SOCIAL STRESS EXACERBATIONS ON ACUTE THEILER'S VIRUS INFECTION: A ROLE FOR INTERLEUKIN-6

A Dissertation

by

ROBIN RANEE JOHNSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Psychology

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ABSTRACT

Social Stress Exacerbations on Acute Theiler's Virus Infection: A Role for Interleukin-6. (August 2006)

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Neurodegenerative diseases, such as multiple sclerosis (MS), are adversely affected by both stress and inflammation. Theiler's murine encephalomyelitis virus infection is an excellent animal model of MS, allowing examination of central nervous system inflammation during the acute phase of infection. Social disruption stress exacerbates acute Theiler's virus infection. Both social disruption stress and Theiler's virus infection elevate the pro-inflammatory cytokine, Interleukin-6 (IL-6). The current study examined the necessity and sufficiency of IL-6 in mediating the negative effects of social disruption stress in acute Theiler's virus infection. Experiment 1 blocked IL-6 function with a neutralizing antibody administered simultaneously with social disruption stress. All mice were then infected, and measures of illness, motor impairment and physiological signs of disease were collected up to 21 d post-infection. Experiment 2 administered exogenous IL-6 for one week (replacing social disruption with the cytokine treatment), followed by infection. Measures

identical to those collected in Experiment 1 were collected for up to 21 d post-infection. Results indicate that IL-6 is necessary for the development of the sickness, motor impairment, and immunological effects of social stress in acute Theiler's virus infection. In contrast, IL-6 alone can induce some, but not all, of the sickness behavior exacerbations, and was not sufficient for the development of either motor impairment or immunological effects previously associated with social disruption stress. These results have many important implications for further research in the effects of social stress on Theiler's virus infection, as well as clinical implications for both MS and other inflammatory mediated diseases, such as Alzheimer's disease and Parkinson's disease.

DEDICATION

To my mom- who understands why I ask: why not? To Frankie Lynn Martinez- the best friend anyone could ever have, she answers when I call, she reminds me of who I am, and she also asks: why not? To Patrick Bridegam- the other best friend anyone could have, for ensuring that I have a social life.

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NOMENCLATURE

"	Inch
AbTx	Antibody to IL-6 Treatment
ACTH	Adrenocorticotropic Hormone
ANOVA	Analysis of Variance
°C	Degrees Celsius
CNS	Central Nervous System
CO_2	Carbon Dioxide
CRF/CRH	Corticotrophin Releasing Factor or Hormone
CTL or CD8+	Cytolytic T-cells
d	Day
dB	Decibel
ELISA	Enzyme-Linked Immunosorbent Assay
g	Gram
GC	Glucocorticoids
GCR	Glucocorticoid Resistance
h	Hour
HBSS	Hank's Balanced Salt Solution
HPA axis	Hypothalamic-Pituitary-Adrenal Axis
IL	Interleukin
L2 or L-cell	Lung Tumor Cells

LPS	Lippopolysaccharide
min	Minute
μL	Microliter
mL	Milliliter
μM	Micromole
mm	Millimeter
mo	Month
N	Newton
NE	Norepinephrine
NF-κB	Nuclear Factor- Kappa B
ng	Nanogram
NIH	National Institutes of Health
NINDS	National Institute of Neurological Disorders
NON	Non-stressed, or Infected Control
pfu	Plaque Forming Unit
pi	Post-infection
pnd	Post-natal Day
s	Second
SAM axis	Sympathetic-Adreno-Medullary Axis
SDR	Social Disruption Stress
SEM	Standard Error of the Mean
Th or CD4+	T-helper Cells

TMEV	Theiler's Murine Encephalomyelitis Virus
TNF	Tumor Necrosis Factor
ULACC	University Lab Animal Care Committee
VEH	Vehicle

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1. INTRODUCTION

Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis (MS) are a growing health concern. Alzheimer's disease is expected to increase in prevalence by 44% by 2050 (Alzheimer's Association, 2005). Parkinson's disease prevalence will also increase as our population ages (NINDS, 2005b). MS has increased in prevalence by 50% in women in the past 20 years, and is expected to continue on this trajectory (NINDS, 2005a). These and other neurological disorders have at least two important factors in common. First, stress has been associated with the onset and exacerbation of symptoms of all of these diseases (Ackerman et al., 1998; Ackerman et al., 2000; Backer, 2000; Mohr et al., 2000; Rabin, 2002; Rodriguez et al., 2003; Smith et al., 2002; Warren et al., 1982; Warren et al., 1991; Wilson et al., 2003). Second, inflammatory processes contribute to the disease courses of all three of these neurodegenerative disorders (Matyszak, 1998; McGeer and McGeer, 1995). Because of these similarities, examining the roles of stress and inflammation in one of these disease models may result in greater knowledge and understanding across many inflammatory-mediated neurodegenerative diseases.

MS is a common inflammatory demyelinating condition of the central nervous system (CNS) (Oleszak et al., 2004). Demyelinating lesions are associated with inflammatory cell infiltrates consisting of plasma cells

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This dissertation follows the style of the Journal of Neuroimmunology.

(activated B-cells), macrophages/microglia, and T-cells. The disease develops between the ages of 15 and 50 and results in the loss of motor, sensory, autonomic, and neurological function. Most commonly, the disease manifests through loss of motor control or sensation in the limbs. Other clinical signs include optic neuritis, sexual dysfunction, cognitive dysfunction, loss of bowel or bladder control, and neuropathic pain. Although the etiology of MS is uncertain, research indicates that several environmental factors interact with genetic factors to cause disease (Kurtzke, 1993; Kurtzke and Hyllested, 1987; Noseworthy et al., 2000; Sospedra and Martin, 2005). Potential environmental risk factors include, but are not limited to, viral infection and stress. Epidemiological studies suggest that exposure to viruses, such as Herpesvirus-6 and Epstein Barr virus, in adolescence is associated with later, adult, development of MS (Acheson, 1977; Ascherio et al., 2001; Kurtzke, 1993; Sospedra and Martin, 2005). While infection with these or other viruses may be necessary, it is not sufficient, as additional work has shown that the majority of the general population has antibodies to these viruses, and yet do not develop MS (Hernan et al., 2001). Therefore, stress may be an important co-factor that interacts with viral infection to determine the development of MS.

That stress, a psychological process, could have such potent effects on immunological processes is no longer controversial. Seyle's original theory of stress, the general adaptation syndrome, included a triad of immunologically significant consequences. These included atrophy of the thymus and spleen,

primary immune organs, and enlarged adrenal glands (Selye, 1946). Research over the past few decades has demonstrated that many mechanisms for cross-talk are in place between the immune and stress systems. Stress activates two primary physiological axes, the hypothalamic-pituitary-adrenal axis (HPA-axis) and the sympathetic-adrenal medullary axis (SAM-axis). HPA-axis activation results in the release glucocorticoids (GCs) from the adrenal gland cortex. In addition, the SAM-axis releases norepinephrine from sympathetic neurons, and both norepinephrine and epinephrine from the medulla of the adrenal glands. Virtually every immunological organ and all immune cells types have receptors for one or more of these products (McEwen et al., 1997).

HPA-axis activation occurs when the paraventricular nucleus of the hypothalamus is stimulated, resulting in the release of corticotrophin releasing factor (CRF, also commonly referred to as corticotrophin releasing hormone or CRH), onto the pituitary gland. The pituitary gland then releases adrenocorticotropic hormone (ACTH), which travels through the blood stream to the adrenal gland. The adrenal gland cortex is then stimulated to release GCs. In humans the main GC is cortisol, while in rodents it is corticosterone. The hypothalamus is originally stimulated differently, based on the type of stressor. If the stressor is systemic or physical, such as hemorrhage or surgery, the central nucleus of the amygdala is activated (Dayas et al., 2001; Herman and Cullinan, 1997). In contrast, psychological or processive stressors, such as noise or restraint activate the medial nucleus of the amygdala, the bed nucleus

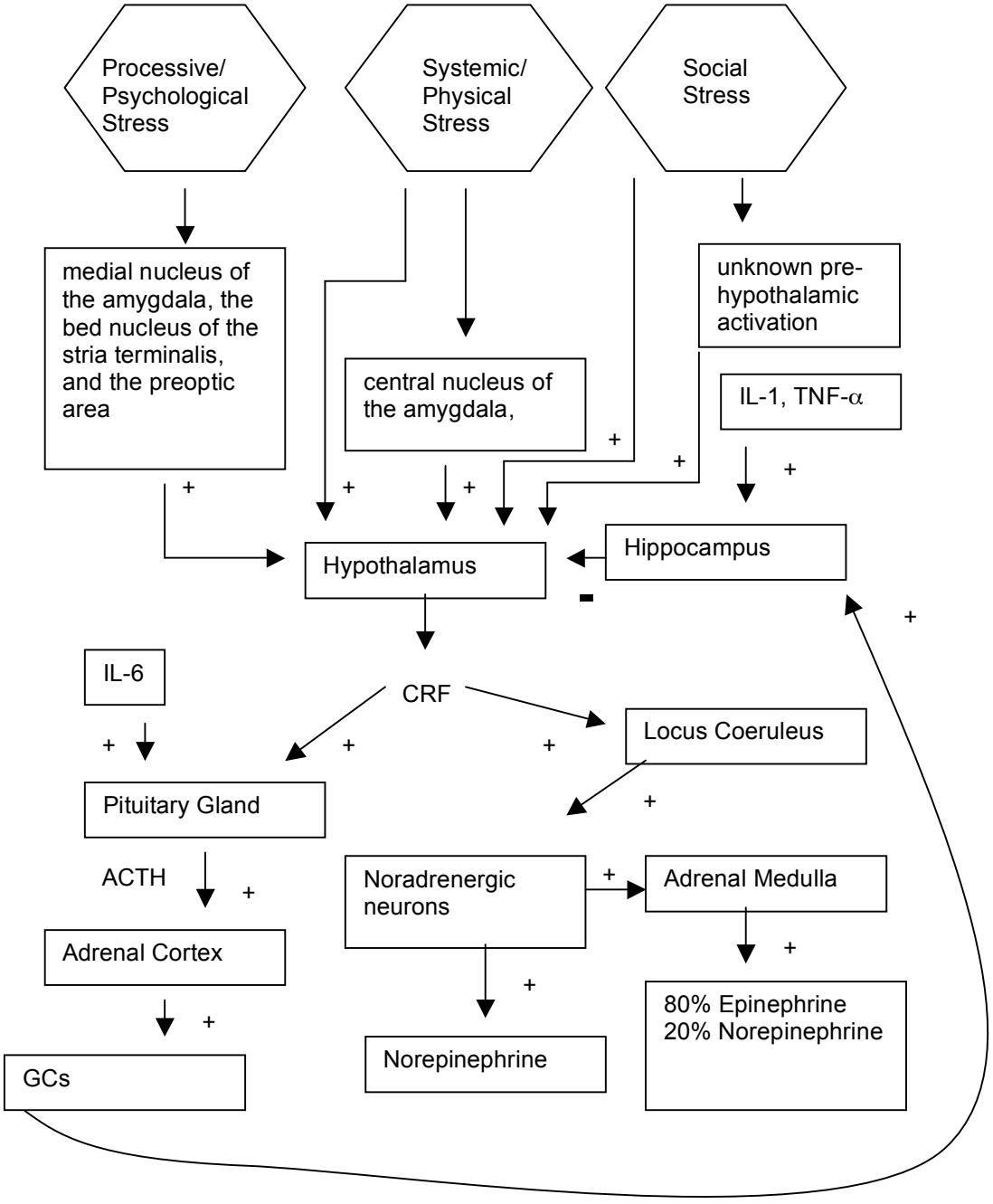


Fig 1.1. Diagram of Stress Axis.
This figure depicts the activation pathways of both the HPA and SAM axes. + signs indicate increased release or action, - signs indicate decreased release or action.

of the stria terminalis, and the preoptic area (Dayas et al., 2001; Herman and Cullinan, 1997). The “pre-hypothalamic” activation areas for social stressors have not been investigated, and are unknown at this time. These pathways are depicted in Fig 1.1.

