the brain, and the serum.

Yang et al. (2005) reported SCI severity dependent up-regulation of IL-1 β , IL-6, and TNF- α mRNAs and proteins in the spinal cord, up to 6 hours post injury. In contrast, we found no effect of injury severity on cytokine or chemokine levels either centrally or peripherally; only the expected spinal cord increases in contused subjects overall relative to intact subjects. The absence of an injury severity effect is likely due to differences in the time of tissue collection. In our study, we examined central inflammation (spinal cord and hippocampi) 25 days post SCI, long after the injury dependent effects on inflammation would have subsided, according to Yang et al. (2005). Only serum levels were assessed in the acute phase of injury, at 24 hours post SCI. Based on

immunohistochemical analyses, Yang et al.(2005) suggested that the increased inflammation they observed was due to endogenous cells of the spinal cord, not peripheral. Therefore, we might not observe injury severity dependent increases in pro-inflammatory molecules in the serum. Indeed, our results suggest that injury severity dependent changes in inflammation may only be present transiently, at the level of the spinal cord, and may not transfer to an injury severity dependent change in inflammation in the periphery, or a long-term injury severity dependent change in inflammation in the central nervous system.

While inflammation did not increase significantly as a function of injury severity, we found that peripheral and central inflammation differed among psychological well-being groups. Relative to the healthy group, the depressed group showed higher levels of several pro-inflammatory cytokines (TNF- α , IL-1 β), anti-inflammatory cytokines (IL-10) and chemokines (GM-CSF, GRO-KC, MIP-2, Fractalkine, and LIX) in the serum. In turn, the anxious/depressed group showed higher levels of two pro-inflammatory cytokines (IL-1 α and TNF- α) in the hippocampus, relative to the healthy group. The anxious/depressed group also showed higher levels of spinal cord pro-inflammatory cytokines (IL-18, IFN- γ , and TNF- α) and chemokines (MCP-1) relative to the healthy group. Importantly, the majority of these differences remained even after removing the intact subjects from analyses, thus confirming that the differences between the depressed and/or anxious/depressed groups and the healthy group were not due simply to a lower number of contused subjects in the healthy group.

The central increase of pro-inflammatory cytokines in the group of subjects identified as anxious/depressed could be explained in two ways. First, it could be explained by a unidirectional relationship between the spinal cord and the brain- spinal cord inflammation, through a series of mechanisms, resulting in anxiety-like behavior. We know that SCI results in short-term increases of pro-inflammatory cytokines at the level of the spinal cord (Yang et al., 2005; Yip & Malaspina, 2012). Furthermore, it has been shown, both in rodent SCI models and in humans, that in some SCI subjects, long-term increases in serum and spinal cord pro-inflammatory cytokine levels occur as well (Beck et al., 2010; Hayes et al., 2002), and it is known that inflammatory stimuli, even peripheral, can prime brain microglia to produce pro-inflammatory cytokines (Audet & Anisman, 2013; Dantzer et al., 2008). In our experiment, the increase in spinal cord pro-inflammatory cytokines immediately following SCI could have primed brain microglia to produce pro-inflammatory cytokines. This sustained SCI inflammation could, in turn, have caused anxiety-like behavior. As described in Chapter I (Introduction), the chronic elevation of cytokines can induce chronic HPA axis activation, resulting in chronic elevation of glucocorticoids and eventually glucocorticoid resistance, loss of HPA axis feedback loop inhibition, uncontrolled inflammation, and further HPA axis activation. Such a unidirectional mechanism could explain the increased levels of pro-inflammatory cytokines in both the spinal cord and the hippocampi of the subset of contused subjects identified as anxious/depressed.

However, the relationship between inflammation in the spinal cord and the brain could also be bi-directional. That is, in addition to the spinal cord inflammation

triggering inflammation supraspinally and activating the stress response as summarized above, the chronic activation of the stress response and the resulting uncontrolled inflammation could have caused generalized increases in central nervous system inflammation, including the spinal cord. In this case, the increased levels of pro-inflammatory cytokines observed in the spinal cord of anxious/depressed subjects could be due both to the SCI itself, and chronic stress. Our experimental design does not allow us to determine whether the relationship between SCI and increased inflammation in the central nervous system is unidirectional or bidirectional. It will be necessary, in the future, to conduct pharmacological studies, for example administering anti-inflammatory agents at different time points following SCI while assessing psychological well-being as well as central and peripheral inflammation, to determine whether anxiety results strictly from spinal cord inflammation, or is the result of both spinal cord inflammation and perpetuation of inflammation by chronic stress via the brain. It will likely be a combination of both. It will also be useful in the future to examine brain levels of serotonin, dopamine, and norepinephrine, to examine how the monoamines traditionally associated with depression are affected in the psychological well-being subgroups we identified, and examine brain levels of glucocorticoids and glucocorticoid receptors to determine whether these are altered in the anxious/depressed subjects.

This experiment is the first to assess, simultaneously, spinal cord, brain, and serum levels of cytokines and chemokines following a spinal cord contusion injury in a rodent model. We have shown that SCI results not only in increased levels of cytokines in the spinal cord, but also in the brain, and the serum. Importantly, we demonstrate that

two sub-groups of contused rodents, those identified as exhibiting depression- and anxiety-like signs, show increases in cytokine and chemokines both peripherally and centrally, even 25 days after the injury. Although our experiment does not allow us to verify a causal role of inflammation in depression and anxiety, the results are of particular clinical relevance, as it suggests that in a sub-group of spinal cord injured patients, there is an association between chronic inflammation and symptoms of depression and anxiety. To improve the mental health of these patients, it may eventually be possible to provide them with treatments for the immune dysregulation, in addition to the conventional treatments for depression.

CHAPTER V

**ENRICHING THE SOCIAL ENVIRONMENT TO PROMOTE
PSYCHOLOGICAL WELL-BEING AND RECOVERY POST SPINAL CORD
INJURY**

Introduction

This experiment explored the effects of group housing on psychological well-being, recovery of function, and peripheral inflammation after spinal cord injury (SCI). The impact of the social environment on inflammation and general health has been noted both in animal and human studies. Chronic social stress, for example, can lead to a blunted glucocorticoid response and an elevated immune response, both in rodents and humans (Avitsur et al., 2009; Miller et al., 2008; Powell et al., 2013). Moreover, in animal models, group housing has been shown to modulate supraspinal brain-derived neurotrophic factor (BDNF) levels (Berrocal et al., 2007; Oztan, Aydin, & Isgor, 2011; Ravenelle, Santolucito, Byrnes, Byrnes, & Donaldson, 2014; Venna, Xu, Doran, Patrizzi, & McCullough, 2014), as well as corticosterone (Moncek, Duncko, Johansson, & Jezova, 2004; Ravenelle et al., 2014), 5-HT (Beck & Luine, 2002), and pro-inflammatory cytokine levels (Krugel et al., 2014; McQuaid, Audet, Jacobson-Pick, & Anisman, 2013). Conversely, social isolation has been found to increase immobility on the forced swim test (Brenes & Fornaguera, 2009; Martin & Brown, 2010), increase anxiety assessed with open field activity and the elevated plus maze (Chourbaji, Zacher, Sanchis-Segura, Spanagel, & Gass, 2005; Ruis et al., 1999), and decrease BDNF levels

in the dorsal hippocampus and frontal cortex of pigs (De Vry et al., 2012). There is also strong evidence that social isolation can lead to further excitation of the immune system, exacerbating depression and anxiety-like signs. For example, Jaremka et al. (2013) found that healthy adults who perceived themselves as lonely show a great inflammatory response in lipopolysaccharide stimulated peripheral blood mononuclear cells following acute stress (Jaremka et al., 2013). Recent studies have also identified social isolation as a risk factor for diseases such as stroke (Venna et al., 2012). Inflammation may affect psychological well-being after SCI, as shown in the preceding chapters, but the inverse may also be true: psychological well-being, as the studies above indicate, may affect inflammation, and thus, SCI recovery.

The role of the social environment has been well studied in the clinical human spinally injured population. Following SCI, social support is positively associated with physical and mental health (Muller, Peter, Cieza, & Geyh, 2012). Individuals with strong social support are less likely to develop chronic pain following SCI (Goossens, Dousse, Ventura, & Fattal, 2009; Raichle, Hanley, Jensen, & Cardenas, 2007) and less likely to develop secondary complications such as pressure ulcers and urinary tract infections (Muller et al., 2012). Individuals with low perceived social support are more likely to develop symptoms of depression and anxiety following SCI (Boekamp et al., 1996; Chan, Lee, & Lieh-Mak, 2000b; Elliott et al., 1991; Muller et al., 2012). The association between social support and secondary complications following SCI could be due to greater compliance with physicians' directions in patients having social support (Bombardier et al., 2004). However, the association between social support, chronic

pain and mental health suggests that the positive effects of the social environment are due to more than simply improved treatment compliance. These observations are of particular importance, for SCI leads many patients to experience a decrease in psychological well-being, and an increase in perceived loneliness (Lohne & Severinsson, 2005): the perception of few people who understand them, and in whom they can confide. These psychiatric-epidemiological studies highlight the potential impact of a positive environment in the recovery of patients following SCI.

Given these animal and human findings, we hypothesized that group housing could reduce depression and anxiety-like signs in spinally contused rodents. We hypothesized that rats pair-housed following a contusion injury would show reduced anxiety- and depression-like signs post injury, lower levels of peripheral inflammation, reduced sensitivity to pain, and greater recovery of locomotor function, relative to single-housed contused animals. Notably, there have been no reports on the effects of group housing (as the sole intervention) on recovery of function or inflammation following SCI. The experiment reported here, therefore, also provides an initial systematic analysis of the effects of pair-housing on recovery of motor and sensory function after SCI.

Methods

Experimental design

Rats underwent a moderate spinal cord contusion injury, as described in the General Methods. There were five experimental groups (n=8): (1) contused animals

single housed (CONT-SH), (2) contused animals with contused cagemate (CONT-CC), (3) contused animal with intact cagemate (CONT-CI), (4) intact animal with contused cagemate (INTACT-CI), and (5) intact animal with intact cagemate (INTACT-II). This last group housing condition allowed us to control for the possible confounding effect of being exposed to an injured cagemate. Prior to spinal cord contusion surgery, all rats were housed in pairs. Post-surgery, they either remained pair housed or were single housed, depending on the condition to which they were assigned.

For all subjects, psychological well-being was assessed at baseline with a comprehensive battery of tests as well as during Test Phase 1 (days 5 and 10) and Test Phase 2 (day 20-21) post injury (See Figure 2). The intact subjects were tested at ages compatible with those of the contused subjects. The procedures for each of the tests are described in detail in the General Methods section (see *Behavioral tests*). Locomotor function was assessed throughout the 21-day recovery period with the BBB scale (Basso et al., 1995). Tests of sensory function (girdle, tactile and thermal reactivity) were conducted during Test Phase 1 (day 11) and Test Phase 2 (day 22). Blood was also collected during each test phase (pre-injury, and days 1, 10, and 24). At the end of the assessment period, subjects received a lethal injection of beuthanasia (100 mg/kg, i.p.).

Results

We first used a series of statistical analyses to determine the psychological well-being of the subjects in the experiment. As described in Chapter III, a principal component analysis was used initially to determine which behavioral measures were

correlated with each other. Next, we ran a hierarchical cluster analysis on the behavioral measures retained by the principal component analysis in order to identify the subject clusters in our sample. The hierarchical cluster analysis identified subjects as exhibiting depression, anxiety/depression, or as being healthy. After identifying the psychological well-being groups, we ran post-cluster repeated measure analyses of variance (ANOVAs) on the behavioral measures retained by the principal component analysis in order to confirm the hierarchical cluster group identifications. Following confirmation of the hierarchical cluster groups, we conducted repeated measure ANOVAs on additional test variables (not included into the principal component and hierarchical cluster analyses), to look for main effects of group housing and psychological well-being, as well as interaction effects, on anxiety, pain, recovery of function and physiological markers.

Principal component analyses: assessment of test validity for identifying psychological well-being

The principal component analysis produced two components, which cumulatively explained 69.19% of the variance between subjects. The first component contained the open field activity, social exploration and the shock probe burying test. The sucrose preference test loaded on the second component (see Table 6). Both components had Eigenvalues greater than 1, and explained a significant proportion of the variance between subjects. They were retained for subsequent analyses.

Behavioral tests	Components	
	1	2
Sucrose preference	.014	.972
Social exploration	.779	.034
Open field activity	.807	.206
Shock probe burying	.689	-.217

Table 6 - Components of principal component analyses in Group Housing experiment. Component loadings are shown for the behavioral tests retained. To be retained, behavioral tests had to load with >.03 on one component only.

Hierarchical cluster analyses: classification of psychological well-being

Average scores (derived from both Test Phases 1 and 2), for each of the behavioral tests retained in the principal component analysis (sucrose preference, social exploration, open field activity and shock probe burying), were used in the hierarchical cluster analysis. The dendrogram produced by this analysis showed that subjects separated into three psychological well-being clusters, with 12 subjects in Hierarchical Cluster (HC) Group 1, 11 subjects in HC group 2, and 16 subjects in HC group 3 (see Figure 34). To identify the psychological well-being characteristics of each cluster group, the three groups were compared on each of the behavioral measures retained in the principal component analysis, across Test Phase 1 and 2, using repeated measure ANOVAs. Based on their performance on these tests, subjects in Group 1 were labeled as exhibiting depression-like signs (or DEP), subjects in Group 2 as exhibiting anxiety and depression like signs (or ANX/DEP) and subjects in Group 3 as being healthy (or HEALTH). Baseline differences were significant only for the open field activity test, $F(2,36)=9.26, p=.001$. For this variable only, repeated measures analyses of covariances were conducted. All *post-hocs* were conducted with multiple comparison Sidak tests.

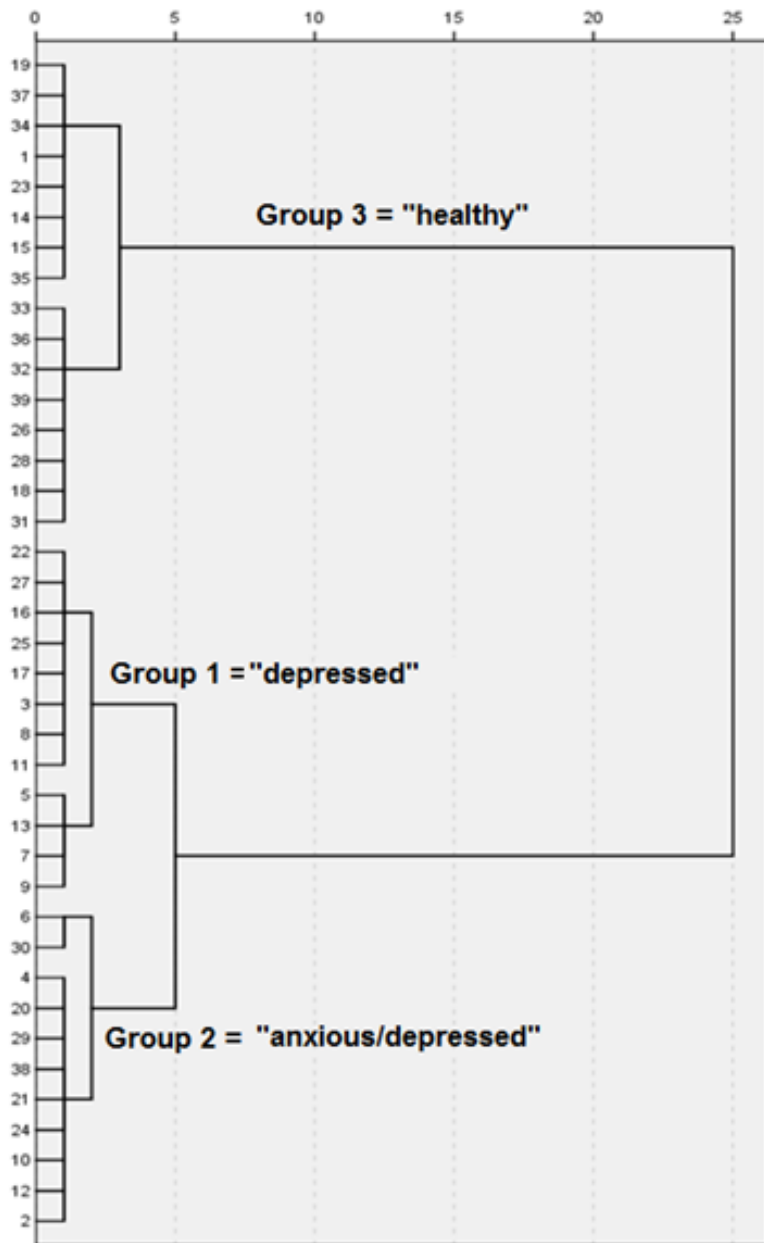


Figure 34- Hierarchical cluster dendrogram, Group Housing experiment. The dendrogram illustrates the results of the hierarchical cluster analysis, which separated the sample into three main clusters (Group 1 (n=12), Group 2 (n=11), and Group 3 (n=16)), based on the subjects' average performance on the behavioral tests retained by the principal component analysis. The numbers on the y-axis represent the subjects.

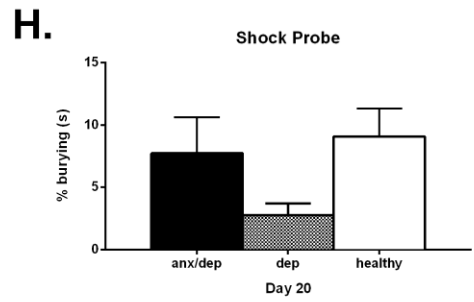
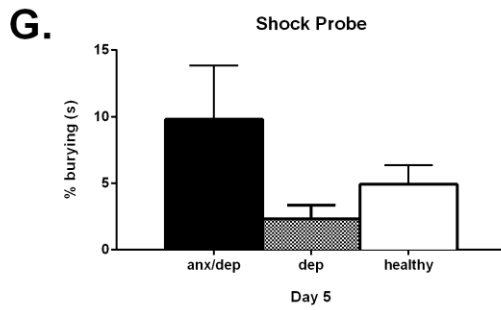
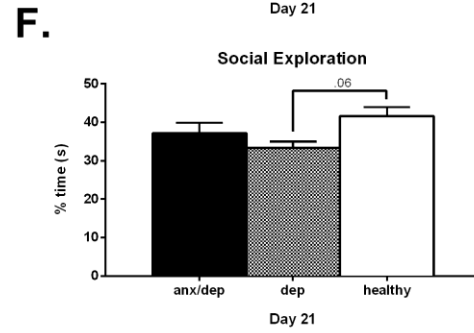
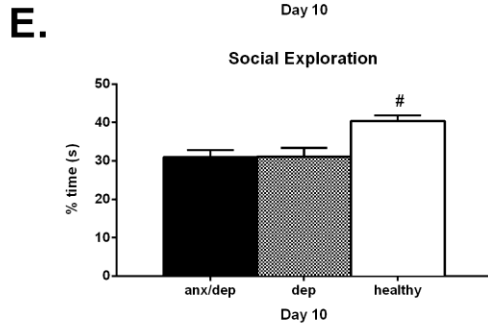
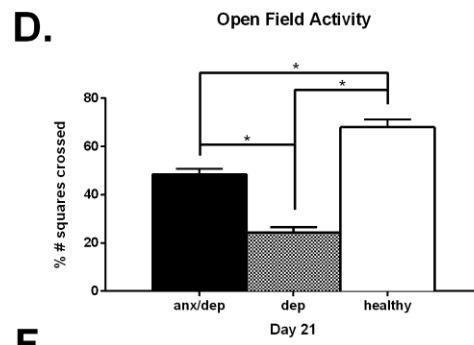
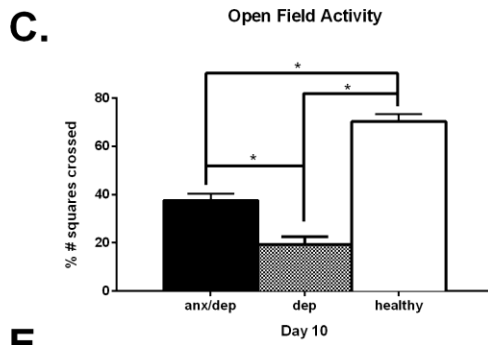
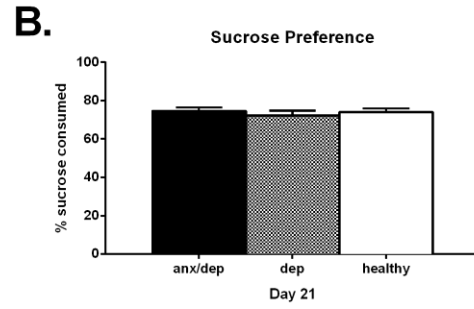
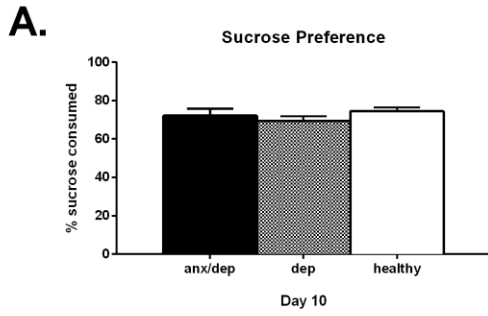
Post hierarchical cluster comparisons

Repeated measure ANOVAs revealed a main effect of psychological well-being (HC group) for the open field activity test and the social exploration test [$F(2,35)=59.45$, $p=.000$ and $F(2,36)=9.41$, $p=.001$, respectively]. Differences also approached significance for the shock probe burying test, $F(2,26)=3.36$, $p=.050$ (Figure 35G and Figure 35F). No differences were found for the sucrose preference test (Figure 35A and Figure 35B). For open field activity, subsequent ANOVAs on Days 10 and 21 confirmed a main effect of psychological well-being at both time points [$F(2,35)=39.58$, $p=.000$, $F(2,35)=37.17$, $p=.000$, for Day 10 and 21 respectively]. *Post-hoc* analyses showed that on both days, the DEP group and the ANX/DEP group displayed a lower number of squares crossed in an open field compared to the HEALTH group. Moreover, the DEP group showed reduced open field activity relative to the ANX/DEP group, $ps \leq .05$ (Figure 35C and Figure 35D). For social exploration, ANOVAs on Days 10 and 21 separately confirmed that the HC groups differed on social exploration on Days 10 and 21, [$F(2,36)=8.66$, $p=.001$ and $F(2,36)=3.21$, $p=.05$ respectively]. *Post-hoc* analyses revealed that both the DEP group and ANX/DEP group showed decreased interaction time compared to the HEALTH group on Day 10, $ps \leq .05$, Figure 35E. On day 21, the DEP group showed decreased interaction time relative to the HEALTH group, $p = .06$, Figure 35F.

Figure 35- Post HC behavioral differences. The average (\pm standard error of the mean) performance of the ANX/DEP, DEP, and HEALTH group are shown on each task. On Day 10 and 21, the DEP and ANX/DEP groups displayed decreased open field compared to the HEALTH group. The DEP group further showed reduced open field activity relative to the ANX/DEP group (C and D). Both the DEP and ANX/DEP group showed decreased social exploration relative to the HEALTH group on Day 10 (E). Finally, on Day 21, the DEP group showed a trend towards decreased social interaction relative to the HEALTH group (F). * $p < .05$, # significant from all others.

Test Phase 1

Test Phase 2



Differences in psychological well-being, pain, recovery of function and physiological markers

Repeated measure ANOVAs were conducted to identify main effects of group housing and psychological well-being on Days 10 and 21 separately. Baseline differences were significant only for open field activity and center activity [$F(2,36)=9.26$, $p=.001$ and $F(2,36)=5.22$, $p=.01$, respectively]. For these variables, repeated measure analyses of covariances were conducted. However, it is important to note that including the baseline as a covariate does not correct fully for the differences pre-contusion on these two variables. Following the comparisons across time, one-way ANOVAs were run for both independent variables separately, on Days 10 and 21. In this experiment, one-way ANOVAs were run for both independent variables separately, on Days 10 and 21, regardless of whether main effects were found for group housing and psychological well-being. One-way ANOVAs were conducted regardless, because of the exploratory nature of this experiment. Finally, *post-hocs* were conducted with multiple comparison Sidak tests to identify group differences on specific test days. Note that for sucrose preference, open field activity, social exploration, and shock probe burying, psychological well-being effects are not reported here, as these were already reported in the post-hierarchical cluster ANOVAs used to characterize the hierarchical cluster subgroups. Psychological well-being group membership by group housing condition is shown in Figure 36.

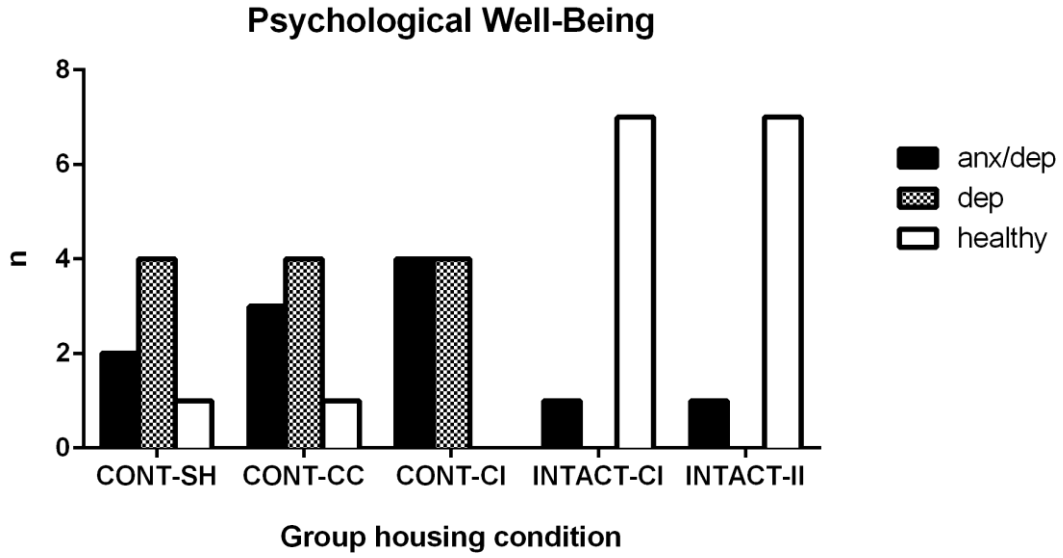


Figure 36- Psychological well-being membership by group housing condition. This figure shows group membership by group housing condition.

Depression measures

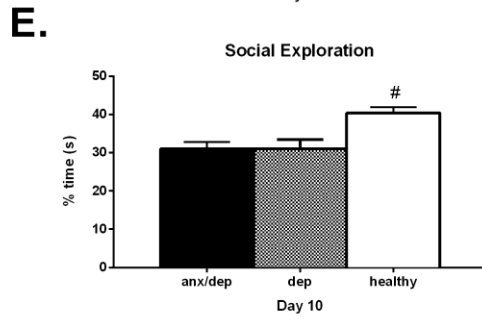
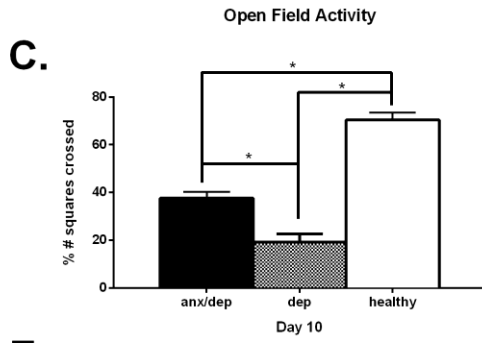
In addition to the main effects of open field activity and social exploration found by psychological well-being, as per the post hierarchical cluster repeated measure analyses run in the previous section (Figure 37), repeated measure ANOVAs also found several main effects of group housing on measures of depression.

Sucrose preference. A repeated measure ANOVA found no main effect of group housing across time for the sucrose preference test. However, subsequent one-way ANOVAs, comparing sucrose preference on Days 10 and 21 separately, found a main effect of group housing on Day 21 only, $F(4, 34)=4.49$, $p=.005$. *Post-hoc* Sidak comparisons by group housing indicated that the CONT-CC group showed significantly higher sucrose preference on Day 21 than the CONT-CI group, and the INTACT-CI group, $ps \leq .05$.