Simultaneous to HPA-axis activation, the SAM-axis is also triggered. Among other possible neural circuits, CRF projections from the hypothalamus lead to the locus coeruleus resulting in the activation of sympathetic neurons. The sympathetic neurons directly release norepinephrine, and the innervation of the medulla of the adrenal glands results in the release of primarily epinephrine, but also lesser amounts of norepinephrine into the blood. SAM-axis can have faster action because norepinephrine is released through this direct neuronal activation as well as through hormonal activation of the adrenal medulla. Since HPA-axis release of GCs requires hormonal activity, the SAM-axis action is usually detectable more quickly than HPA-axis action.

The SAM-axis appears to act mainly through norepinephrine to alter immune function, either by directly innervating immune tissue or through receptors on immune cells. Sympathetic innervation occurs throughout lymphoid tissue, including the lymph glands, thymus, spleen and bone marrow (Felten et al., 1998). Norepinephrine can also influence the development and function of T-cells, and T-cell responses. When naïve CD4+, or T-helper cells are exposed to norepinephrine, they are more likely to become Th-1 cells (these

helper T-cells aid cytolytic T-cell activity) (Madden et al., 1995). However, norepinephrine is also more likely to recruit and aid in the function of Th-2 cells (these helper T-cells aid in B-cell activity) (Ramer-Quinn et al., 1997). Thus, norepinephrine has divergent effects on T-cell function, depending upon when in the immune response it becomes part of the milieu.

The HPA-axis affects the immune system through both CRF and GC. Inflammatory cells (such as macrophages and mast cells) have CRH receptors (Webster et al., 2002; Webster et al., 1998). GCs act primarily through cellular receptors. There are 2 subtypes of receptors, the mineralocorticoid (type 1) and the GC (type 2). While the type 1 receptors have a higher affinity for endogenous GCs, immune cells, including monocytes and lymphocytes express mainly type 2 (GC) receptors. Monocytes (macrophages, microglia) have the highest concentration, overall, followed by lymphocytes (B-cells, T-cells), while neutrophils have the lowest concentration (McEwen et al., 1997). It should be noted that while this overall pattern has been verified, individual cells within a subtype population vary greatly in the concentration of receptors. When GCs bind to a receptor in an immune cell, they act through the NF-κB transcription factor to decrease inflammatory gene transcription (Adcock et al., 2004). NF-κB mediated transcription occurs for a broad range of cellular functions. However, GCs only appear to alter gene transcription for cytokine production. How this specificity occurs considering the commonality of NF-κB transcription in multiple cellular functions is unknown at this time. The end result of GC action in

immune cells is to switch off the production of pro-inflammatory cytokines (IL-1, IL-6, and TNF-α), while simultaneously turning on production of anti-inflammatory cytokine (IL-4, IL-10). This appears to be the primary mechanism through which GCs are immunosuppressive (Adcock et al., 2004).

The communication between the immune system and the stress systems is bidirectional (Watkins et al., 1995b). The immune system can alter both of the stress axes as well. The pro-inflammatory cytokine, IL-1β can bind to receptors in the hippocampus and act to turn off the HPA-axis, acting as part of the negative feedback loop that normally regulates HPA-axis activation (Watkins et al., 1995b). In addition, peripheral IL-1β or TNF-α can bind to the vagus nerve and cause the release of IL-1β or TNF-α centrally (Fleshner et al., 1995; Goehler et al., 1997; Maier et al., 1998; Watkins et al., 1995a; Watkins et al., 1995b). Therefore, cytokine activity in the periphery can have concurrent effects centrally, inducing cytokine sickness behavior syndrome (Dantzer, 2001; Fleshner et al., 1998). Cytokine sickness behaviors include body temperature changes, anorexia, weight loss, decreased motor activity, mechanical hypersensitivity, and anhedonia- a loss of interest in pleasurable activities. (Dantzer, 2001; Watkins and Maier, 2000). These measures are known to be altered by IL-1β (Fleshner et al., 1995; Rachal Pugh et al., 2001), as well as TNF-α (Fleshner et al., 1995; Goehler et al., 1997; Maier et al., 1998; Watkins et al., 1995a; Watkins et al., 1995b). Finally, activated CD4+ (T-helper) cells can

cause the release of norepinephrine from sympathetic neurons (Kohm et al., 2000).

The pro-inflammatory cytokine, IL-6 has a particularly interesting role in the bidirectional communication between the immune and stress systems. Various stressors are able to increase plasma IL-6, in the absence of any immunogenic compound, such as LPS (LeMay et al., 1990; Maes, 2001; Shizuya et al., 1998; Steptoe et al., 2001; Takaki et al., 1994; Zhou et al., 1996). IL-6 may also be produced by non-immunogenic tissue, including both neuronal and adipose tissue (Murphy et al., 1999; Papanicolaou and Vgontzas, 2000). All cytokines act primarily in a paracrine manner, but IL-6 also has many endocrine-like actions (Papanicolaou and Vgontzas, 2000). Interestingly, IL-6 can, in contrast to IL-1, activate the HPA-axis (independent of CRF), during either an immune or stress response (Bethin et al., 2000). IL-6 can also induce some of the cytokines sickness behaviors (fever and increased mechanical sensitivity). However, more significant effects on sickness behavior occur through IL-6 synergism with IL-1β and TNF-α, allowing these cytokines to develop more significant levels of sickness behavior (Bluthe et al., 2000; Bluthe et al., 1994).

Based on the known interactions between the immune and stress systems, the previously stated hypothesis that stress may interact with exposure to viruses to increase the likelihood of the development of MS becomes more viable. Evidence for this theory exists in both human and animal work. In humans, elevated periods of conflict and disruption of routine are associated

with increased MS lesion development, and stress often precedes initial symptom diagnosis onset in MS, as well as further exacerbations of symptoms (Mohr et al., 2000; Mohr et al., 2004; Mohr and Pelletier, 2005). Animal models of MS have also found that stress exacerbates the disease course. Previous work from our laboratory has demonstrated the restraint stress exacerbates the early, acute phase of Theiler's virus infection (Campbell et al., 2001; Mi et al., 2004; Sieve et al., 2004), and these exacerbations can lead to more severe chronic phase (the MS-like phase) as well (Sieve et al., 2004). Acute stress also exacerbates another animal model of MS, experimental allergic encephalomyelitis (EAE) (Chandler et al., 2002).

Theiler's virus infection (TMEV) is a biphasic disease that causes an *acute* CNS inflammatory phase followed by a *chronic* neuroinflammatory/ autoimmune demyelination phase similar to MS in mice. The *chronic* phase of the disease has many similarities, both behaviorally and physiologically with the chronic progressive type of MS (Lipton, 1975; Oleszak et al., 2004). Previous work from our laboratory has found that social disruption stress (SDR) prior to infection with Theiler's virus infection leads to a more severe disease course (Johnson et al., in submission; Johnson et al., 2004). Given that preceding periods of stress negatively impact both MS and Theiler's virus infection, Theiler's virus appears to provide an important model to examine this issue in MS. In addition, acute Theiler's virus infection is highly inflammatory within the

CNS, adding relevance to any findings not only for MS, but the other inflammatory mediated neurodegenerative diseases (e.g. Alzheimer's disease).

Past studies have demonstrated that the social stressor, social disruption (SDR) alters the disease process of both *acute* and *chronic* TMEV infection in several ways when SDR was applied prior to infection. In the *acute* phase, SDR resulted in greater motor impairment, induced GC resistance (GCR), elevated CNS inflammation, and altered viral clearance compared to non-stressed animals (Johnson et al., 2004). Failure to clear the virus in the *acute* phase is necessary (although not sufficient) for the development of the *chronic*, demyelinating phase. In the *chronic* phase, SDR was associated with early onset of chronic phase motoric impairment, elevated antibodies to TMEV and elevated auto-antibody development to myelin proteins (Johnson et al., in submission). Finally, previous studies our laboratory have found that the severity of the *acute* phase is highly correlated with both the time of onset and the severity of the *chronic* phase (Johnson et al., in submission). These results occurred when SDR was applied prior to infection. When SDR is applied concurrent with infection, disease severity in both the *acute* and *chronic* phase of TMEV was alleviated compared to non-stressed animals. Taken together, these studies indicate that interventions that reduce the severity of the *acute* phase are likely to have positive effects for the *chronic* MS-like phase.

IL-6 may be an important molecule mediating or moderating the effects of SDR in *acute* TMEV infection. In humans, psychological stressors such as

examination stress, the Stroop color-word conflict task, and mirror tracing all significantly elevate IL-6 in circulation (Maes, 2001; Steptoe et al., 2001). In addition, caretakers for Alzheimer's patient's have elevations in IL-6 levels that do not dissipate even once care giving is terminated (Kiecolt-Glaser et al., 2003). A broad range of stressors also induce IL-6 in animals including foot shock, restraint, and immobilization (LeMay et al., 1990; Shizuya et al., 1998; Takaki et al., 1994; Zhou et al., 1993). Recently, social stressors have been shown to be powerful stimuli for inducing pro-inflammatory cytokines (Gaab et al., 2005; Merlot et al., 2003; Merlot et al., 2004; Stark et al., 2002; von Kanel et al., 2005; von Kanel et al., 2006). When rats went from individual housing to didactic pairings, IL-1β became elevated, particularly in the dominant animal within each pair (Fano et al., 2001). SDR is also associated with elevated IL-6 and TNF-α in circulation (Avitsur et al., 2005; Stark et al., 2002). In addition, SDR causes lippopolysaccharide (LPS)-stimulated spleen cells to release greater levels of IL-1β, IL-6, and TNF- α (Avitsur et al., 2002). The ability of LPS-stimulated cells to release increased levels of pro-inflammatory cytokines is not altered by the introduction of increasing concentrations of glucocorticoids (Avitsur et al., 2002).