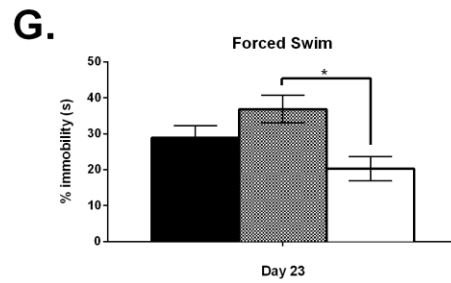
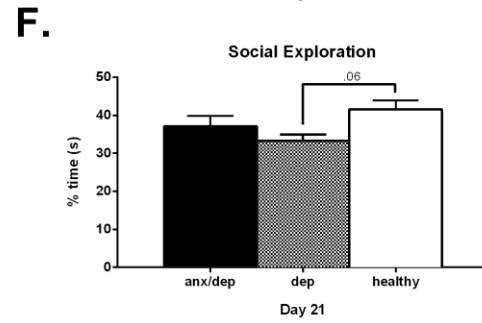
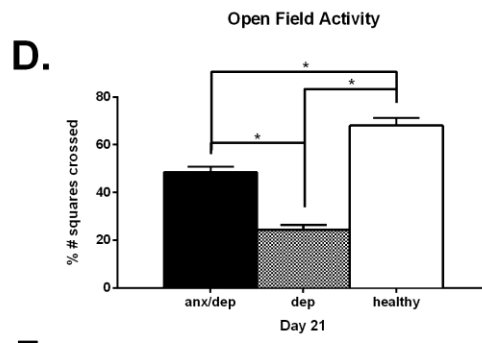
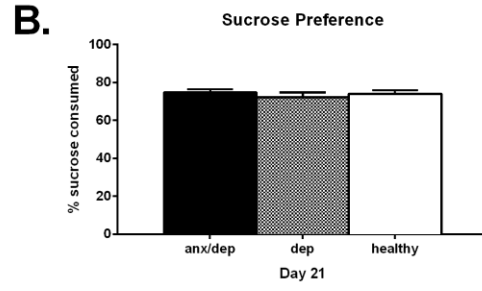
Open field activity. A repeated measure ANOVA revealed a main effect of group housing across time for the open field activity test, $F(4,33)=10.736$, $p=.000$. Subsequent one-way ANOVAs on Days 10 and 21 separately, found a main effect of group housing on Days 10 and 21, [$F(4,33)=13.70$, $p=.000$, $F(4,33)=5.71$, $p=.001$, on Days 10 and 21 respectively]. *Post-hoc* Sidak comparisons by group housing indicated that on Day 10, the CONT-CC and CONT-CI groups displayed decreased open field activity relative to the INTACT-CI and INTACT-II groups, $ps \leq .05$.

Figure 37- Depression measures by psychological well-being. The average (\pm standard error of the mean) performance of the ANX/DEP, DEP, and HEALTH groups are shown on all depression measures. The DEP and the ANX/DEP groups showed decreased open field activity relative to the HEALTH group on Days 10 and 21 (C and D). The DEP group also showed decreased open field activity relative to the ANX/DEP group on both days. The ANX/DEP (on Day 21) and the DEP groups (on Days 10 and 21) showed decreased social exploration relative to the HEALTH group (E and F). Finally, the DEP group showed greater immobility on the forced swim test than the HEALTH group (G). * $p < .05$, # significant from all others.

Test Phase 1



Test Phase 2



Furthermore, on Day 21, the CONT-SH, CONT-CC and CONT-CI groups displayed decreased open field activity relative to the INTACT-CI and INTACT-II groups, $ps \leq .05$, Figure 38.

Social exploration. A repeated measure ANOVA revealed a main effect of group housing across time for the social exploration test, $F(4,34)=2.92$, $p=.035$. Subsequent one-way ANOVAs, comparing social exploration on Days 10 and 21 separately, confirmed a main effect of group housing on Day 10, $F(4,34)=6.62$, $p=.000$. *Post-hoc* Sidak comparisons by group housing indicated that on Day 10, the CONT-CC group displayed significantly less social exploration than the INTACT-CI and INTACT-II groups, $ps \leq .05$. Furthermore, the CONT-CI group exhibited significantly less social exploration behavior than the INTACT-II group, $p < .05$, Figure 38.

Forced swim. One-way ANOVAs, comparing immobility by psychological well-being and group housing separately, found a main effect of psychological well-being and group housing on Day 24, [$F(2,33)=3.511$, $p=.041$, $F(4, 31)=11.54$, $p=.000$, respectively]. *Post-hoc* Sidak comparisons by psychological well-being indicated that the DEP group spent significantly more time immobile at the forced swim test than the HEALTH group, $p=.046$, Figure 37G. *Post-hoc* comparisons by group housing indicated that the INTACT-II group spent significantly less time immobile at the forced swim test than the CONT-SH, CONT-CC, CONT-CI and INTACT-CI groups on Day 24, $ps \leq .05$, Figure 38.

Test Phase 1

Test Phase 2

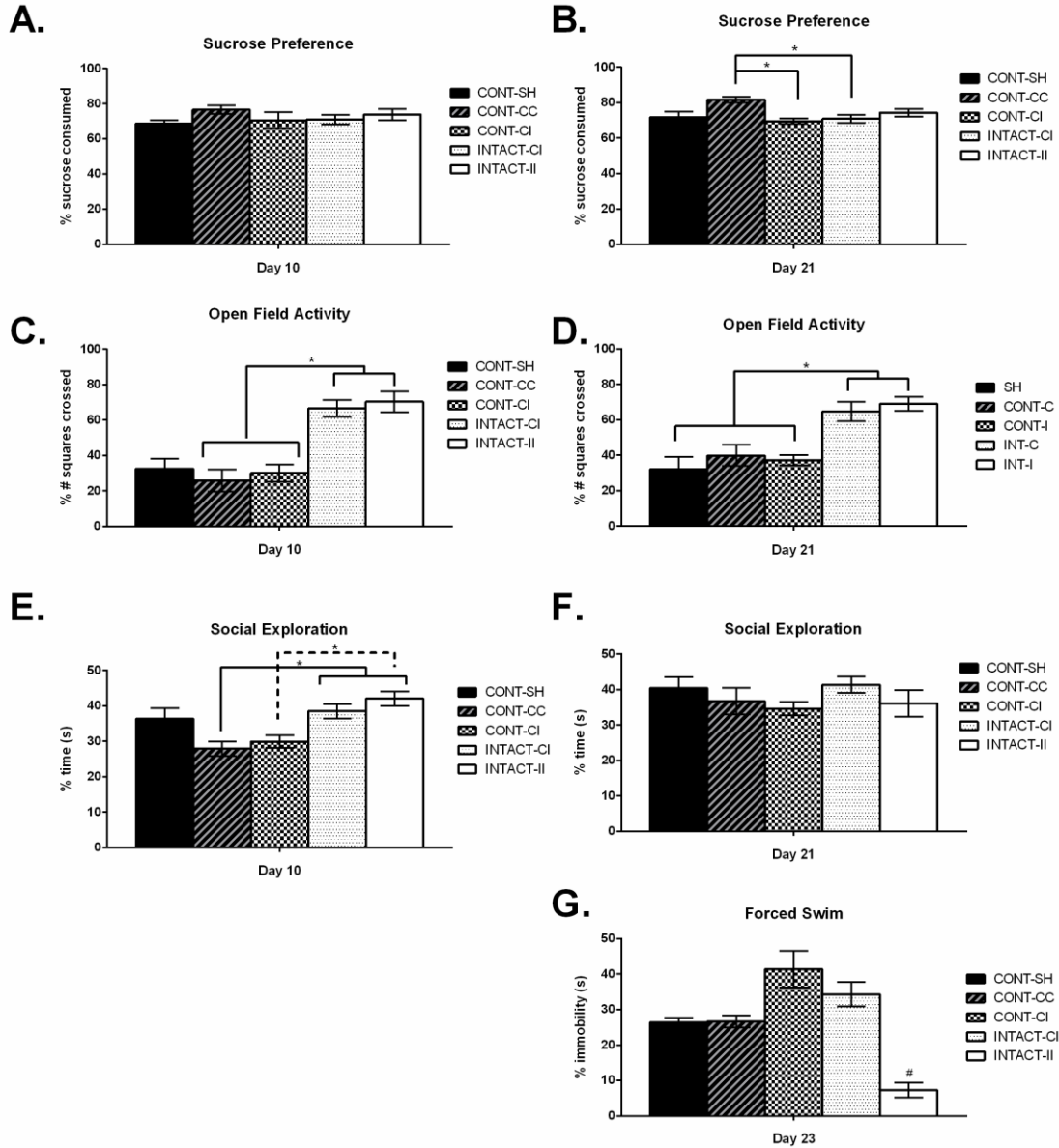


Figure 38- Depression measures by group housing condition. The average (\pm standard error of the mean) performance of the CONT-SH, CONT-CC, CONT-CI, INTACT-CI and INTACT-II groups are shown on all depression measures. The CONT-CC and CONT-CI groups showed decreased open field activity relative to the INTACT-CI and INTACT-II groups on Days 10 and 21 (C and D). On Day 21, the CONT-SH also displayed decreased open field activity relative to the INTACT-CI and INTACT-II groups (D). The CONT-CC group displayed decreased social exploration relative to the INTACT-CI and INTACT-II groups (E). Furthermore, the CONT-CI group also showed decreased social exploration relative to the INTACT-II group (E). Finally the INTACT-II showed decreased immobility on the forced swim test relative to all other groups (G). * $p < .05$, # significant from all others.

Anxiety measures

Shock probe burying. While a main effect was found for shock probe burying by psychological well-being (Figure 39), a repeated measure ANOVA found no main effect of group housing across time for the shock probe burying test. However, subsequent one-way ANOVAs, comparing percent time burying on Days 5 and 20 separately, indicated a main effect of group housing at both time points [$F(4,18)=4.96, p=.007, F(4,25)=3.33, p=.026$, on days 5 and 21 respectively]. *Post-hoc* Sidak comparisons by group housing condition did not find any significant group differences on Days 5 or 20.

Center activity. A repeated measure ANOVA revealed a main effect of group housing across time for center activity in an open field, [$F(4,33)=7.32, p=.000$], as well as a main effect of psychological well-being [$F(2,35)=6.82, p=.003$]. Subsequent one-way ANOVAs, comparing center activity on Days 10 and 21 separately, found a main effect of psychological well-being and group housing [HC groups: $F(2,35)=10.67, p=.000, F(2,35)=3.57, p=.039$, for Day 10 and 21, respectively; GRH groups: $F(4,33)=4.94, p=.003, F(4,33)=7.52, p=.000$, for Day 10 and 21, respectively]. *Post-hoc* Sidak comparisons by psychological well-being indicated that on Day 10, both the DEP group and the ANX/DEP group showed significantly less center activity than the HEALTH group, $ps \leq .05$. By Day 21, the DEP continued to show significantly less center activity than the HEALTH group, $p=.034$. *Post-hoc* Sidak comparisons by group housing indicated that on Day 10, the CONT-CC and the CONT-CI groups showed lower center activity than the INTACT -II, and on Day 21, the CONT-SH, CONT-CC, CONT-CI and

INTACT-CI groups showed lower center activity than the INTACT-II group, $ps \leq .05$, (Figure 40).

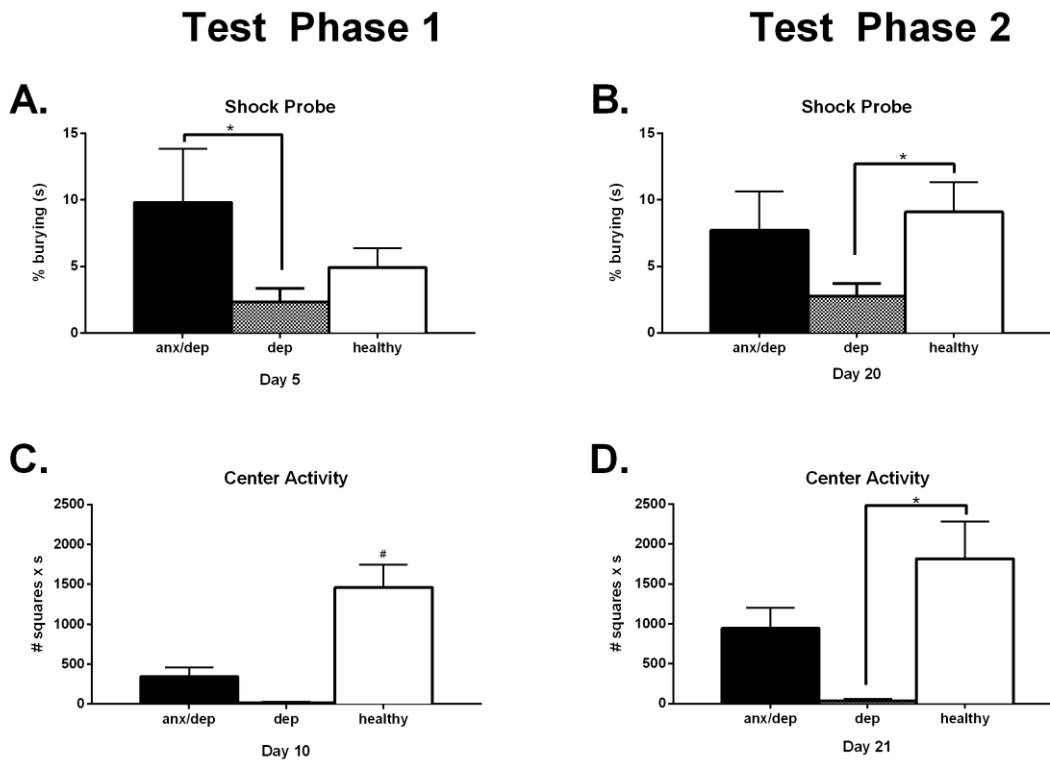
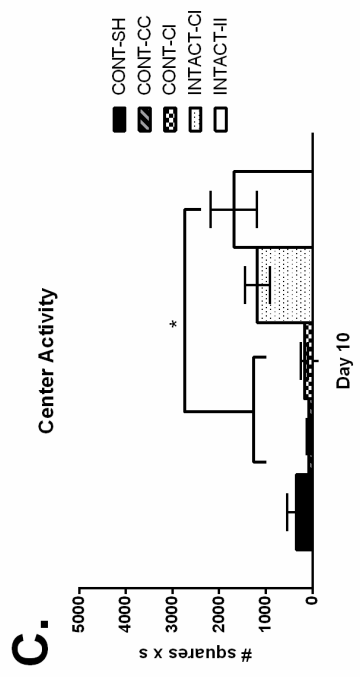
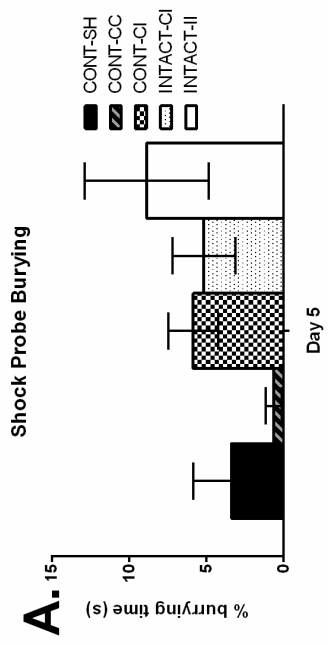


Figure 39- Anxiety measures by psychological well-being. The average (\pm standard error of the mean) performance of the ANX/DEP, DEP, and HEALTH group, are shown on all anxiety measures. The ANX/DEP group showed greater shock probe burying than the DEP group (A and B). The DEP group showed significantly less shock probe burying than the HEALTH group (B). On Day 10, the ANX/DEP and DEP showed decreased center activity relative to the HEALTH group (C). On Day 21, the DEP continued to show decreased center activity relative to the HEALTH group (D). * $p < .05$, # significant from all others.