Although the immune and stress systems have many mechanisms for co-regulation, this regulation can fail. The failure of corticosteroids to regulate immune function is referred to as GCR. As noted previously, GCs are normally immunosuppressive, primarily by suppressing pro-inflammatory production and

inducing anti-inflammatory cytokine production. In GCR, the GCs fail to down-regulate immune function. This dysregulation can manifest through multiple functional outcomes. For example, in parental caregivers of pediatric cancer patients, serum IL-6 levels do not decrease with exposure to the synthetic GC, dexamethasone (Miller et al., 2002), indicating that IL-6 regulation is no longer under the control of GC. Additionally, depressed patients often have elevated corticosteroid levels concurrent with elevated pro-inflammatory cytokines, and GCR may be an important in the relationship between depression and cytokine-sickness behavior. In recent years, rodent models of social stress consistently induce GCR. Paired fighting, social defeat, and social disruption all induce GCR in the splenic macrophages (Avitsur et al., 2001; Bailey et al., 2004; Stark et al., 2001). GCR appears to develop exclusively in *social* stress animal models, with other types of stress (e.g. restraint) failing to induce this phenomena (Avitsur et al., 2001). Interestingly, pro-inflammatory cytokines can increase the expression of the beta subtype of the GC receptor, which has been associated with the development of glucocorticoid resistance (Necela and Cidlowski, 2004). Thus, elevated proinflammatory cytokines may be related to the development of GCR, while GCR also results in the production of elevated proinflammatory cytokines.

In the SDR model, GCR develops in a well-characterized manner. Social stress activation of the HPA-axis and the SAM-axis leads to the trafficking of GC-insensitive macrophages from the source bone marrow to the spleen (Engler et al., 2005). This subtype of macrophage is normally present within the

organism, but does not typically leave the bone marrow. Because bone marrow is innervated with noradrenergic neurons, norepinephrine may be an important signal for the altered trafficking pattern of macrophages; however this is unknown at this time. Over time (at least four nights of SDR), the GC insensitive macrophages become the dominant cell type in the spleen (Avitsur et al., 2002). Normal splenic macrophages, when stimulated with LPS, will survive at higher rates than non-stimulated macrophages. If the LPS stimulated cells are then exposed to increasing concentrations of corticosteroid, cell proliferation is reduced in a dose dependent manner. The GCR macrophages maintain the LPS alone levels of proliferation when exposed to increasing levels of corticosteroids. The failure of corticosteroid to reduce cell proliferation occurs because the macrophages no longer react to the corticosteroid's regulation. The regulation is no longer effective because while the GCR macrophages have normal GC receptors, the receptor-GC bound complex does not translocate into the nucleus. Because the bound complex does not translocate, the NF-κB transcription pathway is not induced, and the GCs have no regulatory power (Quan et al., 2003).

Macrophages are an important source of pro-inflammatory cytokines (IL-1, IL-6, TNF-α) during both innate and acquire immune responses. These cytokines recruit and signal other cells (including other macrophages, microglia, etc.) to the site of infection, and stimulate surrounding monocytes to proliferate. In the CNS, microglia (the native CNS monocyte and immune cell) become

activated, allowing them to become antigen presenting cells, furthering the persistence of the inflammatory-immune response (Mack et al., 2003). Receptor bound GCs that translocate to the nucleus of macrophages act to switch the production away from proinflammatory cytokines to anti-inflammatory cytokines. The anti-inflammatory cytokines signal the other area macrophages to stop proliferating and signal other cells to stop migrating to that area. Thus, GCR macrophages will not switch "off", and production of proinflammatory cytokines continues. In previous SDR-TMEV studies, inflammation was exacerbated by social stress, possibly through GCR related dysregulation of the macrophages and microglia. The relationship of social stress to inflammation becomes highly relevant in light of the aforementioned inflammation-neurodegeneration and neurodegeneration-stress interactions in neurodegenerative diseases, including MS.

Our laboratory has previously demonstrated that SDR applied prior to infection lead to exacerbated motor impairment in both the acute and chronic phase of TMEV (Johnson et al., in submission; Johnson et al., 2004). In addition, SDR applied prior to infection also inhibited viral clearance and elevated inflammation in the acute phase (Johnson et al., 2004). The current studies examine the role of IL-6 in the acute phase effects of SDR applied prior to infection. Others have shown that both IL-6 and TNF- α are elevated by SDR (Avitsur et al., 2005; Stark et al., 2002). Our laboratory has also shown that IL-6 is slightly elevated at day 9 post-infection (pi) in circulation (Johnson et al., in

submission), while previous attempts to examine TNF-α by our laboratory have failed to find any changes in this cytokine due to either SDR and/or infection. TMEV infection is also associated with elevated IL-6. IL-6 is an essential signal allowing microglia to activate and become competent antigen presenting cells in TMEV infection (Mack et al., 2003; Olson et al., 2001; Palma et al., 2003). IL-6 production is also elevated in brain tissue as a whole and in cultured astrocytes from TMEV infected mice (Rubio and Sierra, 1993).

The *objective* of Experiment 1 was to determine the *necessity* of IL-6 by blocking IL-6 with a neutralizing antibody and then replicating our previous acute phase experiments. Others have used this technique to block IL-1, IL-6, and TNF-α in both rats and mice (Herx et al., 2000; Jean Harry et al., 2003; Laflamme et al., 1999; Lebel et al., 2000). We *hypothesized* that by blocking IL-6, we would block the negative effects of SDR. In the current study, mice were cannulated and given daily intracerebral ventricular cannula injections of the neutralizing antibody to IL-6 several hours prior to administration of our typical SDR protocol. Following the SDR/antibody administration, animals were infected and examined for the development of sickness behaviors (anhedonia, reduced activity, reduced body weight, hyperalgesia) and motor impairment (hind limb impairment score, activity, vertical activity, sensitivity to physical stimuli, and stride length). In order to compare immunological outcomes with our previous work, viral clearance and inflammation in both brains and spinal cords was examined in separate subsets of mice.

The *objective* of Experiment 2 was to examine the *sufficiency* of IL-6 in replicating the effects of SDR, by administering exogenous IL-6 instead of SDR. We hypothesized that replacing SDR with IL-6 would fail to replicate the effects of SDR, as IL-6 is only one of many inter-related factors that may be responsible for the negative effects of SDR. Once again, the sickness, motor impairment, and immunological measures taken similar to Experiment 1 were collected to allow comparison and consistency across both the current and previous studies using SDR applied prior to TMEV infection.

2. GENERAL METHODS

2.1. Animals

Male Balb/cJ mice were acquired from Jackson Labs (Bar Harbor, ME). Mice arrived from the breeder at post-natal day (pnd) 23 and were weighed and assigned to individual cages. On pnd 24 mice were cannulated (see below). Following cannulation, mice continued to be individually housed for 48 h. On pnd 26, mice were placed in group-housing, 3/cage, counterbalanced by weight. Mice were maintained on a 12-h light/dark cycle (0500-h/ 1700-h) with ad libitum access to food and water, with the exception of the 2-h SDR sessions.

Intruders for the SDR were retired male breeders, 6-to-8 mo of age, housed with sterilized females to increase territoriality and aggressive behavior. The intruders were selected based on latency to attack both peers and adolescents. All intruders consistently attacked peers within 30 s and adolescents within 2 min on 3 separate occasions. All animal care protocols were in accordance with NIH Guidelines for Care and Use of Laboratory Animals and were approved by the Texas A&M University Laboratory Animal Care and Use Committee (ULACC).

2.2. Cannulation surgery

Mice were anesthetized with isoflurane gas (2-5%), heads shaved, eyes coated with petroleum jelly, and placed in a mouse adapted stereotaxic device (fitted with a mouse nose cone (#51625, #51609, Stoelting, Wood Dale, IL). The isoflurane delivery system was purchased from Vet Equip (#901806,

Pleasanton, CA), and the gas recovery system was purchased from Surgivet (model AES, Waukesha, WI). An incision was made longitudinally along the midline of the skull and the skull was exposed (hemostats were not needed). The periosteum was removed with a sterile cotton swab, and 2% lidocaine was applied to the wound. Bregma was located and noted. Rostral-caudal leveling was accomplished by measuring the vertical position (z plane) of lambda and bregma and ensuring that they were equalized. The skull was swabbed dry, and the cannulation hole was drilled at +1 mm lateral to bregma and -.4 mm rostral to bregma over the left lateral ventricle (based on Paxinos & Franklin). Guide cannula (33g) purchased from Plastics One (Roanoke, VA, C315GS-2/SPC), were pre-cut to a depth of 1.75 mm, and implanted and secured with cyanoacrylic gel according the manufacturer's suggestion. Mice were then wrapped with bubble wrap to aid in insulation to combat hypothermia induced by anesthesia, and placed back in the home cage that was warmed over a heating pad. Mice were monitored until fully awake and ambulatory. Once ambulatory, bubble wrap was removed, and mice were returned to the animal colony. For pain control post-surgery, mice were provided with water treated with Tylenol (325 mg/ 2000 mL). To aid in recovery mice were also provided with food softened with the Tylenol treated water in the cage. Mice were allowed to recover approximately 6 d prior to any further procedures.

2.3. Social disruption (SDR) stress

For the stressed mice, intruders were introduced into the cage of resident mice at dark cycle onset (1700 h) for a period of 2 h for a total of 6 SDR sessions the week prior to infection, beginning on pnd 30. SDR occurred for 3 consecutive sessions, then 1 night off, followed by 3 additional consecutive sessions (Avitsur et al., 2001; Stark et al., 2001), in a separate procedure room. Control mice (NON) remained in the colony room, undisturbed for the duration of SDR. SDR sessions were monitored to ensure that the intruder attacked the residents and that the residents demonstrated submissive behaviors. Intruders that did not attack within 10 min of session initiation were replaced, and the session continued for the remaining 2 h.

2.4. Virus and infection

The BeAn strain of Theiler's virus (obtained from Dr. H.L. Lipton, Department of Neurology, Northwestern University, Chicago, IL.) was propagated and amplified in L- cells. The culture supernatant containing infectious virus was aliquoted, titrated and stored at –80º C before use (Welsh et al., 1987). Mice were anesthetized on day 0 pi/ pnd 37 with isoflurane (Vedco Inc., St. Joseph, MO) and injected intracranially (ic) with a 27-g needle into the right mid-parietal cortex (depth approximately 1.5 mm) with 5×10^4 pfu of the BeAn strain of Theiler's virus in a 20-µl volume as previously described (Campbell et al., 2001; McGavern et al., 1999; McGavern et al., 2000; Rose et

al., 1998; Theil et al., 2000). Inoculation for all subjects occurred at 2100 h, following the last SDR session.