Test Phase 1



Test Phase 2

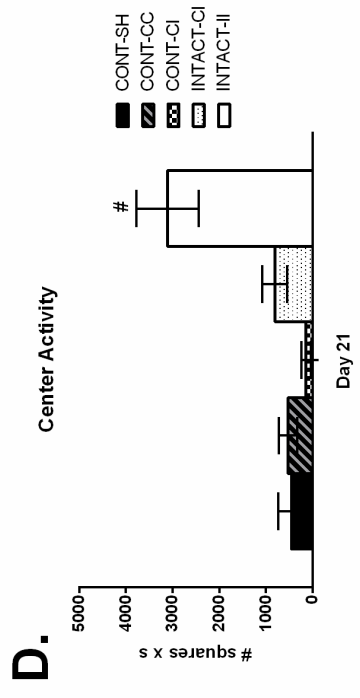
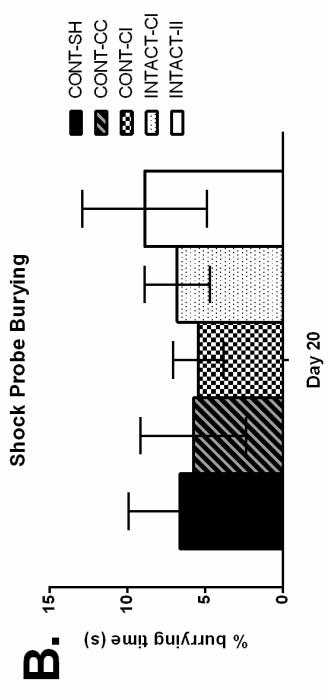


Figure 40- Anxiety measures by group housing condition. The average (\pm standard error of the mean) performance of the CONT-SH, CONT-CC, CONT-CI, INTACT-CI and INTACT-II groups are shown on all anxiety measures. On Day 10, the CONT-CC and the CONT-CI groups showed lower center activity than the INTACT -II (C), and on Day 21, the INTACT-II group showed greater center activity than all other groups (D). * $p < .05$, # significant from all others.

Pain measures

Tactile test- paw withdrawal threshold. Repeated measure ANOVAs found a main effect of group housing for the tactile test (paw withdrawal threshold) [$F(4,34)=4.93$, $p=.003$], but no main effect of psychological well-being. However, subsequent one-way ANOVAs, comparing paw withdrawal thresholds on Days 11 and 22 separately, found a main effect psychological well-being and group housing on Day 11, [$F(2, 36)=5.24$, $p=.010$, $F(4,34)=4.53$, $p=.005$]. *Post-hoc* Sidak comparisons by psychological well-being indicated that on Day 11, the DEP group showed a lower threshold on the motor tactile test than the ANX/DEP and the HEALTH groups, $ps \leq .05$, Figure 41A. *Post-hoc* Sidak comparisons by group housing indicated that on Day 11, the CONT-CC group showed a lower threshold on the motor tactile test than the CONT-SH and the INTACT-II group, $ps \leq .05$, Figure 42A.

Tactile test- vocalization threshold. Repeated measures ANOVAs found no main effect of psychological well-being (HC group) or group housing (GRH group) effect for number of vocalizations on the tactile test, [$F(2, 36)=1.71$, $p=.196$, $F(4, 34)=1.79$, $p=.153$, by HC group and GRH group respectively]. Subsequent one-way ANOVAs did not find a main effect of group housing or psychological well-being.

Tail-flick. Repeated measure ANOVAs found a main effect of group housing (GRH group) for withdrawal latency [$F(4,34)=4.18$, $p=.007$], but no main effect of psychological well-being. Subsequent one-way ANOVAs, comparing withdrawal latency on Days 10 and 22 separately, found a main effect of group housing on both

Days, [$F(4,34)=2.77, p=.043, F(4,34)=3.46, p=.018$, Days 10 and 22 respectively], but no main effect of psychological well-being. *Post-hoc* Sidak indicated that on Day 11, the INTACT-II group displayed greater withdrawal latency than the CONT-CI group, $p=.038$, Figure 42C. On Day 22, the INTACT-II group displayed greater withdrawal latency than the CONT-CC group, $p=.016$, Figure 42D.

Girdle test. Repeated measure ANOVAs found a main effect of group housing (GRH group) for the number of vocalizations on the girdle test [$F(4,34)=8.62, p=.000$], but no main effect of psychological well-being. Subsequent one-way ANOVAs, comparing number of vocalizations on Days 11 and 22 separately, found a main effect group housing on Day 11 only, $F(4, 34)=5.27, p=.002$. *Post-hoc* Sidak comparisons by group housing indicated that the CONT-SH group displayed a significantly higher number of vocalizations on the girdle test than the CONT-CC group, the CONT-CI group, the CONT-II group, the INTACT-CI group and the INTACT-II group, $ps \leq .05$, Figure 42E.

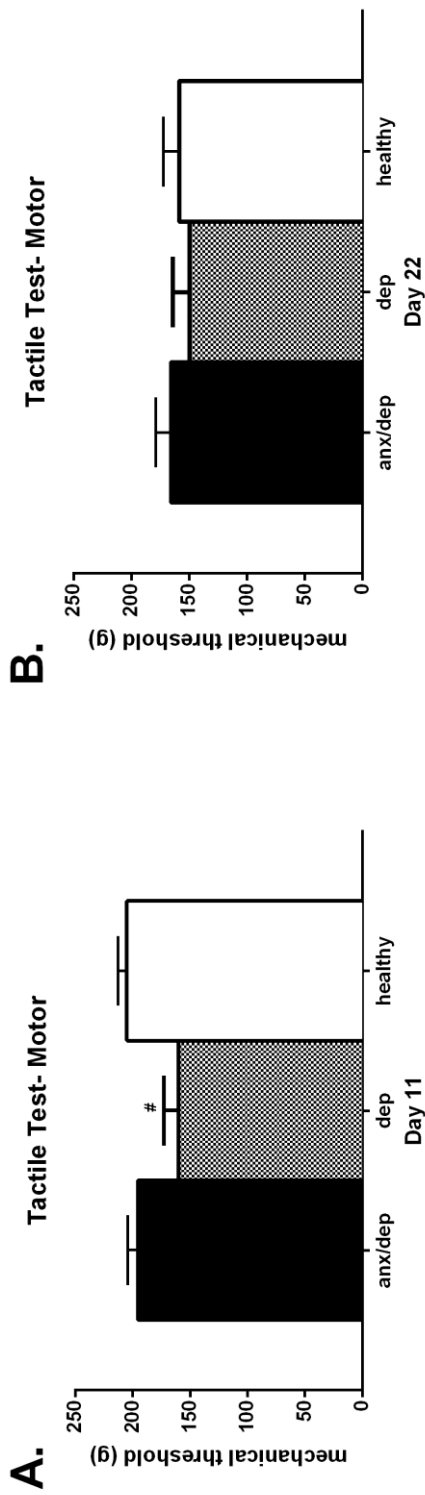


Figure 41- Pain measures by psychological well-being. The average (\pm standard error of the mean) performance of the ANX/DEP, DEP, and HEALTH group, are shown the tactile tests. On Day 11, the DEP group showed a lower threshold on the motor tactile test than the ANX/DEP and the HEALTH groups (A). * $p < 0.05$, # significant from all others.

Test Phase 1

Test Phase 2

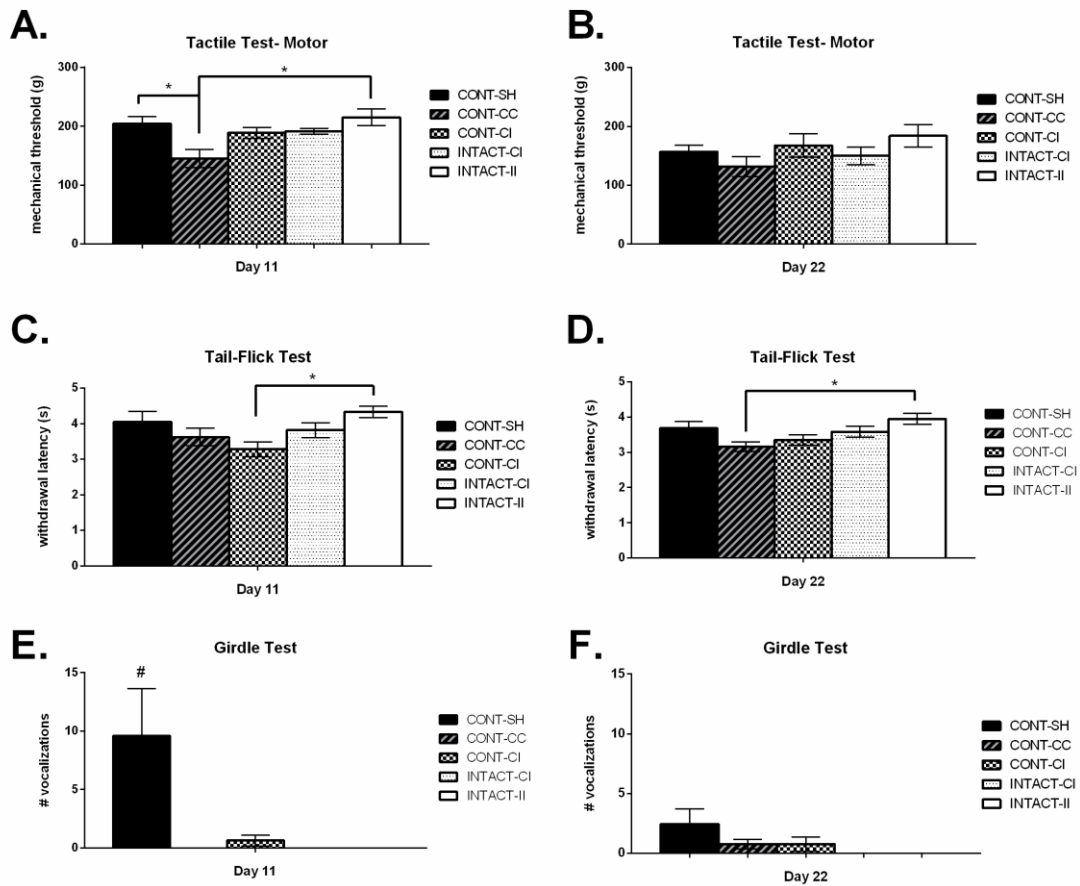


Figure 42- Pain measures by group housing condition. The average (\pm standard error of the mean) performance of the CONT-SH, CONT-CC, CONT-CI, INTACT-CI and INTACT-II groups are shown on the tactile tests. On Day 11, the CONT-CC group showed a decreased paw withdrawal threshold relative to the CONT-SH and INTACT-II group (A). On Day 11, the INTACT-II showed a higher vocalization withdrawal threshold relative to the CONT-CI group (C), and on Day 22, relative to the CONT-CC group (D). Finally, the CONT-SH group showed a greater number of vocalizations on the girdle test than all other groups on Day 11 (E). * $p < .05$, # significant from all others.

Recovery measures

Locomotor recovery of function. Repeated measure ANOVAs revealed a main effect of group housing (GRH group), $F(4,34)=54.43$, $p=.000$, but no main effect of psychological well-being (when excluding the intact subjects). Subsequent one-way ANOVAs also did not find a main effect of psychological well-being. *Post-hoc* Sidak comparisons by group housing confirmed that the INTACT-CI and the INTACT-II groups displayed a significantly higher recovery scores (BBB) than the CONT -SH, CONT -CC and CONT-CI, $ps \leq .05$, Figure 43.

Weight gain. Repeated measure ANOVAs found a main effect of group housing [$F(4,34)= 54.36$, $p = .000$], and psychological well-being [$F(2,36)= 19.63$, $p = .000$]. However, subsequent one-way ANOVAs, comparing weight gain by psychological well-being and group housing separately, found a main effect of group housing, $F(4,34)= 54.36$, $p = .000$ and a main effect of psychological well-being, $F(2,36)= 19.63$, $p = .000$. *Post-hoc* Sidak comparisons by psychological well-being group revealed that the ANX/DEP and DEP groups showed significantly less weight gain than the HEALTH group, $ps \leq .05$, (Figure 44A). However, Figure 44B indicates that this effect disappears when intact subjects are removed. *Post-hoc* Sidak comparisons by group housing revealed that the INTACT-CI and the INTACT-II groups showed greater weight gain than the CONT-SH, CONT-CC and CONT-CI groups, $ps \leq .05$, Figure 45.

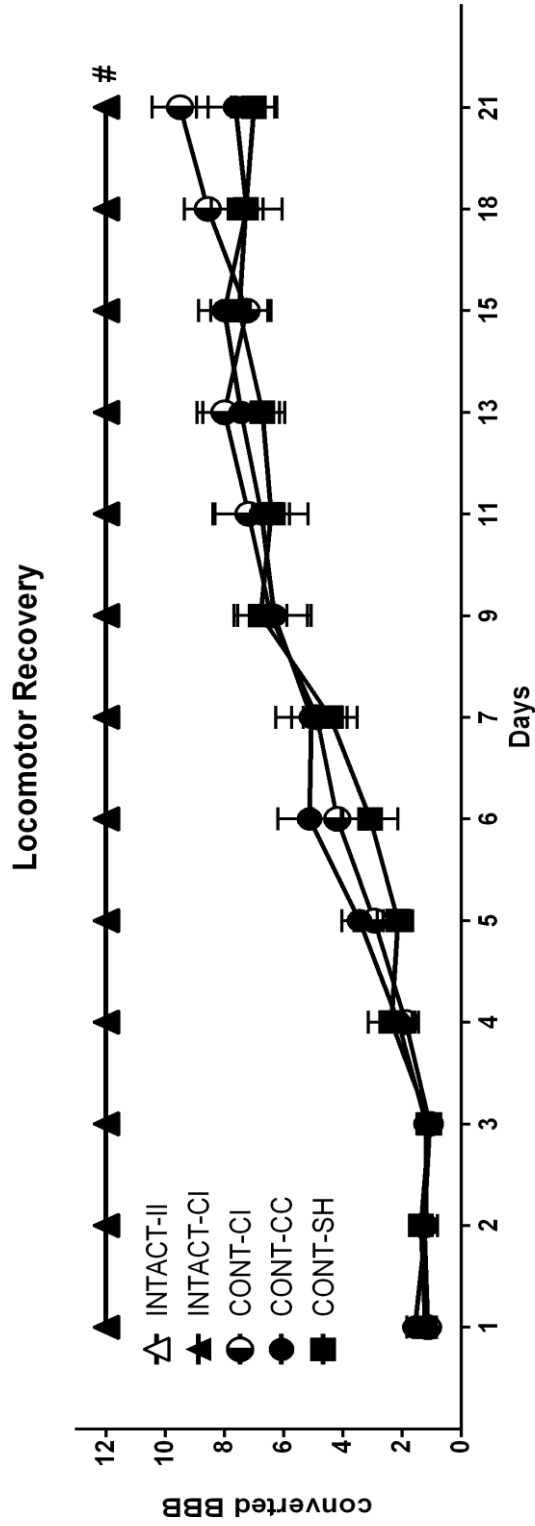


Figure 43- Locomotor recovery of function by group housing condition. The average (\pm standard error of the mean) performance of the CONT-SH, CONT-CC, CONT-CI, INTACT-CI and INTACT-II groups are shown on the converted BBB test in the 21 days post injury. The INTACT-II and INTACT-CI showed higher converted BBB scores than all other groups throughout the recovery period. * $p < .05$, # significant from all others.

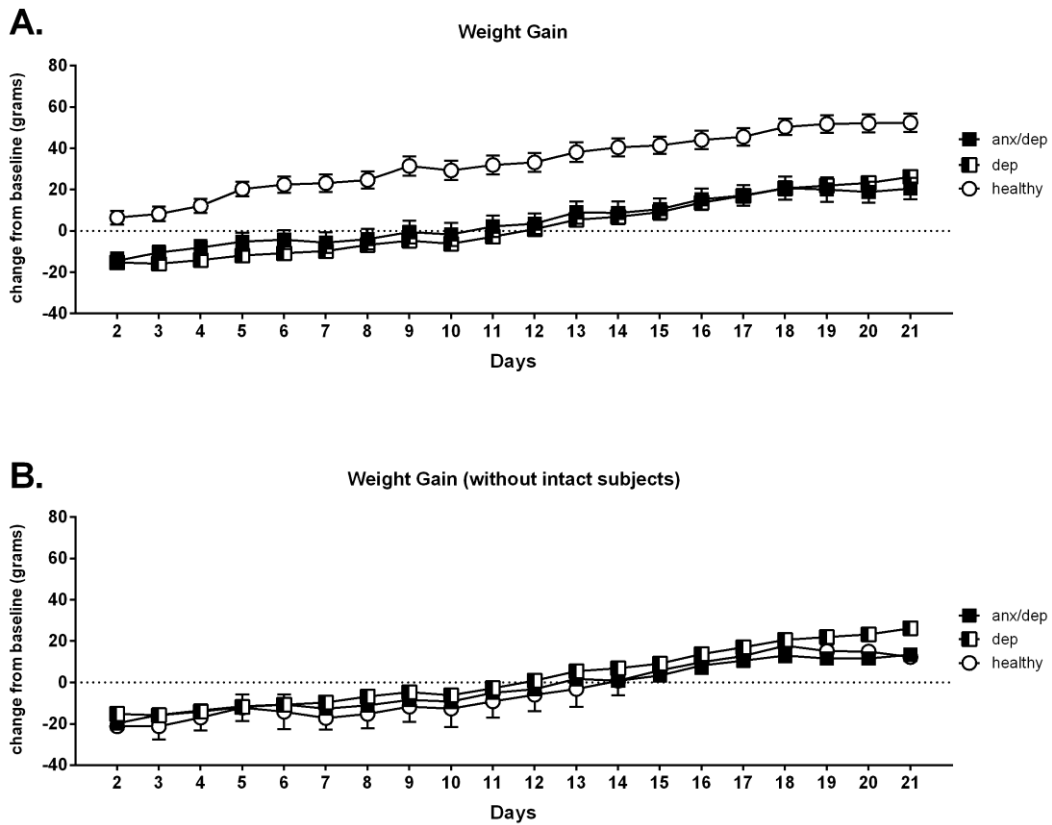


Figure 44- Weight gain by psychological well-being. The average (\pm standard error of the mean) weight gain of the ANX/DEP, DEP and HEALTH groups in the 21 days post injury are shown, with intact subjects included (A) and removed (B). Post-hoc Sidak comparisons by psychological well-being group revealed that the ANX/DEP and DEP groups showed significantly less weight gain than the HEALTH group (A). However, B indicates that this effect disappears when intact subjects are removed. * $p < .05$, # significant from all others.

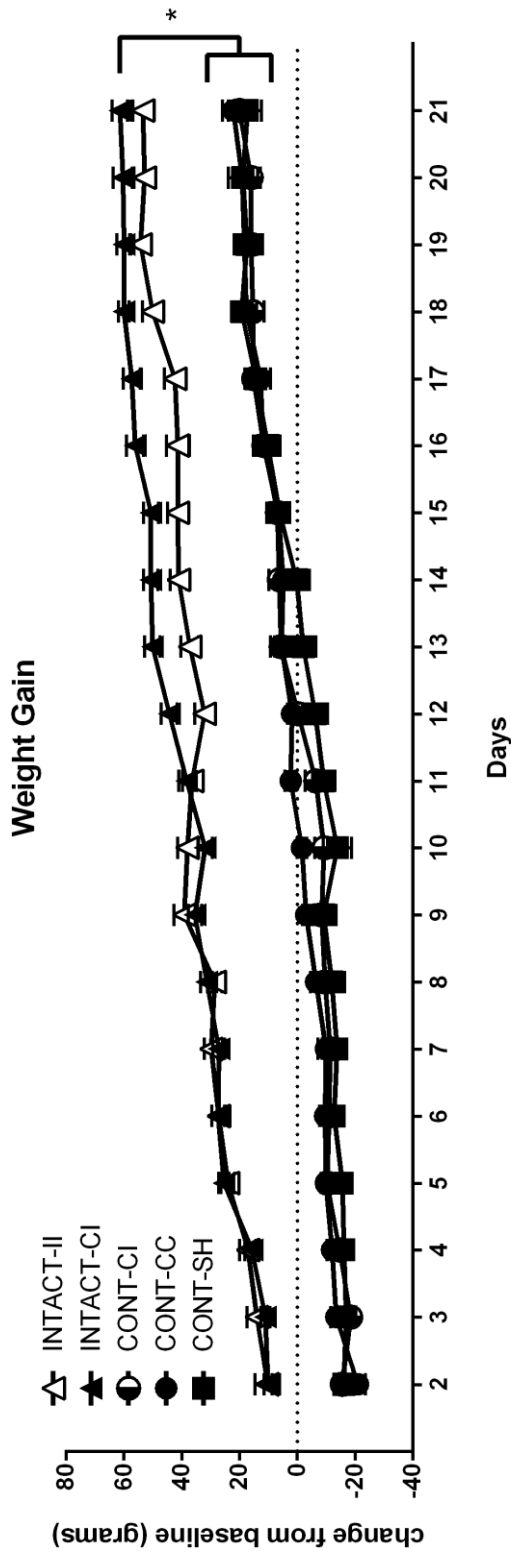


Figure 45- Weight gain by group housing condition. The average (\pm standard error of the mean) weight gain of the CONT-SH, CONT-CC, CONT-CI, INTACT-CI and INTACT-II groups are shown across the 21 days post injury. The INTACT-CI and the INTACT-II groups showed greater weight gain than the CONT-SH, CONT-CC and CONT-CI groups. * $p < .05$, # significant from all others.

Physiological measures

Alpha-2 macroglobulin. It was expected that levels of alpha-2 macroglobulin would be extremely low in intact subjects. However, they were not excluded from these analyses, because all but two intact subjects fell into the HEALTH group, therefore creating highly unequal groups. This is an inherent limitation of the study given the non-orthogonality between the two independent variables. Additionally, because alpha-2 macroglobulin is an acute phase protein, we expected group differences to be evident shortly after the spinal cord injury only. Therefore, we conducted ANOVAs on days 1, 10 and 21 separately. One-way ANOVAs for Days 1, 10 and 21 separately revealed a main effect of psychological well-being on Day 1, $F(2,33)= 13.91, p = .000$. A main effect of group housing was also found on Day 1, $F(4,31)= 21.34, p = .000$. *Post-hoc* Sidak comparisons by psychological well-being indicated that on Day 1, the DEP and ANX/DEP groups showed higher levels of serum alpha-2 macroglobulin than the HEALTH group, $ps \leq .05$, (Figure 46A and Figure 46B). *Post-hoc* Sidak comparisons by group housing indicated that on Day 1, the CONT-SH, CONT-CC, and CONT-CI groups showed significantly higher levels of serum alpha-2 macroglobulin than the INTACT-CI and INTACT-II groups, $ps \leq .05$, (Figure 47A and Figure 47B).

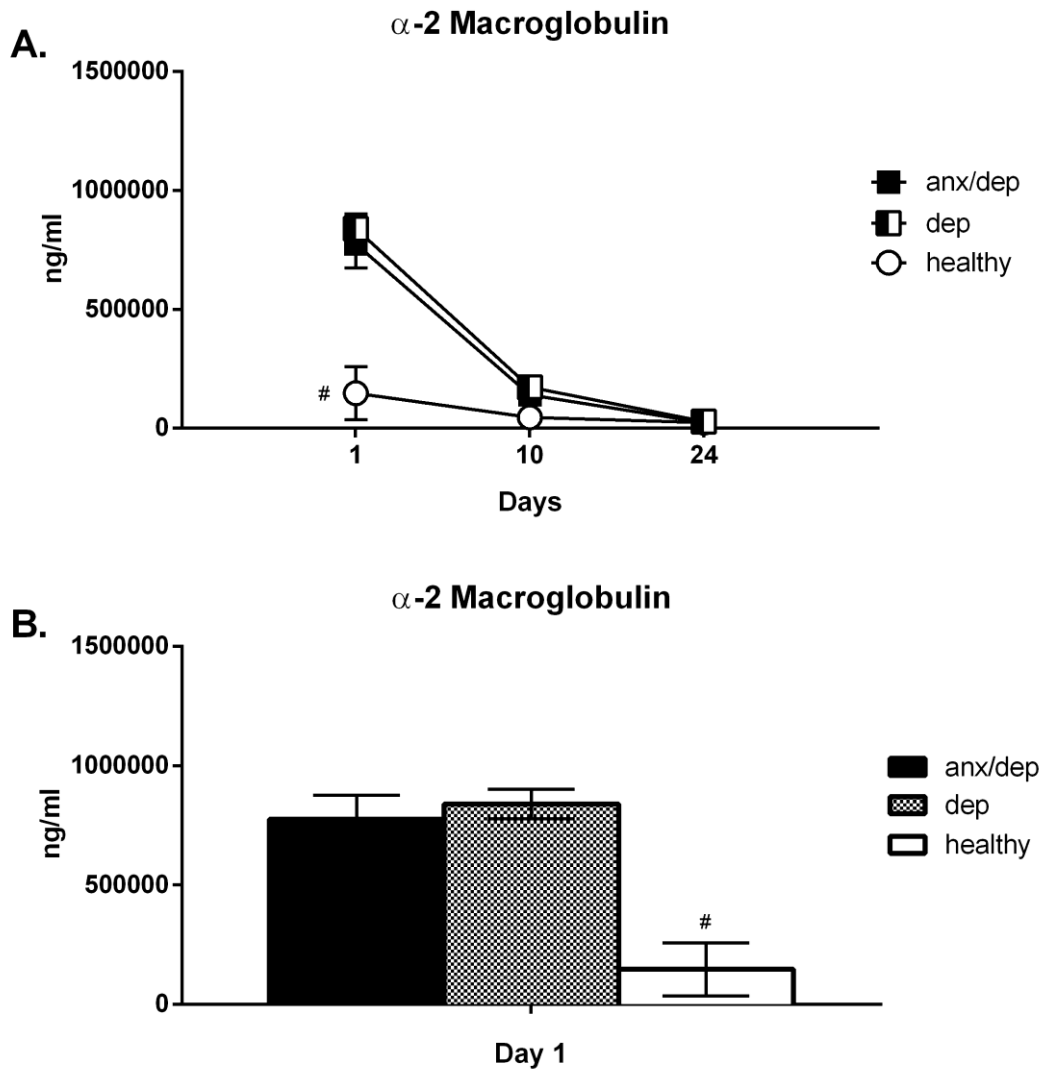


Figure 46- Serum alpha-2 macroglobulin levels by psychological well-being. The average (\pm standard error of the mean) alpha-2 macroglobulin levels of the ANX/DEP, DEP and HEALTH groups are shown across the recovery period (A) and for Day 1 post SCI specifically (B). On Day 1, the DEP and ANX/DEP groups showed higher levels of serum alpha-2 macroglobulin than the HEALTH group (B). * $p < .05$, # significant from all others.

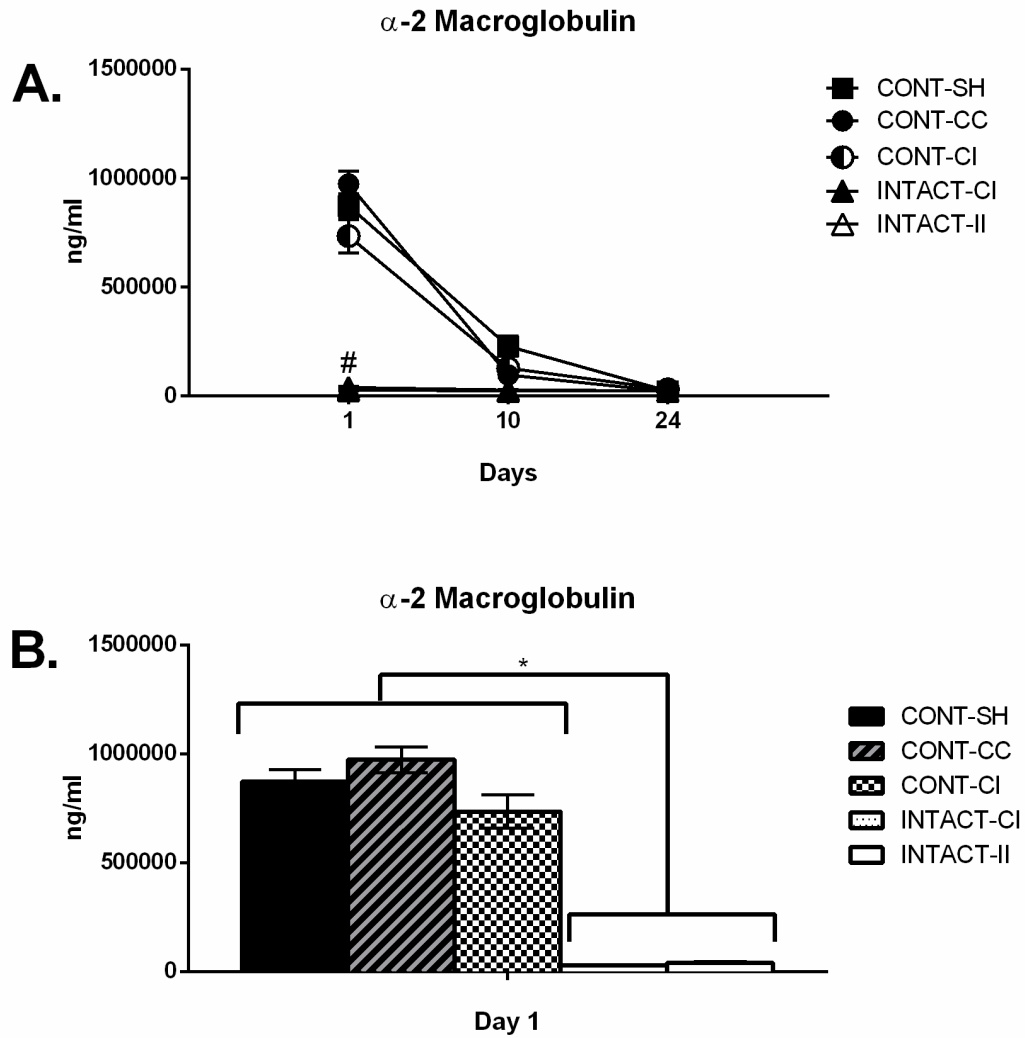


Figure 47- Serum alpha-2 macroglobulin levels by group housing condition. The average (\pm standard error of the mean) alpha-2 macroglobulin levels of the CONT-SH, CONT-CC, CONT-CI, INTACT-CI and INTACT-II groups are shown across the recovery period (A) and for Day 1 post SCI specifically (B). On Day 1, the contused groups showed higher levels of serum alpha-2 macroglobulin than the two intact groups (B). * $p < .05$, # significant from all others.