2.5. Illness measures

CNS inflammation is associated with a range of sickness behaviors/measures, very similar to cytokine-induced sickness syndrome. These include body temperature changes, anorexia, weight loss, decreased motor activity, mechanical hypersensitivity, and anhedonia- a loss of interest in pleasurable activities (Barak et al., 2002a; Barak et al., 2002b; Dantzer, 2001; Pollak et al., 2000; Pollak et al., 2003a; Pollak et al., 2003b; Watkins et al., 1995b). These responses are associated with the secretion of both peripheral and central proinflammatory cytokines.

2.5.1. Body temperature

Body temperature changes are common with acute phase responses due to infection. In contrast to other mammals, mice commonly develop hypothermia during at least the first 24 h of an immune response. Body temperature can be expected to decrease by .5 - 4 °C pi under normal circumstances (Dybing et al., 2000; Leon, 2002; Leon et al., 1998; Wang et al., 1997). Body temperature was measured daily at 0900-h, beginning the morning prior to infection for baseline, and continuing through day 7 pi. Body temperature was measured using a laser temperature gun (Ex-Tech 47257), set to Celsius and sensitive to .1°C. Each mouse was scruffed, and the laser guide was aimed at the abdomen, and the

temperature indicated on the gun was recorded. Each mouse was tested three times each morning, and the average analyzed.

2.5.2. Sucrose preference

Sucrose preference was used to assess anhedonia from day -1 pi through day 7 pi. Mice were provided with a 2% sucrose solution and plain tap water beginning at day -11 pi in identical 500 mL water bottles. Assessment of preference began at day -7 pi, by weighing each bottle to determine intake from each bottle every 24 h, at 0900-h. Preference was determined by comparing sucrose water intake to total fluid intake (g of sucrose/ (g of sucrose + g of tap water). Preference was acquired when 60% or more of fluid intake was sucrose water. Only cages that met criteria *prior* to infection were included in analysis.

2.5.3. Weight loss

Sickness behavior related weight loss was assessed from days -1 through 1 pi (Experiment 1), or days -1 through 7 pi (Experiment 2) . Each mouse was weighed using a scale sensitive to .01 g, each morning at 0900-h.

2.5.4. Motor activity

To assess sickness behavior related activity decreases, open field horizontal activity at day 1 pi was measured. The apparatus consisted of six optical beam activity monitors (Model RXYZCM-16), equipped with two banks of eight photocells on each wall, were used to measure locomotion activity, and The system utilizes a multiplexer-analyzer and Versamax software (Model DCM-4, Omnitech Electronics, Columbus, OH), used to measure locomotion activity,

located in an adjacent room. White noise (64 dB) was continually present to mask extraneous disturbances. Each box was modified for use with mice by placing a bench paper liner under a false Plexiglas floor, and using 2 Plexiglas dividers that separated the larger device into four equal sized, smaller chambers. These adaptations allowed two mice to be placed in each larger chamber for each session, one in the lower left chamber, and another in the upper right chamber. Mice were habituated in the chambers for 1-h, on pnd 28, prior to baseline measure collection at 1500-h. Baseline assessment occurred on pnd 29. All testing was conducted in the dark beginning at 1500-h, for 30 min. The baseline level for each animal was subtracted from the day 1 pi levels for assessment, to control for any individual differences across animals.

2.5.5. Mechanical sensitivity

Mice were habituated to the sensitivity apparatus for 20 min prior to assessment. The apparatus consisted of a 2 mm gauge screen placed 4”-to-6” from a countertop. Each screen was large enough to accommodate 2-to-3 mice. Each mouse was placed under a one pint sized Rubbermaid container during habituation and testing. Multiple screens were set up each session to accommodate running six mice at a time. After the 20 min habituation period, von Frey filaments (Stoelting) were applied to the pad of each hind limb starting with the smallest filament. Each filament was applied to the left, then right, then right, then left hid paw in an ABBA manner. Each filament was tested on each mouse prior to continuing with successive sized filaments (ascending). Each

mouse was tested until the first withdrawal response occurred, and the size of that filament was recorded. For the second trial, the order of filaments was reversed (descending), and the last filament to elicit a withdrawal response was recorded. Each mouse was tested for three trials, ascending, descending, and then the third trial is ascending again. For sickness related mechanical hypersensitivity, mice were tested at day 1 pi, and greater sensitivity was expected.

2.6. Behavioral assessment of motoric impairment

Multiple measures of psychomotor behavior were examined. These included: hind limb impairment ratings, locomotor activity (in the vertical and horizontal planes), mechanical sensitivity, and footprint stride length.

2.6.1. Hind limb impairment (HLI)

Mice were assessed for hind limb impairment beginning at day -1 pi and continuing through days 7 or 21 pi, as appropriate. The hind limb impairment scale has been presented elsewhere (Johnson et al., 2004). A rater blind to the subject's experimental conditions assessed hind limb impairment. All subjects underwent the exact same scoring procedure independent of symptoms or experimental condition. Individual mice were placed on a floor grid, with 1 mm wide bars running in parallel every 10 mm. The mice were inverted on the grid, so that they were hanging upside-down. The rater then assessed hind limb weakness or paralysis. A separate numeric score was given for each hind limb, based on the symptoms of impairment the mice display (0=healthy; 1=slight

weakness in grip; 2=clear weakness in grip; 3=slight paralysis; 4=moderate paralysis, 5=complete paralysis with muscle tone, 6= complete paralysis with no muscle tone (Johnson et al., 2004). These separate scores were then added together and the combined score analyzed.

2.6.2. Open field activity monitoring

Motor impairment related activity decreases, open field horizontal and vertical activity, were collected on days 4,7,11, and 18 pi. The apparatus consisted of six optical beam activity monitors (Model RXYZCM-16), equipped with two banks of eight photocells on each wall, were used to measure locomotion activity, and The system utilizes a multiplexer-analyzer and Versamax software (Model DCM-4, Omnitech Electronics, Columbus, OH), used to measure locomotion activity, located in an adjacent room. White noise (64 dB) was continually present to mask extraneous disturbances. Each box was modified for use with mice by placing a bench paper liner under a false Plexiglas floor, and using 2 Plexiglas dividers that separated the larger device into four equal sized, smaller chambers. These adaptations allowed two mice to be placed in each larger chamber for each session, one in the lower left chamber, and another in the upper right chamber. Mice were habituated in the chambers for 1-h, on pnd 28, prior to baseline measure collection at 1500-h. Baseline assessment occurred on pnd 29. All testing was conducted in the dark beginning at 1500-h, for 30 min. The baseline level for each animal was

subtracted from each day pi levels for analysis, to control for any individual differences across animals.

2.6.3. Mechanical sensitivity

Mice were habituated to the sensitivity apparatus for 20 min prior to assessment every day of assessment (days 1, 7, 14, 21 pi). The apparatus consisted of a 2 mm gauge screen placed 4”-to-6” from a countertop. Each screen was large enough to accommodate 2-to-3 mice. Each mouse was placed under a one pint sized Rubbermaid container during habituation and testing. Multiple screens were set up each session to accommodate running six mice at a time. After the 20 min habituation period, von Frey filaments (Stoelting) were applied to the pad of each hind limb starting with the smallest filament. Each filament was applied to the left, then right, then right, then left hid paw in an ABBA manner. The filament was tested on each mouse prior to continuing with successively larger sized filaments (ascending). Each mouse was tested until the first withdrawal response occurred, and the size of that filament was recorded. For the second trial, the order of filaments was reversed (descending), and the last filament to elicit a withdrawal response was recorded. Each mouse was tested for three trials, ascending, descending, and then the third trial is ascending again. For motor impairment, mice were expected to require stimulation with larger filaments in order to elicit a response.

2.6.4. Stride length

Footprint stride length and spread were assessed using a method similar to that used by McGavern et al. (1999, 2000) at day 20 pi. This time point has previously been shown to be sensitive to the effects of SDR in the acute phase of TMEV infection (Johnson et al., 2006; Johnson et al., 2004). Briefly, hind limbs were painted with blue paint while forelimbs were painted with red paint. Mice were then allowed to walk down a 2.5" by 36" runway lined with paper to record limb placement. Mice were then cleaned thoroughly using a soft bristled paint brush and a mild soap and water solution, and dried. Once the paint dried on paper strips, the stride length was measured in mm, for the first six steps. This has been shown to provide a reliable and valid measure of virally-mediated nerve damage and demyelination (McGavern et al., 1999; McGavern et al., 2000).

2.7. Sacrifice

Mice were sacrificed at either day 7 or 21 pi, for either histology or viral titer assay. Each mouse was overdosed with ketamine (100mg/kg)/xylazine (5 mg/kg), and bled from the brachial artery. For histology, mice were perfused with 10 mL of PBS followed by 10 mL of 10% formalin. For viral titer assay, mice were perfused only with PBS. Spleen, thymus, brain and spinal cord were harvested appropriately and weighed. For histology brains and spinal cords were placed in 10% formalin overnight, and sectioned as in previous studies (Johnson et al., 2004). For the mice that were used for viral titer assays,

spleens were used for the corticosteroid sensitivity assay to assess GCR. Brains and spinal cords were flash frozen in liquid nitrogen, and stored at -80 ºC until viral titer assay was preformed.

2.8. Corticosteroid sensitivity assay

On the sacrifice day, spleens were harvested to determine sensitivity of splenocytes to GC regulation (as per Stark, et al., 2001). Briefly, spleens were placed in ice-cold Hank's balanced salt solution (HBSS), and mashed to obtain a single cell solution. Red blood cells were then lysed (red blood cell lysis, Sigma, St. Louis, MO), followed by a wash of HBSS+ 10% heat inactivated fetal bovine serum (FBS-HI, Equitech, Kerrville, TX). Viable cells were then counted using trypan blue dye exclusion and re-suspended at 2.5 X 10^6 cells/ml in supplemented RPMI (Sigma) +10% FBS-HI, (supplementation: 75% sodium bicarbonate, 10 mM Hepes buffer, 100 μg/ml penicillin, 100 μg/ml streptomycin sulfate, 1.5 mM l-glutamine, and .00035% 2-mercaptoethanol). LPS (Sigma # L6529) in 2% ethanol was added at a concentration of 1 μg/ml for mitogen stimulation. GCR was tested by exposing aliquots of each suspension to dilutions of corticosteroid (0-5 μM, Sigma, St. Louis, MO, #C2505) dissolved in 2% ethanol and supplemented RPMI. Cell suspensions were placed in triplicate in flat-bottomed 96-well micro-titer plates in 100 μL aliquots and incubated for 48-72 h at 37°C and 5% CO_2. After incubation, the cell survival assay was performed.

Cell proliferation was assessed following the manufacturer's instructions with the CellTiter 96 Aqueous non-radioactive proliferation assay kit from Promega (Madison, WI). Color changes were quantified by optical density readings at 490 nm from an EMAX ELISA plate reader (Molecular Devices, Sunnyvale, CA). Mean optical density values for the 3 replications for each sample were used, and the percentage of the corticosteroid-exposed cells versus the LPS stimulated cells was determined for statistical analysis.