Being housed with a contused cagemate: the caregiver effect

Given that we observed some unexpected differences between the two group housed intact conditions (intact animals housed with a contused animal versus an intact animal), we ran another series of exploratory ANOVAs using contrasts to examine the differences in behavioral performance post contusion by intact group housing conditions. We first ran repeated measures ANCOVAs (baseline covariate) by Group Housing condition (GRH). Next, we ran ANCOVAs on each post contusion day separately: Day 10, and Day 21. Contrasts were conducted to compare INTACT-CI and INTACT-II groups. In graphs, the average score of all contused groups (CONT-SH, CONT-CC, and CONT-CI) are shown as a point of reference, but they are not included in the analyses.

We found two important differences in behavior in intact animals paired with a cagemate that received a contusion injury (INTACT-CI) relative to intact animals paired with a cagemate that remained intact throughout the experiment (INTACT-II). In the forced swim test, the INTACT-CI group showed significantly greater immobility than the INTACT-II group, $F(1,34)=15.719, p=.000$, Figure 48. In the open field, we found that the INTACT-CI group showed significantly less center activity than the INTACT-II group on Day 21, $F(1,33)=16.772, p=.000$ (Figure 49).

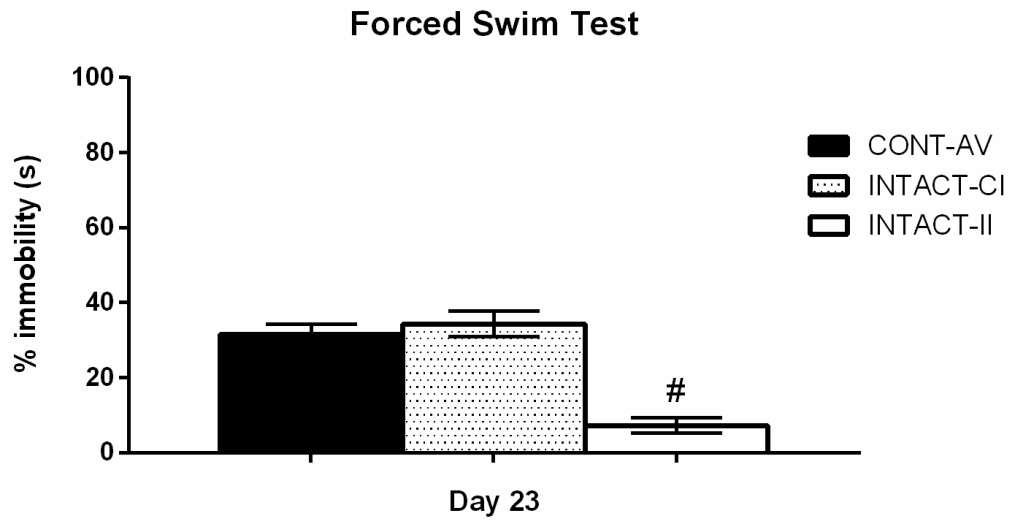


Figure 48- Differences in depression-like behavior in intact subjects. The average (\pm standard error of the mean) immobility at the forced swim test is shown for the INTACT-CI and INTACT-II groups. For reference, the contused groups (CONT-SH, CONT-CC, and CONT-CI) have been collapsed into one bar, CONT-AV. The INTACT-CI group showed immobility levels equivalent to those of the contused subjects, and greater immobility than the INTACT II group. * $p < .05$, # significant from all others.

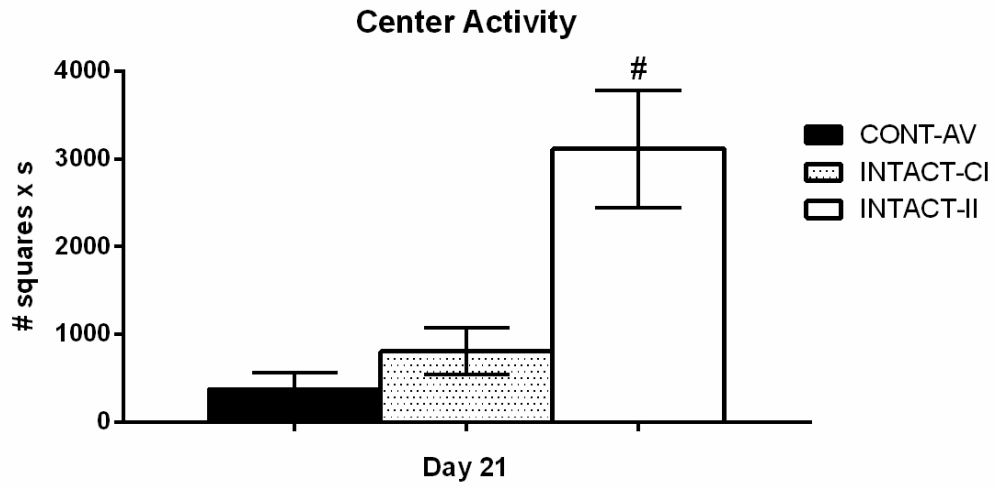


Figure 49- Differences in anxiety-like behavior in intact subjects. The average (\pm standard error of the mean) center activity is shown for the INTACT-CI and INTACT-II groups. For reference, the contused groups (CONT-SH, CONT-CC, and CONT-CI) have been collapsed into one bar, CONT-AV. On Day 21, the INTACT-CI group showed center activity levels equivalent to those of the contused subjects, and less center activity than the INTACT-II group. * $p < .05$, # significant from all others.

Discussion

In this experiment, we again characterized psychological well-being in spinal cord injured rats, using behavioral measures of anxiety and depression, and identified psychological well-being subgroups by conducting a series of statistical analyses. Second, we explored the effect of group housing and psychological well-being on measures of depression, anxiety, pain, recovery of function, and inflammation. We hypothesized that group housing would act as a form of social enrichment, increasing psychological well-being and decreasing inflammation.

Using the principal component and cluster analyses based on behavioral measures of depression and anxiety, we determined that 30.7% of rodents exhibited a depression-like profile, consistent with both the results of an earlier depression study (Luedtke et al., 2014), and the results of the Injury Severity and Stress experiment (Chapter 2). Another 28.2% of rodents exhibited an anxiety and depression-like profile. The subjects identified as depressed were equally distributed among the contused groups: CONT-SH, CONT-CC and CONT-CI. In fact, no intact subject (INTACT-CI or INTACT-II) was identified as depressed by the hierarchical cluster analyses. The majority of subjects identified as anxious/depressed were also contused subjects. Only two intact subjects were identified as anxious/depressed. It is worth noting that although the overall incidence of depression-like signs (30.7%) in this experiment is similar to that of Chapter III, the number of contused subjects falling into non-healthy categories (depressed and anxious/depressed) is markedly higher than in the Injury Severity and Stress chapter (Chapter III). This may in part be due to the fact that in the present

analyses, the principal component analysis and the hierarchical cluster analysis included a much larger number of intact subjects relative to the total sample size than in Chapter III. This high number of intact subjects, most of which generally show higher psychological well-being than contused subjects, may have driven the hierarchical cluster analysis to cluster a larger number of contused subjects into separate groups from the intact subjects, which were then identified, based on ANOVAs, as the depressed and anxious/depressed clusters.

Interestingly, similar to the results of Chapter III, we again found a relationship between anxiety and depression-like signs and mechanical allodynia following SCI. On Day 11, subjects fitting a depressed profile displayed a significantly lower paw withdrawal threshold at the tactile test than the healthy subjects. Moreover, the results indicated that both the anxious/depressed and the depressed subjects showed higher levels of serum alpha-2 macroglobulin levels than the healthy subjects. It must be noted, however, that in this experiment, the intact animals were not excluded from alpha-2 macroglobulin analyses, because all but two intact subjects were in the healthy group. Removing intact subjects would have rendered groups highly unequal and impeded analyses of variance. A significantly larger sample would have been necessary to ensure equal groups. Despite this, and given that the previous behavioral experiment (see Chapter III) found a main effect of psychological well-being for serum alpha-2 macroglobulin levels one day post injury, these data do point again to a relationship between decreased psychological well-being, increased pain sensitivity, and increased peripheral inflammation.

With regards to social enrichment, we found that group housing did not improve recovery following SCI. Our results also indicate that, surprisingly, housing a contused animal with a cagemate does not in itself improve psychological well-being. Depression-like signs did not differ significantly among single housed contused subjects and contused subjects housed with another contused subject or with an intact animal. On measures of anxiety, we found that all contused subjects, particularly the group housed contused subjects (either with a contused cagemate or an intact cagemate), showed increased passive anxiety: decreased center activity in an open field, decreased center time, and/or decreased center squares crossed. As others have suggested (Burke et al., 2007), we also found that group housing did not improve recovery of locomotor function in contused subjects.

While group housing did not affect psychological well-being or recovery for the contused subjects, we did find an unexpected effect of this variable in the intact groups. One of the most interesting findings in the current study was the evidence for a “caregiver effect.” Rather than protecting the contused subject against the decreased psychological well-being effects of a contusion injury, housing a contused subject and an intact subject together triggered depression- and anxiety-like signs in the physiologically intact subjects. For example, we observed significantly less immobility on the forced swim test in intact animals housed with an intact cagemate relative to all other groups, including intact cagemates housed with a contused subject. This suggests that intact animals are affected by the presence of an injured cagemate. Remarkably, being paired with an injured animal results in intact animals showing a level of learned helplessness

comparable to that of contused subjects. Further, we observed that the intact animals housed with an intact cagemate showed significantly more center activity in an open field than all other groups, including the intact animals housed with a contused cagemate. These intriguing results indicate that the psychological well-being of intact animals is affected by the presence of an injured cagemate.

This appears to mirror the effect of caregiving in humans. In effect, providing care for a long-term home care relative suffering from Alzheimer's, cancer, dementia, or SCI, has been shown to be a source of chronic stress, and is associated with depression and anxiety. Forty to 50% of caregivers for spinal cord injured relatives experience significant depressive symptoms (Arango-Lasprilla et al., 2010; Rodakowski, Skidmore, Rogers, & Schulz, 2013). In addition to this, caregivers report general reduction in quality of life (Graca, Nascimento, Lavado, & Garanhani, 2013) and overall psychological well-being (Gajraj-Singh, 2011). Furthermore, caregiving can affect the immune system. A systematic review of 37 dementia caregiver studies found that caregiving was consistently associated with elevated stress and inflammation (Fonareva & Oken, 2014). Caregivers of dementia relatives, for example, show increased levels of pro-inflammatory cytokines such as IL-6 (Kiecolt-Glaser et al., 2003), inflammation markers such as C-reactive protein (Miller et al., 2008), as well as up-regulation of the inflammation-related transcription factor NF- κ B (Hänsel, Hong, Cámara, & von Känel, 2010; Miller et al., 2008). Although we observed no serum alpha-2 macroglobulin level increases in the intact subjects housed with contused cagemates, it would be interesting

to further explore whether inflammation plays a role in the caregiver effect we observed in future studies.

To our knowledge, this experiment is the first to explore the effects of social enrichment on psychological well-being following a spinal cord contusion injury in a rodent model. In contrast to the various human clinical studies reporting a positive association between social support and psychological well-being post SCI, we found that housing a contused rodent with a cagemate did not improve psychological well-being following SCI. One important limitation of this study was the uneven number of subjects in each sub-group (group housing condition by psychological well-being), as evident in Figure 36. Ideally, we would have had an equal number of subject in each sub-group to run 2-way ANOVAs. However, this would have required running a large number of additional subjects, as the psychological group membership of each subject could only be determined on Day 10 post SCI, and the group housing conditions were assigned on the day of surgery. Intriguingly, however, we observed a “caregiver effect”, with decreased psychological well-being in intact animals housed with contused animals. In the future, an experimental animal model of group housing post SCI such as ours may be instrumental in studying the mechanisms underlying the mental and physical health effects associated with caring for family member experiencing SCI or another chronic health condition.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

In this dissertation, we examined two inverse relationships between spinal cord injury (SCI) and psychological well-being: (1) that SCI affects psychological well-being, and (2) that psychological well-being affects SCI. Chapters III and IV focused on the former relationship, and Chapter V explored the latter. To assess whether SCI influences psychological well-being, we first characterized psychological well-being post SCI at the behavioral level as a function of injury severity, and assessed pain reactivity and general peripheral inflammation (Chapter III). We found that psychological well-being decreases significantly following SCI, but not clearly as a function of injury severity. Decreased psychological well-being was associated with increased serum levels of alpha-2 macroglobulin, a biomarker of peripheral inflammation in rats, as well as reduced thymus weight, and increased pain reactivity. These results suggested that SCI may impact the immune system which then contributes to decreased psychological well-being following SCI.

To investigate this further, in Chapter IV, we assessed the levels of 27 cytokines and chemokines peripherally and centrally, in the serum, brain and spinal cord tissue of the subjects from Chapter III. While almost no effects of injury severity were observed in the serum, we noted a clear effect of psychological well-being, with the depressed group showing higher levels of several pro-inflammatory cytokines and chemokines on

Day 1 (GM-CSF, GRO-KC, MIP-2, TNF- α , IL-1 β , IL-10, Fractalkine, and LIX) and Day 24 (GM-CSF and IL-1 β), relative to the healthy group.

In the hippocampi also, no effects of injury severity were observed. Interestingly, however, cytokine and chemokine levels differed significantly by psychological well-being group, with the anxious/depressed group showing higher levels of IL-1 α and TNF- α than the healthy group. Finally, in the spinal cord, we found that, as expected, SCI resulted in an increase in several cytokines and chemokines (IL-1 α , IL-1 β , IL-2, IL-6, IL-18, GRO-KC, MCP-1, and TNF- α), relative to intact animals. Moreover, we found psychological well-being differences similar to those found in the brain: the anxious/depressed group showed higher levels of IL-18, IFN- γ , MCP-1, and TNF- α than the healthy group. These results indicated that in anxious/depressed subjects, anxiety, may maintain high levels of pro-inflammatory cytokines and chemokines in the central nervous system, even 25 days after the injury. Our results confirmed our hypothesis, that SCI results in increased cytokine and chemokine levels, and that these are associated with decreased psychological well-being.

In Chapter V, we explored the inverse relationship; that increased psychological well-being may modulate SCI recovery. We used group housing, as a modulator of psychological well-being, to examine the effects of psychological well-being on recovery from SCI. We found that, surprisingly, housing a contused animal with a cagemate does not in itself improve psychological well-being. Depression-like signs did not differ significantly among single housed contused subjects and contused subjects

housed with another contused subject or with an intact animal. We also found that all contused subjects showed increased passive anxiety. However, intriguingly, we found evidence for a “caregiver effect.” Rather than protecting the contused subject against the decreased psychological well-being effects of a contusion injury, housing a contused subject and an intact subject together triggered depression- and anxiety-like signs in the physiologically intact subjects. These results are reminiscent of the effect of caregiving in humans, where providing care for a long-term home care relative can be a source of chronic stress, and is associated with depression and anxiety.

In sum, our data suggest that SCI, by activating the immune system, may hinder psychological well-being, potentiating the development of depression and anxiety-like signs post injury, as well as pain. Moreover, psychological well-being may in turn influence the physical course of recovery by modulating immune system activation in the long-term. Figure 50 illustrates the main findings of this dissertation, and the relationships we propose to have uncovered between the various components studied within it: SCI, inflammation, depression, anxiety, and pain. In the following sections, we will review the relationships between SCI, psychological well-being, inflammation, and pain based on the results obtained in our three studies (Chapters III, IV, and V), and discuss their clinical relevance.

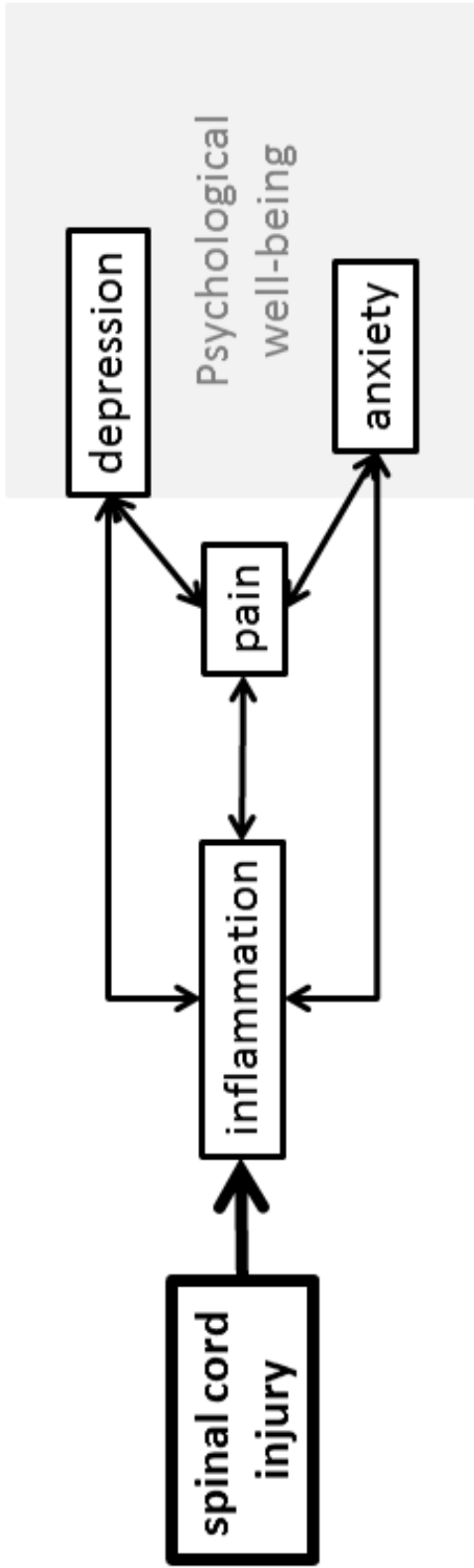


Figure 50- Comprehensive summary of findings. This figure illustrates all the relationships uncovered in this dissertation, between SCI, inflammation, pain, depression, and anxiety. Arrows represent suggested directional relationships based on findings, but do not intend to indicate causality. With the exception of the SCI -> inflammation causal relationship (bold arrow), our experiments were designed to test associations, not causality.

Spinal cord injury results in distinct depression sub-types

Approximately 60% of spinal cord injured patients suffer from depression (Shin et al., 2012) or anxiety (Post & van Leeuwen, 2012) at some point following the injury. Consistent with the clinical reports, in Chapter III, we found that 63.8% of contused rodents presented depression or anxiety-like signs following SCI. Specifically, one subgroup of contused rodents exhibited a purely depression-like profile (30.5% of contused subjects), and a second subgroup of contused rodents exhibited a profile of both depression and anxiety-like signs combined (33.3%). In Chapter V, the number of contused subjects exhibiting a depression-like profile and an anxiety/depression-like profile were higher. Fifty-two percent of contused subjects were identified as depressed, and 39% were identified as anxious/depressed. This may in part be explained by the fact that this experiment included a much larger number of intact subjects relative to the total sample size than Chapter III. This high number of intact subjects, most of which generally show few depression and anxiety-like signs, may have driven the hierarchical cluster analysis to cluster a larger number of contused subjects into separate groups from the intact subjects, which were then identified, based on ANOVAs, as the depressed and anxious/depressed clusters. Nonetheless, in both Chapters III and V, the depressed subgroup showed decreases in various behavioral tests designed to mirror the DSM-IV criteria for depression in humans, such as psychomotor reduction, decreased interest in social interactions, and passive anxiety. In turn the anxious/depressed sub-group showed certain depression-like signs, such as anhedonia, psychomotor reduction, decreased interest in social interactions, as well as active and passive anxiety. Despite the

differences in incidence of depression and anxiety in Chapters III and V, in both chapters, we observed the same three distinct sub-groups: subjects with no depression or anxiety (healthy), subjects with depression-like signs only (depressed), and subjects with a combination of depression- and anxiety-like signs (anxious/depressed).

The incidence of depression in Chapter III was also commensurate with a previous study in our laboratory. Luedtke et al. (2014) found that 34.6% of contused subjects displayed a behavioral profile characteristic of depression. However, Luedtke et al. (2014) did not assess anxiety, and the hierarchical cluster analyses identified only two clusters of animals in these data. In the present experiments, the unrestricted hierarchical cluster analyses separated the data into three clusters with the third cohort representing an anxious/depressed cluster. The identification of a third cluster of subjects in the current study may have been due to one important methodological difference in the two studies. In Luedtke et al. (2014), no blood draws were performed, whereas in the present experiment, blood draws were performed prior to the contusion injury, and on days 1, 10 and 21 post injury. We suspect that the repeated blood draws, in combination with the invasive contusion surgery, may have acted as a stressor to increase anxiety-like behavior.

While methodological differences may have resulted in the manifestation of anxiety-like signs, similarly to Luedtke et al (2014), we found no relationship between psychological well-being and physical function post SCI. Locomotor recovery, as assessed by the BBB scale, did not differ based on psychological well-being.

Furthermore, performance on the behavioral tests of depression- and anxiety-like signs did not decrease as a function of injury severity. In fact, the severe injury group displayed greater movement in some tests, such as the open field activity test. This is of great relevance, for it indicates that the differences we observed between psychological well-being subgroups are not due simply to differences in motor ability.

Spinal cord injury results in increased inflammation

One of the primary aims of this dissertation was to investigate the relationship between inflammation and psychological well-being. As predicted, we found that SCI results in increased inflammation both peripherally (Chapters III, IV and V) and centrally (Chapter IV). In Chapter III, we observed significant increases in serum alpha-2 macroglobulin, an acute phase protein, in contused subjects one day post SCI. These results were replicated in Chapter V, in which all contused subjects, regardless of housing condition, displayed higher levels of serum alpha-2 macroglobulin than the intact animals on Day 1 post SCI. However, in Chapter IV, we found that the cytokine and chemokine levels of the contused subjects were only significantly increased in the spinal cord. Relative to the intact group, the contused groups showed higher levels of IL-1 α , IL-1 β , IL-2, MCP-1, IL-6, GRO-KC, IL-18 and TNF- α . In the serum and the hippocampi, although some trends emerged, we found no significant increases in cytokine and chemokine levels overall in the contused groups relative to the intact group, except for serum MCP-1, which was increased in the moderate and severe injury groups relative to the intact group. In contrast, Hayes et al. (2002) reported elevated serum IL-2 and TNF- α in chronic spinal cord injured patients (injury experienced 12

months earlier or more) relative to healthy, aged-matched controls. We have found two possible explanations for the discrepancy between these results. First, Hayes et al. (2002) found that the serum increase in the pro-inflammatory cytokines was manifest in a subset of spinal cord injured patients only. Similarly, we found that subsets of SCI rodents showed elevated serum cytokine and chemokine levels 24 days post SCI: the subjects identified as depressed. Therefore, although our results appear, at first glance, to indicate that SCI does not result in peripheral nor brain increases of cytokine and chemokine levels, it does, but only in a subset of contused subjects. Second, Hayes et al. used enzyme-linked immunosorbent assays (ELISAs) to assess serum IL-2 and TNF- α levels. Because we sought to obtain a broad picture of pro- and anti- inflammatory cytokine and chemokine levels post SCI, and given that we were limited by small serum samples, we used multiplex assays, rather than ELISAs. Recent comparison studies and unpublished data presented at the 21st annual meeting of the PsychoNeuroImmunological Research Society (2014) indicate that although multiplex assays provide high-sensitivity detection of multiple cytokines simultaneously, their ability to provide absolute changes in cytokines is limited, and variations between kits are generally higher in multiplex assays than the traditional ELISAs (Breen, Perez, Olmstead, Eisenberger, & Irwin, 2014; Breen et al., 2011). It is therefore possible that differences in serum and brain cytokines and chemokines would have been observed among injury severity groups had we used ELISAs. However, the clear differences we observed among psychological well-being groups in these same samples, as well as in

the spinal cord, reinforce the reliability of the assays conducted in this dissertation, and suggest this explanation is unlikely.

Further, despite significant increases in spinal cytokine expression with SCI, we did not find that cytokine nor chemokine levels increased as a function of injury severity. This contrasts with our original hypothesis, but was likely affected by the time of tissue collection. In our study, we examined central inflammation (spinal cord and hippocampi) 25 days post SCI. Only serum levels were assessed in the acute phase of injury, at 24 hours post SCI. Yang et al. (2005) reported increases in inflammation as a function of SCI severity in spinal cord samples; however, these were collected in the hours following injury. Furthermore, Yang et al.'s immunohistochemical evidence suggested that the increased inflammation observed was due to endogenous cells of the spinal cord, as opposed to cells in the periphery. Our results suggest that such injury severity dependent changes in inflammation may only be present transiently, at the level of the spinal cord, and may not transfer to an injury severity dependent change in inflammation in the periphery, or a long-term injury severity dependent change in inflammation in the central nervous system.

However, similar to Hayes et al. (2002), who found that a subpopulation of spinal cord injured patients show increased serum levels of pro-inflammatory cytokines more than one year after the injury, we found elevated serum, hippocampal, and spinal cord cytokine and chemokine levels in subsets of SCI rodents even 25 days post SCI. Our studies extend the clinical findings to a rodent model of SCI, not only verifying that

in rats, like in humans, a subset of subjects show long-term increases in peripheral inflammation, but also demonstrating that this inflammation extends to the brain and the spinal cord. Moreover, we have shown that the subsets of subjects with increased cytokine levels also exhibit a distinct behavioral profile associated with depression and anxiety.

Site of inflammation varies by depression sub-type

In Chapter III, we found both depression and anxiety to be associated with higher levels of an acute phase protein, alpha-2 macroglobulin on Day 1 post SCI. Consistent with the literature indicating that thymus weight decreases with depression (Leonard & Song, 1996; Song & Leonard, 2000), we also found that at the time of sacrifice, the thymus weight of depressed subjects was significantly smaller than that of the healthy subjects. Similarly, human studies have found depression and anxiety to be associated with higher levels of circulating C-reactive protein, an acute phase protein equivalent to alpha-2 macroglobulin in rats (Copeland, Shanahan, Worthman, Angold, & Costello, 2012; Howren et al., 2009). However, when we examined the inflammation changes post SCI by psychological well-being sub-group more closely in Chapter IV, we found two interesting and distinctive patterns: the purely depressed sub-group showed increased inflammation in the periphery, whereas the anxious/depressed group showed increased inflammation in the central nervous system.

Depression post spinal cord injury is associated with peripheral inflammation

Juengst et al. (2014) have reported increased levels of inflammation in the cerebrospinal fluid of traumatic brain injury patients exhibiting posttraumatic depression. In Chapter III, we found that the sub-group of subjects identified as exhibiting purely depression-like signs showed increased serum levels of several pro-inflammatory cytokines and chemokines in both the acute and chronic phase of injury (Days 1 and 24 post SCI respectively) only. On Day 1, relative to the healthy group, the depressed group showed higher serum levels of GM-CSF, GRO-KC, MIP-2, TNF- α , IL-1 β , IL-10, Fractalkine, and LIX. On Day 25, relative to the healthy group, the depressed group showed higher serum levels of GM-CSF and IL-1 β . These results fit well the body of literature both in humans and rodents suggesting an inflammatory basis for depression (Audet & Anisman, 2013; Dantzer et al., 2008; Maes, 1999; Maes et al., 1997; van West & Maes, 1999; Walker et al., 2014), particularly after a central nervous system injury or illness (Juengst et al., 2014). Surprisingly, we did not find any increases in cytokines and chemokines in the hippocampi of depressed subjects. In hindsight, it is likely that this depression sub-type is associated with changes in brain areas other than the hippocampi, as hippocampal changes are mostly observed in human depressed patients, who often have comorbid anxiety, and in animal models in which depression is induced with chronic stress exposure (Marsden, 2013; Price & Drevets, 2010). In a meta-analysis of 23 human voxel-based morphometry studies examining brain changes associated with major depressive disorder, Bora et al. (2012) found that whereas depression with comorbid anxiety was characterized by significantly less gray matter in

areas of the limbic system, depression without comorbid anxiety was characterized by significantly less gray matter in the frontal cortex and the anterior cingulate cortex. In future studies, it will be necessary to examine inflammation levels in brain regions known to be affected in depression with, and without, comorbid anxiety.