2.9. Histological evaluation of CNS inflammation

Brains and vertebral columns (containing intact spinal cord) were sectioned transversely into 4 and 12 pieces, respectively (see Campbell et. al., 2001 for further detail). Tissues were then processed for routine hematoxylin and eosin (H&E) staining. Lesions of both the brain and spinal cord were scored in a similar manner by raters, blind to experimental condition. Lesions evaluated included perivascular cuffing (accumulation of lymphocytes and macrophages around blood vessels), meningitis (accumulation of lymphocytes and macrophages in the meninges), and microgliosis (presence of increased microglia/macrophages within the parenchyma of the brain and spinal cord). Raters used stereo navigator software to assess the total perimeter and area available, and the percent of each effected by lesions. Briefly, the perimeter was drawn around each slice or section of tissue, and closed. This allowed the program to determine the length of the perimeter and the area enclosed. For microgliosis, incidents of inflammatory cells were traced with a separately

defined line (to distinguish this data from the overall perimeter/area data), with multiple layers being traced over for as many layers of inflammatory cells as occurred. For perivascular cuffing and microgliosis, the area affected is traced around (using a third line type), and enclosed. Because perivascular cuffing and microgliosis often occur in close proximity, the entire area is used, and the area affected includes both types of inflammation.

2.10. Viral load and clearance

Prior studies have shown that viral titers in CNS are maximal at 1-2 wk pi and by 3-4 wk pi the virus is apparently cleared to non-detectable levels (Welsh et al., 1987; 1989). To evaluate the impact of SDR on CNS viral clearance, brains and spinal cords were removed on the day of sacrifice, homogenized, and stored at –80°C. The viral content of the supernatant from the homogenized brains and spinal cords were determined by plaque assay on L2 cells (Welsh et al., 1987). The plates were incubated at 37°C at 5% CO_2 for 72 h, and then the plates were stained with .1% crystal violet to visualize the plaques that were formed. Plates were scored based on the number of plaques formed on the L2 cells and calculated per gram of tissue harvested (Welsh et al., 1987).

2.11. Statistical analyses

Data are presented as mean $\pm$ SEM. Analysis of variance (ANOVA) was used to evaluate differences across SDR and infection conditions; and repeated measure ANOVA were used for analysis over time as appropriate. These

analyses were followed by *post hoc* mean comparisons using Duncan's New Multiple Range Test. For effect sizes, r^2 was used.

3. EXPERIMENT 1

3.1. Introduction

Experiment 1 examined the necessity of IL-6 by neutralizing this cytokine with antibodies. This experiment had two steps. The first determined if the antibody treatment (AbTx) was able to block IL-6. Previous studies have also shown that SDR induced IL-6 does not interfere or alter SDR induced GCR. Therefore, we also examined the development of GCR prior to infection in these animals. A 2 (AbTx, vehicle) x 1 (SDR) design was used for this step. Once we established that IL- 6 was in fact elevated by SDR and blocked by the AbTx, a 2 (SDR, NON) x 2 (AbTx, vehicle) design was used to examine how blocking IL-6 with antibodies altered the effects of SDR on the acute phase of TMEV infection.

3.2. Methods

3.2.1. Antibody to IL-6

Polyclonal antibody to mouse IL-6 was purchased from R&D Systems (AF-406-NA, Madison, WI). To provide the stock solution, the original 100 μg was dissolved in 1 mL sterile PBS. The stock solution was then further diluted by 1:20 to result in a 5 ng/μL solution.

3.2.2. Antibody administration

The 5 ng/μL solution of neutralizing antibody was administered using a 25-μL Hamilton syringe and a pump set to administer 60 μL/hour through a 36-g cannula (Plastics One, Roanoke, VA, C315IDC/SPC). 2 μL was then administered over 2 min, followed by a 30 s delay to prevent backwash of the

solution before the cannula was removed. The total dose per animal was then 10 ng. This dose was based on the 50% neutralizing dose information provided by the manufacturer. Control (NON-SDR) animals were administered mouse immunoglobulin (Ig, Santa Cruz Biotechnology, Inc #SC-2025) in the same volume of sterile saline as the vehicle, in order to account for generalized responses to proteins.

3.2.3. Efficacy of antibody treatment in blocking IL-6

In order to assess the efficacy of the antibody dose in blocking SDR induced IL-6, 12 mice were used. These animals arrived from breeder, were cannulated and underwent SDR as described in the general methods section. A 1 (SDR) x 2 (AbTx, vehicle) was used. At 1300-h daily, 4-h prior to SDR, antibody or vehicle administration occurred. Following AbTx/SDR sessions, all animals were sacrificed and spleens were harvested for GCR assessment, while brain tissue and sera were assessed for IL-6 levels, using an ELISA assay (timeline for these procedures is depicted in Fig 3.2..1.).

3.2.4. IL-6 ELISA

ELISA for IL-6 assay was purchased from R & D Systems (R & D Systems Madison, WI), and sera (frozen at -80°C between sacrifice and the time of the assay) was assessed according to the manufacturer's instructions. Brain tissue was homogenized in 1 mL of Dulbecco's Eagle Medium (Sigma) and flash frozen. Tissue was stored at -80°C until the time of the assay. Tissue was then

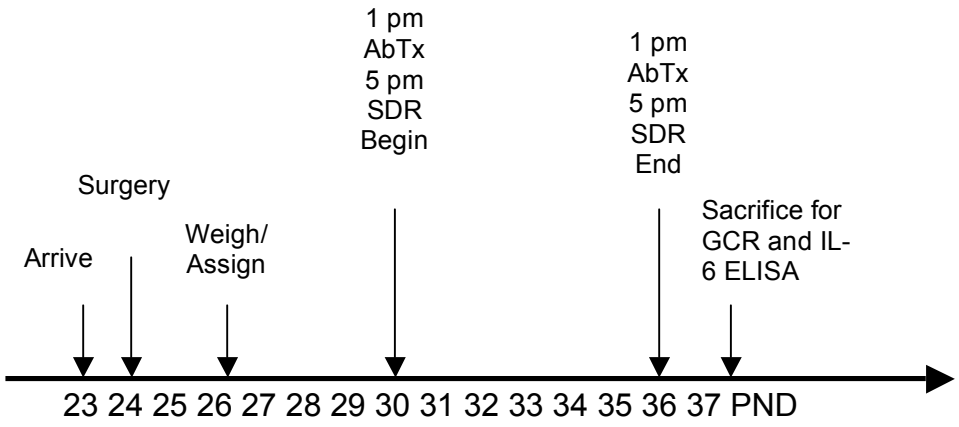


Fig 3.1. Procedure for Verification of Antibody to IL-6.
Time line for all experimental procedures for the verification of the antibody to IL-6 in blocking IL-6 activity in Experiment 1.

thawed, centrifuged at 2000 rpm for 5 min. 50 μL of the supernatant per well was used to assess IL-6 levels in the brain.

3.2.5. Procedure

Upon arrival, mice were weighed and caged individually. The next morning, cannulation surgeries occurred. Mice were allowed to recover in individual housing for 2 d. On pnd 27, mice were weighed again and assigned to cages in a counterbalanced manner, based on weight. The Tylenol water was removed and sucrose and plain water were added for each cage. Baseline measures were taken during this time period, with the exception of footprint. On pnd 30-36, antibody or vehicle was administered daily beginning at 1300, and then the stressed animals were administered SDR at from 1700-1900. After the final SDR session, at 2100, all mice were infected. Illness measures were then taken through day 7 pi on all animals. Motor function measures were taken only in those animals sacrificed at 21 pi. Mice were sacrificed at either day 7 pi or day 21 pi, for either viral clearance or histology. Mice sacrificed on day 7 pi for viral clearance were also tested for GCR. The timeline events table for all procedures for this experiment are depicted in Table 3.1.

Table 3.1. Procedural Event Table: Experiment 1.

Arrive																
Surgery																
Weigh/ Assign																
Open Field Habituation																
Open Field Baseline																
Illness Behaviors																
AbTx																
SDR																
Footprint-Baseline																
INFECT																
Motor -B																
Sacrifice- 7																
Footprint-20																
Sacrifice -21																
PND	23	24	26	27	29	31	33	35		37						
PI									0		3	5	7	14	20	21

Time line of all experimental procedures in Experiment 1, blocking IL-6 with neutralizing antibody to IL-6.

3.3. Results

3.3.1. Verification of antibody treatment

In order to verify that IL-6 was elevated due to SDR in the current study, and that this elevation was blocked by the neutralizing antibody treatment, an ELISA for IL-6 was used to test both sera and brain homogenate. Figs 3.2.A and 3.2.B depict IL-6 levels in brain homogenate and sera, respectively. ANOVA confirmed that SDR alone elevated IL-6 levels, and antibody treatment effectively reduced free IL-6, F (1,9) = 11.974, $p < .01$ in brain homogenate, and F (1.9) = 9.783, $p < .05$ in sera. In addition, GCR developed normally in response to SDR, regardless of antibody treatment (Fig 3.2. C). ANOVA verified that no significant reduction in proliferation of spleen cells occurred across increasing corticosteroid treatment, F (4,32) = 1.733, $p = .2$, indicating that SDR induced GCR. Finally, there was no interaction between corticosteroid concentration with antibody treatment, F (4,32) = 1.508, $p = .16$, or main effect for antibody treatment, F (1,8) = .048, $p = .8$.

3.3.2. Illness measures

Illness measures were assessed by analysis of data either within the first 24 h pi, or comparing data collected in the first 24 h pi to pre-infection data. The next five figures will present a series of illness-measure related data. Measures taken included body temperature, percent sucrose preference (as a measure of anhedonia, body weight, activity in an open field, and mechanical sensitivity. Overall, the SDR-VEH animals had equal or greater illness behaviors compared

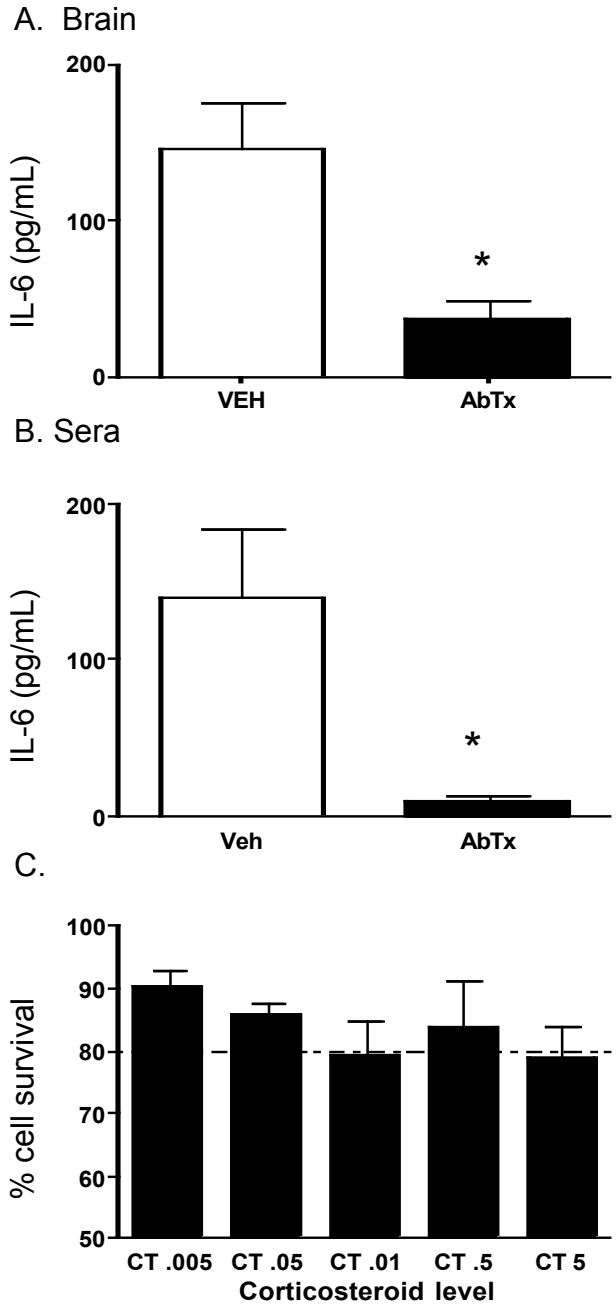


Fig 3.2. Verification of Neutralizing Antibody to IL-6 Treatment. Panels A and B are IL-6 levels in brains and sera, respectively, following SDR, but prior to infection. Panel C shows GCR prior to infection, the line is at 80% cell survival. Asterisks (*) indicate significant differences of SDR-AbTx compared to group not exposed to the SDR-VEH group.

to animals that did not receive SDR. In contrast, treatment with antibody to IL-6 blocked the effects of SDR, and occasionally infection.

3.3.2.1. Body temperature. Body temperature (°C) was examined 12 h pre-infection and 12 h pi (Fig 3.3.). Following infection, only the SDR-AbTx animals had significant change from pre-infection levels. ANOVA confirmed a 3-way interaction (SDR x AbTx x time), F (3, 98) = 5.925, p < .05. *Post hoc* comparisons determined that the interaction was primarily due to the elevation in body temperature in the SDR-AbTx animals, while significant changes in body temperature were not evident in the other groups.

3.3.2.2. Sucrose preference. Sucrose preference was examined daily, as infection is usually associated with a loss of interest in pleasurable activities. Within the first 7 days pi, the SDR-VEH group has significantly less preference for sucrose water, and the antibody treatment (SDR-AbTx) restored sucrose preference (Fig 3.4.). ANOVA confirmed a 2-way interaction (SDR x AbTx), F (1, 30) = 18.396, p < .001. *Post hoc* means comparisons showed that the SDR-VEH group had significantly less preference compared all three other groups.

3.3.2.3. Body weight. Body weight loss is a common side effect of infection, therefore, body weight was monitored pre-infection and 12 h pi. Significant body weight loss occurred within 12 h pi, in all groups except the SDR-AbTx animals (Fig 3.5.). A 3-way interaction (SDR x AbTx x time) was confirmed with ANOVA, F (3, 98) = 6.304, p < .05. In addition, a significant

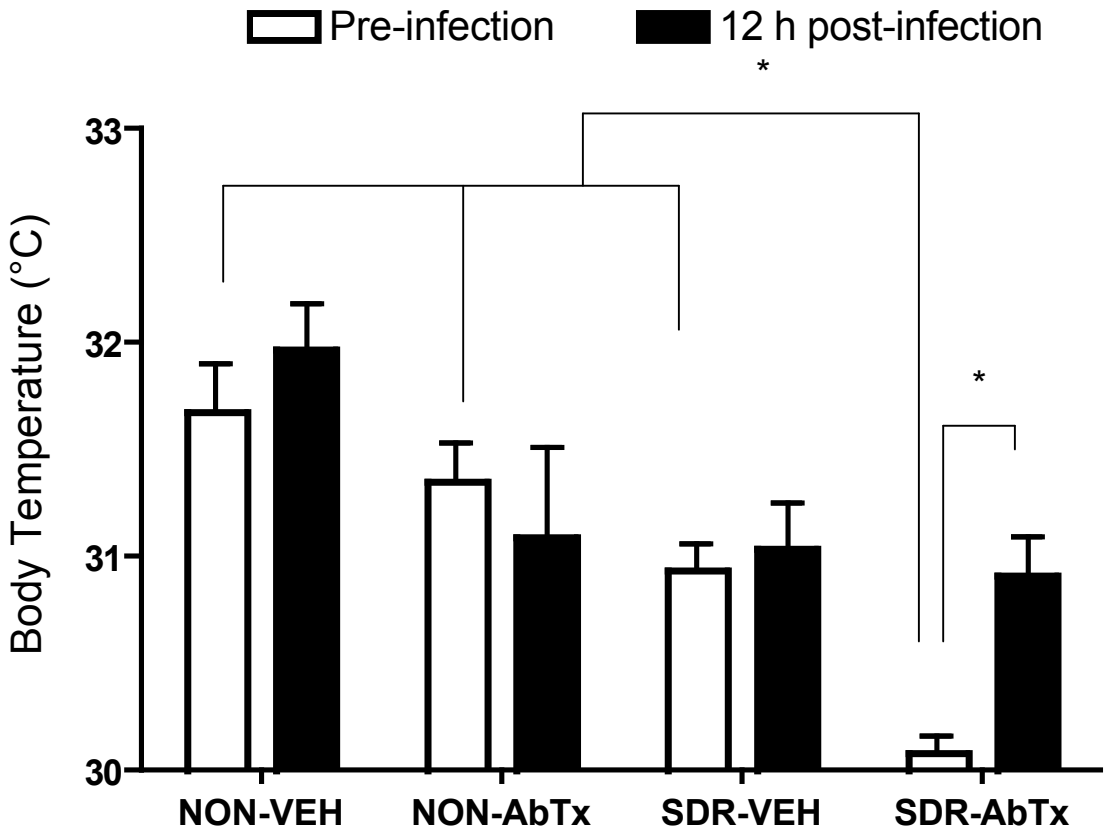


Fig 3.3. Body Temperature.
Body temperature changes pre-infection compared to post-infection in all groups (n = 24 per group). Only the SDR-AbTx group had a significant change in body temperature. Pre-infection, SDR combined with antibody treatment appeared to induce hypothermia. This hypothermia was resolved post-infection, in this group. Significant differences are indicated with asterisk (*).

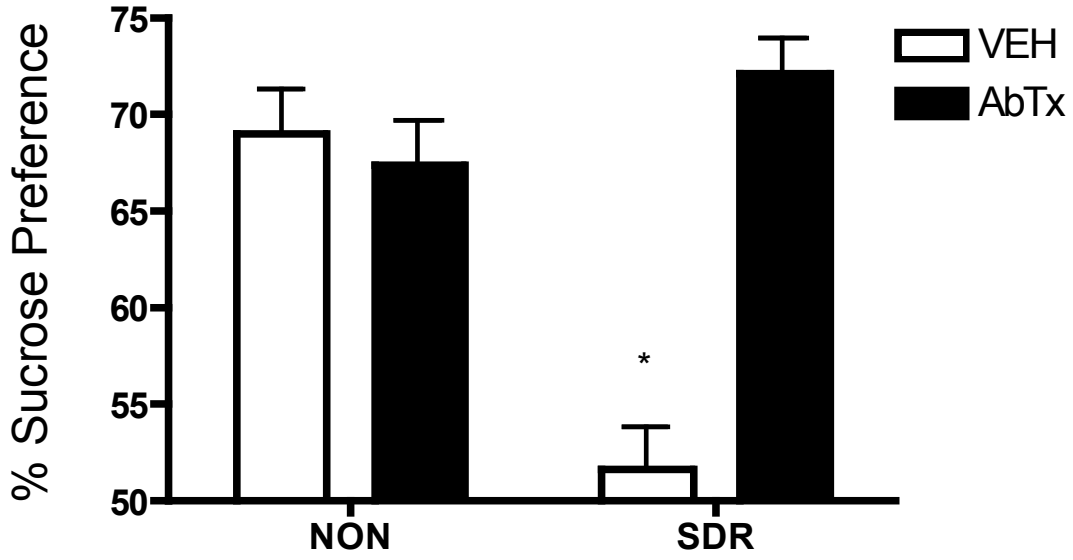


Fig 3.4. Sucrose Preference.
Sucrose preference was assessed by percentage of total fluid intake during the first 24-h pi. All cages included in the analysis met a criterion of a minimum of 60% sucrose preference prior to infection to be included in the analysis. The SDR-VEH group was the only group to loose sucrose preference post-infection. All other groups, including the SDR-AbTx group maintained a preference for sucrose water. Significant differences are indicated with asterisk (*).

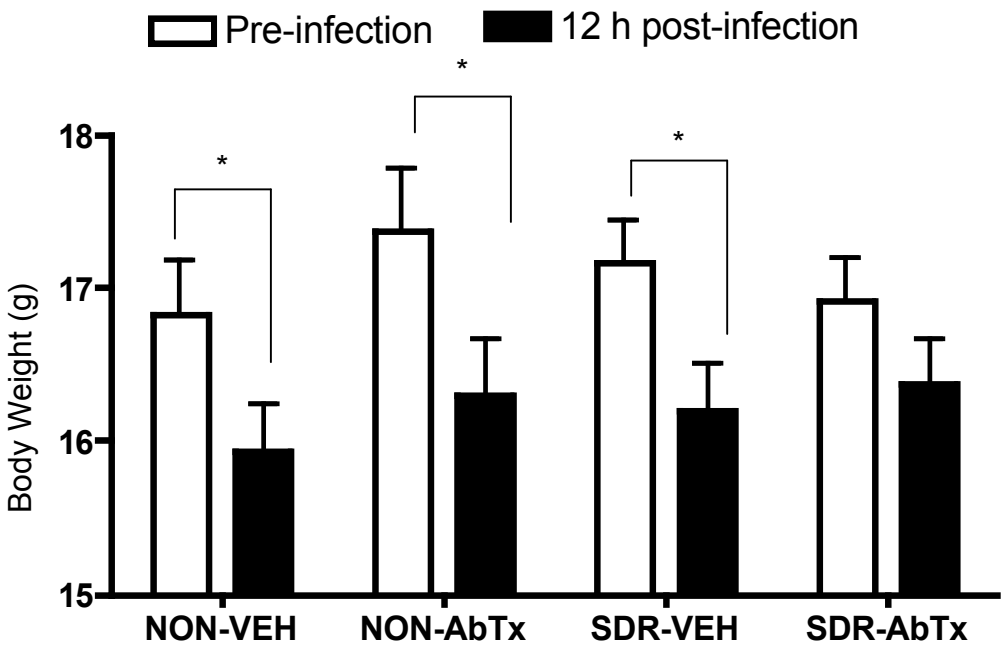


Fig 3.5. Body Weight.
Body weight loss was determined by comparing pre-infection weights (open bars) to post-infection weights (closed bars). All groups lost a significant amount of weight post-infection with the exception of the SDR-AbTx mice. No between group differences were found. Significant differences are indicated with asterisk (*).

main effect of time, F (1, 101) = 3.782, p < .0001, indicated that body weight was reduced in all groups post-infection. However, *post hoc* comparisons revealed that while most animals lost significant weight post-infection, weight loss was not significant in the SDR-AbTx group.