Although our results do not allow us to state that there is a causal relationship between increased peripheral cytokine levels and depression, they provide a first line of evidence in that direction. As reviewed in the introduction, an extensive literature exists on the causal role of inflammation in depression, both animal and human (Capuron et al., 2001; Maes et al., 2011; van West & Maes, 1999). Human studies of SCI commonly observe depression post SCI, but are unable to identify clear predicting factors, for they are faced with a multitude of psychosocial variables such as financial difficulties and loss of independence. In this dissertation, with the use of an animal model, we avoided the confounding psychosocial factors. We were therefore able to demonstrate that even in a context devoid of psychosocial factors, SCI results in depression in a sub-group of injured subjects. Our results also revealed that increased levels of peripheral pro-inflammatory cytokines post SCI were associated with post SCI depression. Similar changes in serum levels of pro-inflammatory cytokines are reported in a subgroup of patients with depression (Felger & Lotrich, 2013; Howren et al., 2009). A meta-analysis of 51 studies conducted between 1967 and 2008, examining the relationship between C-reactive protein, IL-6, IL-1 and depression found, consistently, a relationship between these variables. Certainly, it must be recognized that publication bias may have prevented negative findings from being published. Nonetheless, taken together, the

existing human literature and our original findings, suggest that in the human SCI population, a sub-group of patients may experience depressive symptoms due to post SCI dysregulations in peripheral inflammation, rather than to purely psychosocial factors.

Importantly, we do not claim that monoamine and hypothalamic-pituitary-adrenal (HPA) axis dysregulation are not involved in depression following SCI. Rather, we propose that sub-types of depression exist, and that in certain sub-types, the molecular mechanisms underlying the development and maintenance of depression involve inflammation. However, it is important to keep in mind that simply using a peripheral biomarker of inflammation, such as C-reactive protein, to identify patients falling into this depression sub-group is not be sufficient. Many recent studies continue to further uncover the complex role of inflammation in depression, for example demonstrating that inflammation is in itself neither necessary nor sufficient to diagnose depression (Raison & Miller, 2013). In effect, many individuals who suffer from inflammatory illnesses, such as, for example, rheumatoid arthritis, are not depressed; and inversely, many individuals suffering from depression may show no dysregulations in inflammatory levels. Nonetheless, biomarkers of inflammation should be incorporated in the current diagnosis of depression post SCI, so that spinal cord injured patients suffering from depression due to post SCI inflammation dysregulation specifically may receive a personalized treatment.

Anxiety post spinal cord injury is associated with central inflammation

Central nervous system inflammation is associated with anxiety-like behavior. Monocyte recruitment to the brain has been shown to result in anxiety-like behavior in a mouse model of chronic social stress, repeated social defeat (Wohleb, Powell, Godbout, & Sheridan, 2013). In a rat model of post-traumatic stress disorder also, anxiety-like behavior was associated with elevated pro-inflammatory cytokines in the hippocampus (Wilson et al., 2013). Similar findings have been reported in the spinal cord. For example, repeated social defeat results in spinal up-regulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), two pro-inflammatory molecules, as well as hyperalgesia, and anxiety in mice (Rivat et al., 2010). Likewise, in our study, the sub-group of subjects identified in Chapter III as exhibiting both depression and anxiety-like signs showed increased levels of several pro-inflammatory cytokines and chemokines in the hippocampi and in the spinal cord (Chapter IV). The anxious/depressed group showed increased levels of IL-1 α and TNF- α in the hippocampi and increased levels of IL-18, IFN- γ , MCP-1, and TNF- α in the spinal cord. These results suggest that in this sub-group of subjects, anxiety maintained high levels of pro-inflammatory cytokines and chemokines in the central nervous system, even 24 days after SCI. To the best of our knowledge, this is the first study to examine changes in brain cytokines following SCI.

As mentioned in the Introduction to this dissertation (Chapter I), a chronic activation of the HPA axis can lead to chronically elevated circulating glucocorticoids. In our study, repeated saphenous vein blood collections combined with SCI may have

acted as a chronic stressor, thus activating the HPA axis chronically. This, in turn, may have caused glucocorticoid resistance, thereby undermining the anti-inflammatory actions of glucocorticoids. As a result, production of pro-inflammatory cytokines would not be inhibited, and inflammation levels would be increased and sustained (see Figure 1). This may, in part, explain the increased levels of pro-inflammatory cytokines in the central nervous system of subjects identified as anxious/depressed. In addition to this, it is possible that the acute increase in inflammation due to SCI, both peripherally and centrally, further activated the HPA axis, reinforcing the cycle described above, as circulating pro-inflammatory cytokines are known to activate the HPA axis (Pace et al., 2007; Rosenblat et al., 2014).

The increase in pro-inflammatory cytokines and chemokines in the spinal cord specifically, provides evidence that, as proposed in Maldonado Bouchard (2014), anxiety may result in increased inflammation post SCI, including in the spinal cord, and it may be maintained into the chronic phase of injury, thereby modulating SCI recovery in the long-term. This has important clinical implications, as it suggests that spinal cord injured patients displaying prolonged and severe signs of anxiety may not only be experiencing mental distress, but that anxiety in turn may, inadvertently, be causing immune changes at the level of the lesioned spinal cord. Such changes could contribute, at the cellular level, to neurotoxicity and apoptosis, and to the development of neuropathic pain. In our studies, we did not observe decreases in locomotor recovery associated with increased inflammation that could be suggestive of apoptosis at the level of the spinal cord. We also did not find a direct correlation between pain measures and

inflammation, although we noted that the subjects who displayed decreased paw withdrawal thresholds on the tactile reactivity test (anxious/depressed) were also the subjects who showed greater central inflammation, both in the hippocampi and the spinal cord. Inflammation is known to be associated with chronic pain (Walker et al., 2014), and most chronic pain is of a spontaneous nature. Some have argued that the conventional tests used to assess pain, which require application of a stimulus, such as von Frey filaments, do not accurately report chronic pain (Sufka, 1994). Because they require tactile stimuli, these tests are thought to only accurately assess evoked pain, not spontaneous pain. The use of a conditioned place paradigm paired with an analgesic agent has been proposed to uncover spontaneous pain without the application of any tactile stimulus (King et al., 2009; Sufka, 1994). In the future, it would be interesting to use this type of conditioned place paradigm with our rodent model of depression and anxiety post SCI to further examine whether an association exists between central inflammation, anxiety, and neuropathic pain.

Interplay between pain, inflammation and psychological well-being

The results obtained in both Chapter III and Chapter V suggest a relationship between inflammation, pain and depression and fit well with psychoneuroimmunological studies proposing that inflammation may be the common factor underlying both depression and pain in patients exhibiting comorbid depression and chronic pain (Walker et al., 2014). In Chapter III, we found that the subjects identified as depressed not only showed decreased psychological well-being, but also showed decreased paw withdrawal

thresholds on the tactile test, and increased peripheral inflammation, as measured by serum alpha-2 macroglobulin levels.

In Chapter V, we found a similar relationship between anxiety and depression-like signs and mechanical allodynia following SCI. On Day 11, subjects fitting a depressed-like profile displayed a significantly lower paw withdrawal threshold at the tactile test than the healthy subjects. Moreover, the results indicated that both the anxious/depressed and the depressed subjects showed higher levels of serum alpha-2 macroglobulin levels than the healthy subjects. Together, these findings point to a relationship between decreased psychological well-being, increased pain sensitivity, and increased peripheral inflammation.

Recent studies have revealed the therapeutic potential of common anti-inflammatory agents, non-steroidal anti-inflammatory agents in particular, such as the common aspirin, for the treatment of mental illness (Berk et al., 2013). By inhibiting COX-1 and COX-2 and triggering lipoxin production (anti-inflammatory eicosanoids), aspirin is thought to result in the inhibition of neutrophil and eosinophil activation, as well as the regulation of NF- κ B activation, a pro-inflammatory cytokine gene transcription factor, thus regulating inflammation and regulating cytokine-induced oxidation, two pathophysiological processes involved in depression (Berk et al., 2013). Supporting this idea, Guan et al. (2014) have found that aspirin administered i.p. following a 15 minute forced swim test session reduced immobility on the forced swim test 24 hours later, as well as reduced serum levels of pro-inflammatory cytokines

following the second test. If our interpretation of our results from this dissertation is correct, and inflammation is at the crossroads between decreased psychological well-being and pain following SCI, it suggests that spinal cord injured patients suffering from depression and chronic increases in peripheral and central inflammation may not only benefit from a personalized therapy to control immune dysregulation for the treatment of depression, but also for the treatment of chronic pain.

Social environment post spinal cord injury and the caregiver effect

In Chapter V, we found that group housing did not improve recovery of locomotor function in contused subjects, nor did it promote psychological well-being. Depression-like signs did not differ significantly among single housed contused subjects and contused subjects housed with another contused subject or with an intact animal. All contused subjects, particularly the group housed contused subjects (either with a contused cagemate or an intact cagemate), actually showed increased passive anxiety. At first glance, these results contradict the studies reporting social enrichment to be associated with beneficial effects, such as decreased anxiety-like signs, increased levels of BDNF in the amygdala and hippocampus (Ravenelle et al., 2014), decreased depression (Grippo et al., 2014; Krugel et al., 2014) and lowered basal heart rate (Azar, Sharp, & Lawson, 2011). However, three possible explanations exist for these findings.

The immediate interpretation is that group housing does not improve psychological well-being in spinally injured rats. Instead, by decreasing the space available in the cage for the contused subject to move, decreasing access to food and

water, and increasing the likelihood of harmful physical contacts post injury, it may be a source of physical stress. This stress may, in fact contribute to the increased incidence of depression and anxiety found in this experiment relative to the experiment of Chapter III and Luedtke et al. (2014). Consistent with this idea, social enrichment, in the form of housing 10 rats in a cage, has been reported to result in increased levels of corticosterone and, increased anxiety-like signs (Moncek et al., 2004). The fact that we observed decreased body weight in group housed contused subjects also supports this explanation, although the literature on the health promoting effects of social enrichment (Ravenelle et al., 2014) would contradict this interpretation.

The second explanation is that because spinal cord contusion is a traumatic injury of a severe nature, the potentially mild benefits of social interventions, even when present, may remain imperceptible. In effect, our findings in Chapter III and IV demonstrate that SCI results in depression- and/or anxiety-like signs in nearly 64% of contused subjects, as well as increases in inflammation in the spinal cord, the serum, and the brain. The positive effects of social enrichment may be too mild to counter the dramatic psychoneuroimmunological changes that take place post SCI.

Finally, a third explanation for our results is that we did not implement social enrichment with the most appropriate form of group housing. Numerous group housing protocols exist. Certain studies provide social enrichment by housing subjects in groups of four in a large pen-like cage (Azar et al., 2011), 6 rats (Berrocal et al., 2007) or even ten (Moncek et al., 2004). Others combine social enrichment with physical enrichment,

providing animals with a variety of objects every day (Ravenelle et al., 2014). Animal density, duration of group housing prior to experimental procedures, and cage size, among others, are all factors that can influence behavior (Burke et al., 2007). Because our experimental subjects were contused and thus technically vulnerable to physical attacks in the days following the surgery, we opted for a group housing paradigm which consisted of only two rats housed together. It may be that in order to observe the beneficial effects of group housing, it would have been necessary, for example, to house a larger number of subjects together, in a larger cage, or begin group housing prior to SCI. Nonetheless, our experiment allowed us to stumble on an interesting and unexpected finding regarding the effect of spinal cord injured subject exposure on intact cagemates.

The caregiver effect

Providing care for a spouse, parent, or child suffering from a long-term disease is a source of chronic stress. The incidence of depressive symptoms in such caregivers is high- up to 50 % (Arango-Lasprilla et al., 2010; Rodakowski et al., 2013)- and frequently accompanied with high levels of inflammation (Fonareva & Oken, 2014), making caregivers prone to illness. In Chapter V, we noticed that intact animals housed with a contused subject displayed levels of learned helplessness and passive anxiety comparable to those of contused subjects. Although we did not observe differences in alpha-2 macroglobulin levels between the two groups, these intriguing results point to an effect of caregiving, possibly mirroring that reported in humans.

We can only hypothesize on the mechanism underlying the decrease in psychological well-being in intact animals housed with a contused cagemate. One possibility, for example, involved kidney malfunction, which often accompanies SCI in both humans and rodent models (Pettersson-Hammerstad, Jonsson, Svennung, & Karlsson, 2008; Rodriguez-Romero, Cruz-Antonio, Franco-Bourland, Guizar-Sahagun, & Castaneda-Hernandez, 2013). Glomerular function can decrease post SCI, and this is associated with the development of proteinuria (Vaidyanathan, Abraham, Singh, Soni, & Hughes, 2014). Therefore, it is possible that traces of acute phase proteins and/or stress hormones are excreted in the urine of contused subjects. Exposure to these proteins may result, for example, in activation of the HPA axis in intact cagemates.

In the clinical setting, the caregiver effect is an important aspect of SCI, and yet, it has been so far overlooked by animal research. An animal model of SCI caregiving, potentially based on our experimental design, may help better understand, at a biological level, the physiological and psychological effects of caring for an SCI family member.

Conclusion

In this dissertation, we tested the hypothesis that SCI-induced inflammation decreases psychological well-being and that, in turn, psychological well-being affects SCI recovery. To test this hypothesis, we conducted three experiments. Chapter III assessed the impact of SCI on psychological well-being at the behavioral level, and examined one biomarker of inflammation in relation to depression and anxiety-like signs post SCI. Chapter IV further examined cytokine and chemokine levels post SCI as a

function of injury and psychological well-being both peripherally (serum) and centrally (brain and spinal cord). Finally, Chapter V explored the inverse relationship; that is, that psychological well-being (modulated with group housing conditions) affects SCI recovery. As illustrated in Figure 50, with the data obtained throughout these three studies, we were able to identify a relationship between (1) spinal cord injury and inflammation, (2) inflammation and psychological well-being (depression and anxiety), (3) inflammation and pain, and (4) pain and psychological well-being. However, the last relationship studied, (5) psychological well-being and spinal cord injury recovery, could not be confirmed.

The experiments in this dissertation are the first to both examine depression and anxiety following SCI, and obtain a tableau of changes in immune system activation post SCI in the spinal cord, the brain, and the serum. Furthermore, we are the first to report a possible caregiver effect in an animal model of SCI. These findings provide confirmatory evidence that inflammation post SCI is associated with depression and anxiety. In the future, it will be necessary to conduct further molecular and pharmacological studies to determine the causal role of inflammation in psychological well-being post SCI. It will also be interesting to investigate the mechanisms underlying the caregiver effect post SCI. With this work, we have demonstrated that the depression and anxiety patients experience following spinal cord injury is not due solely to psychosocial factors, but may also, in part, result from increased immune activation following the injury. This constitutes one important step forward in improving our

understanding of mental health following SCI, and providing more appropriate psychiatric support for spinal cord injured patients.

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