3.3.2.4. Activity. Activity level is commonly reduced due to infection, and animals are less likely to explore the center area of the open field. The change from baseline activity at day 1 pi was examined here to investigate this phenomenon (Fig 3.6. A and B respectively). ANOVA verified a significant 2-way interaction on horizontal activity (SDR x AbTx), F (1, 44) = 20.141, p < .0001. In addition, a significant 2-way interaction (SDR x AbTx) also occurred for center time F (1, 44) = 4.610, p < .05. *Post hoc* comparisons found that the SDR-VEH group lost significantly more activity from baseline compared to the other groups. In addition, the SDR-AbTx group had significantly less loss of activity compared only to the SDR-VEH group, indicating a recovery of activity level to normal levels.

3.3.2.5. Mechanical sensitivity. Infection is often associated with enhanced mechanical sensitivity. Sensitivity to the mechanical stimulus, von Frey filament, was examined within the first 24 h pi (Fig 3.7.). ANOVA verified a significant 2-way interaction (SDR x AbTx), F (1, 44) = 13.765, p < .001. *Post hoc* comparisons found that the SDR-VEH group became significantly more sensitive compared to the other groups. The SDR-AbTx group also became

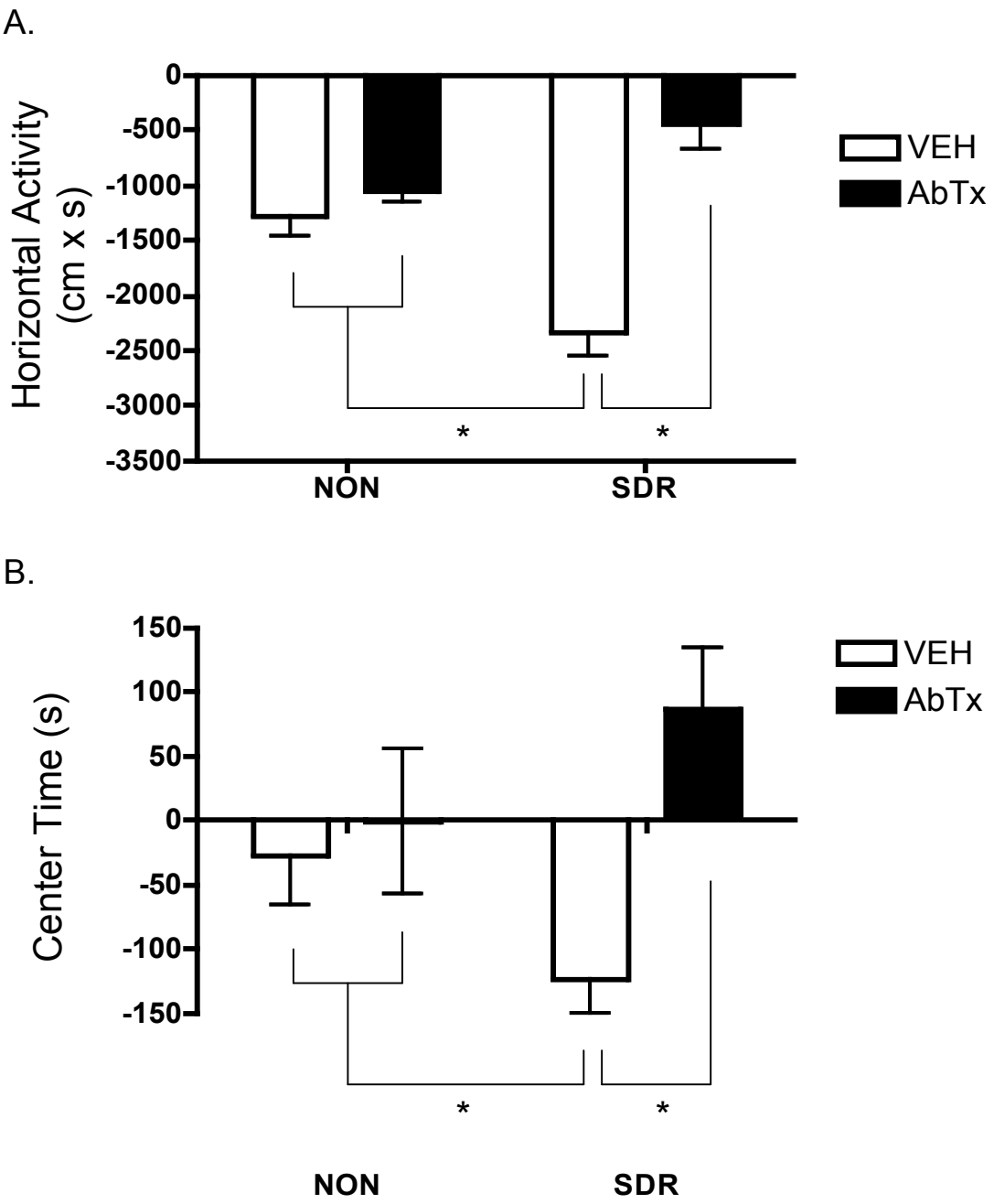


Fig 3.6. Activity: Illness Related.
Activity in the horizontal plane was taken as a measure of activity (A), as well as time spent in the center (B) both of which are often reduced due to illness. The graph depicts the change from baseline activity. The SDR alone (SDR-VEH) group had significantly reduced activity levels compared to the non-stressed groups. In contrast, the antibody treatment (SDR-AbTx) reversed the activity reductions due to SDR. Significant differences are indicated with asterisk (*).

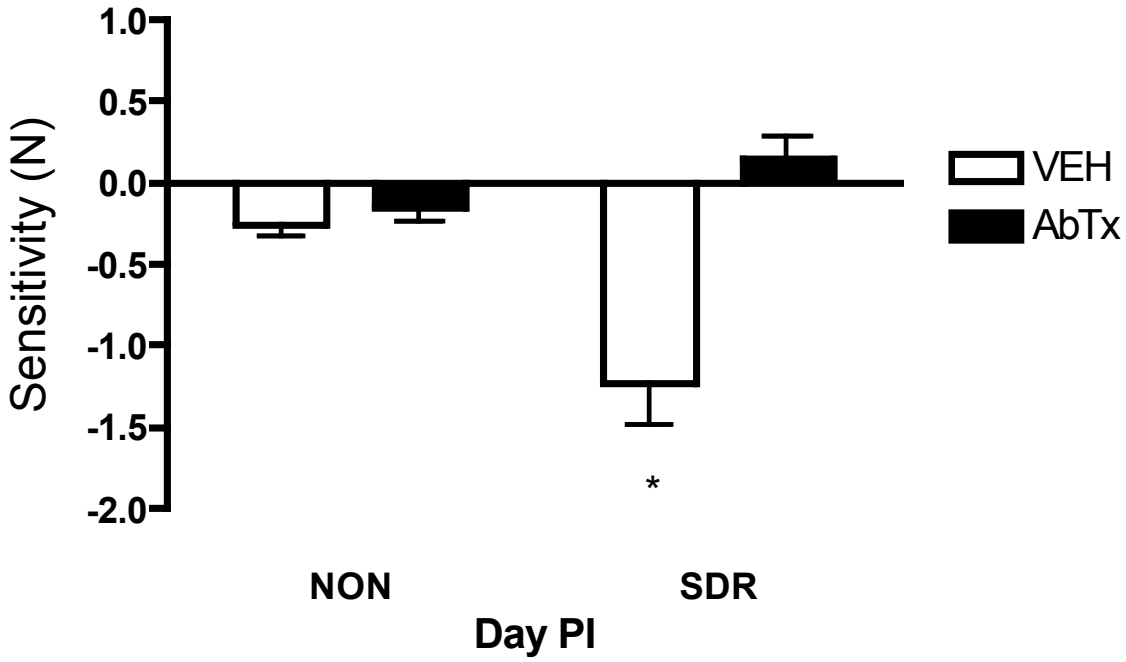


Fig 3.7. Mechanical Sensitivity: Illness Related.
Mechanical hypersensitivity was expected to develop within 24-h post infection. In the SDR-VEH animals, this was what occurred. The graph depicts the change from baseline at day 1 pi on sensitivity to von Frey filament stimulation. Only the SDR-VEH group developed greater sensitivity, compared to the other groups. Significant differences were noted with an asterisk (*).

significantly less sensitive compared only to the SDR-VEH group, indicating a recovery of activity level to normal levels.

3.3.3. Motor impairment measures

TMEV infection causes a polio-like impairment in early infection, particularly in the Balb/cJ strain. Motor impairment was assessed by examining multiple measures, including hind limb impairment, reduced mechanical sensitivity, horizontal activity, vertical activity, and stride length. These measures are presented in the next five graphs. By examining a panel of motoric impairment measures, converging lines of evidence confirmed that motoric impairment occurred in the SDR alone animals (SDR-VEH), as in previous studies. In contrast, SDR plus antibody treatment against IL-6 (SDR-AbTx) reduced motor impairment to non-stressed levels or better.

3.3.3.1. Hind limb impairment score. Hind limb impairment was examined using a scoring system that has previously been shown to be sensitive to the changes in impairment in early TMEV infection in Balb/cJ mice (Johnson et al., 2004). The effect of SDR and antibody against IL-6 on HLI was assessed through day 18 pi. SDR exacerbated infection related hind limb impairment, while antibody against IL-6 reduced this impairment (Fig 3.8.). ANOVA confirmed a main effect of SDR, F (1,52) = 69.227, $p < .0001$. Additionally, a 3-way interaction (SDR x AbTx x Day pi) indicates that HLI levels were restored to better than non-stressed infected animals over time by blocking IL-6 during

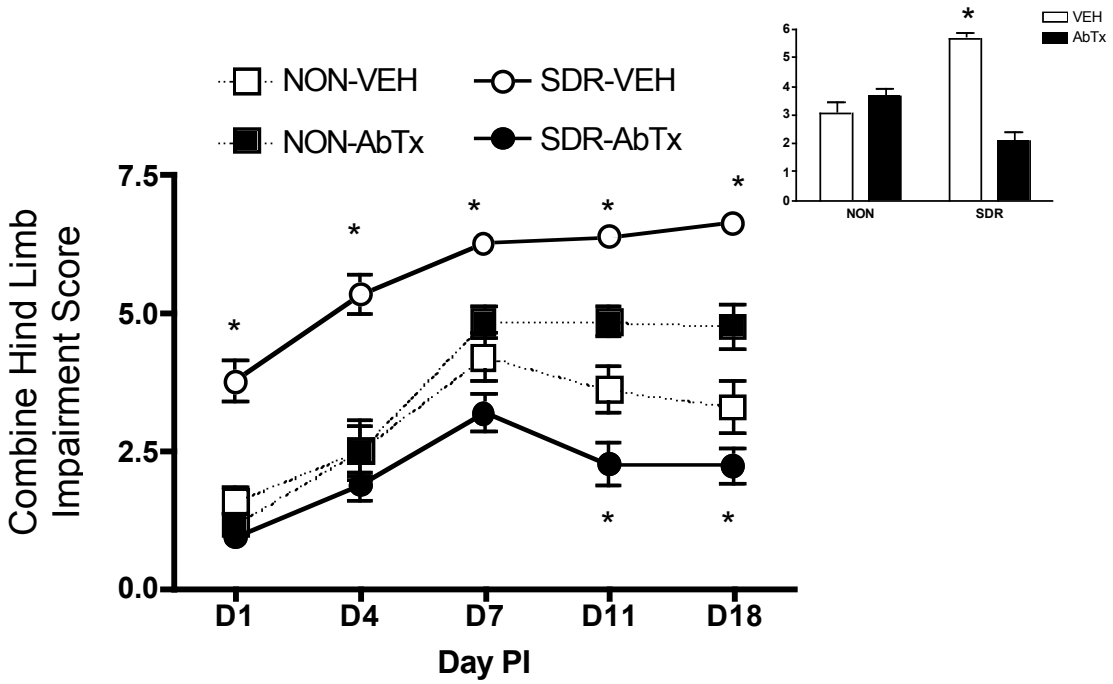


Fig 3.8. Combined Hind Limb Impairment: Experiment 1. Hind limb impairment scores have proven to be a reliable indictor of the motor impairment that develops as a result of TMEV infection. The man graph above depicts the development of hind limb impairment scores over the course of time. The inset depicts these data collapsed over time. The SDR-VEH group developed significantly greater impairment compared to the other groups. In addition, the SDR-AbTx group had significantly less impairment by days 11 and 18 pi. Asterisks (*) indicate significant effects.

SDR, F (4,200) = 4.92, p < .001. *Post hoc* comparisons determined that the SDR-VEH animals had significantly elevated impairment at every time point compared to all other groups. The SDR-AbTx group also had significantly less impairment compared to non-stress groups at days 11 and 18 pi.

3.3.3.2. Mechanical sensitivity. Von Frey measures sensitivity to non-noxious mechanical stimuli, using filaments of increasing size and pressure needed to bend the filament. When animals require larger filaments than control, NON-SDR, animals before making a response, then either sensory or motoric impairment can be inferred. Therefore, the SDR-VEH animals were expected to develop decreasing sensitivity, corresponding to increasing size of filament needed to elicit a response. ANOVA confirmed a 3-way interaction (SDR x AbTx x Day pi) interaction, indicating that the SDR-VEH animals did in fact develop decreasing sensitivity over the course of infection (Fig 3.9.), F (3,132) = 11.763, p < .0001. In addition, a main effect for antibody treatment alone, F (1,44) = 4.757, p < .05, and a 2-way interaction (SDR x AbTx) were also shown through ANOVA, F (1,52) = 6.169, p < .05. *Post hoc* comparison indicated that the SDR-VEH group was significantly less sensitive compared to all other groups at both day 14 and 20 pi. Finally, the SDR-AbTx group was not significantly different from the non-stressed groups at any time point, and these three groups (NON-VEH, NON-AbTx, and SDR-AbTx) did not change over time.

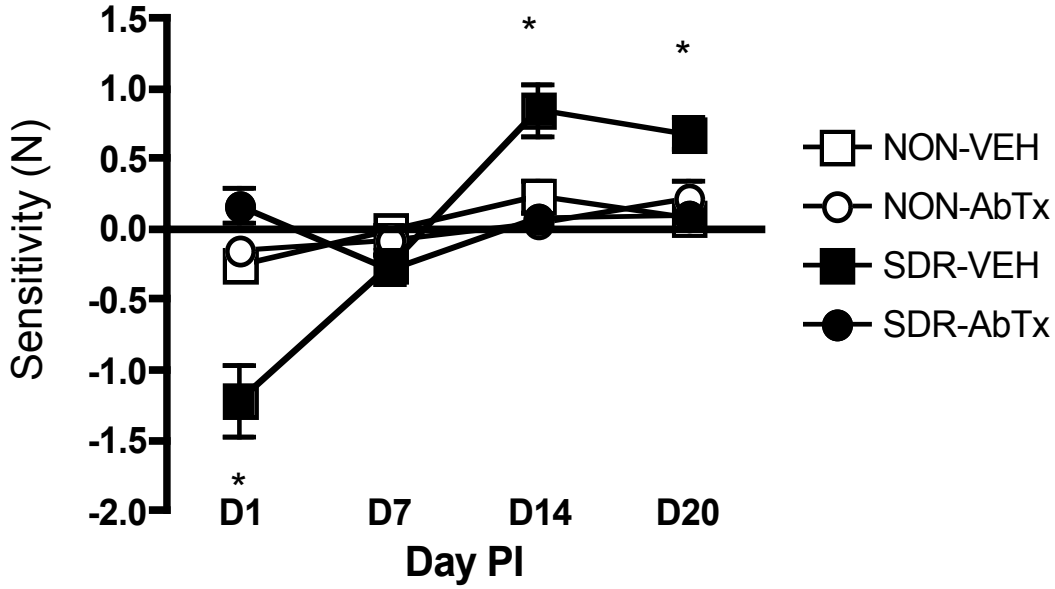


Fig 3.9. Mechanical Sensitivity: Impairment Related, Experiment 1. Motor impairment related mechanical sensitivity was examined over the course of infection. These data are presented as a change from baseline. Only the SDR-VEH group changed over time, and was significantly reduced immediately post infection (see Fig 4..5), however over the course of infection, these animals became less sensitive compared to the other groups, indicating either a motor or sensory impairment. Asterisks (*) indicate significant differences,

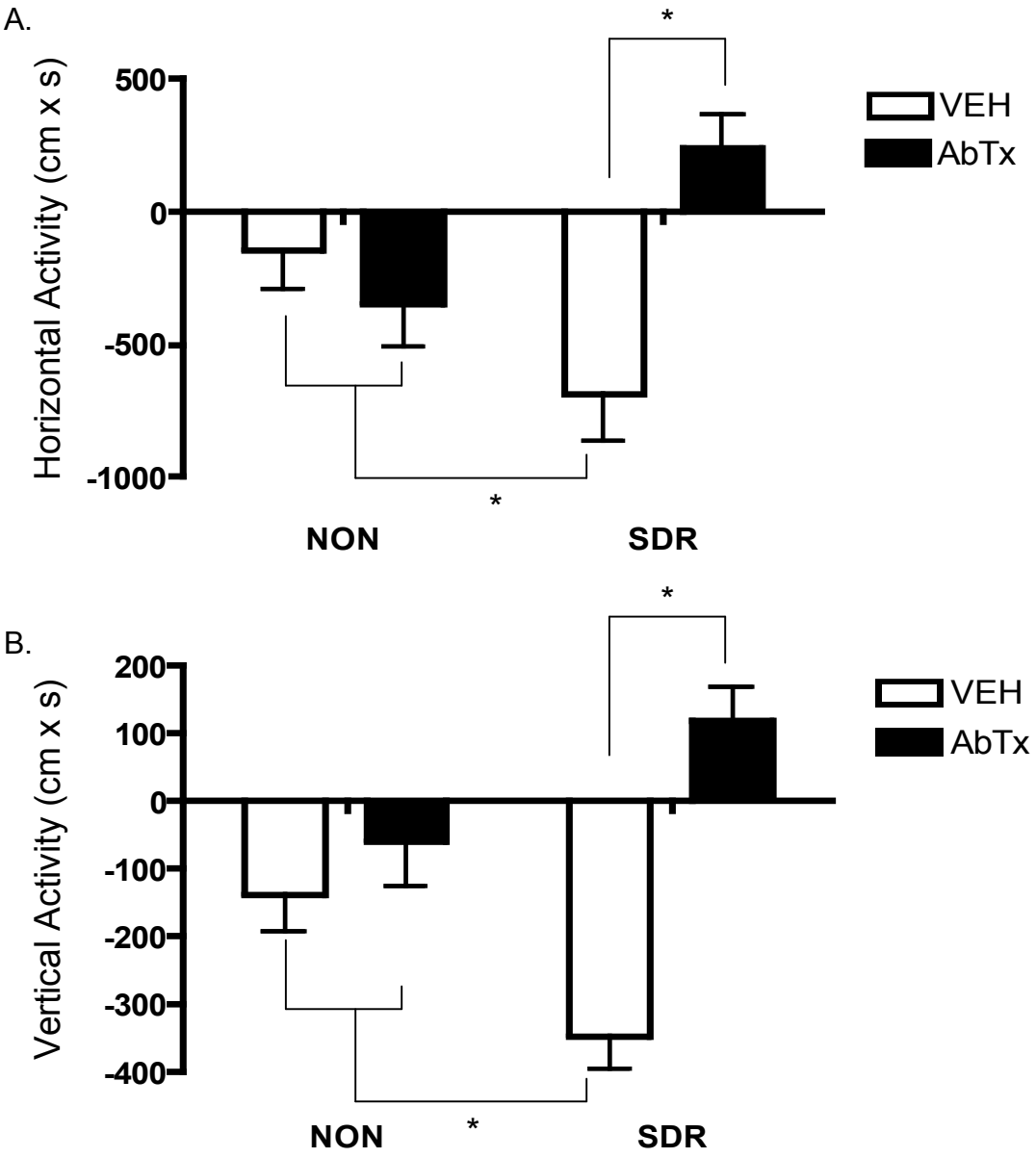


Fig 3.10. Activity Measures: Experiment 1.
Horizontal activity (A), and vertical activity (B) were both analyzed as change from baseline activity levels. Horizontal activity indicates overall motor impairment, while vertical activity is more relevant to hind limb impairment. SDR-VEH mice had significantly reduced activity in both planes, while the antibody treatment reverse these deficits. Significant differences are indicated with asterisks (s).

3.3.3.3. Open field activity. Open field activity was assessed as a change from baseline in both the horizontal and vertical planes. Analyses of open field activity found deficits in both horizontal (Fig 3.10.A) and vertical activity (Fig 3.10.B) due to SDR treatment, and these deficits were reversed when the SDR animals were treated with antibody to IL-6. An ANOVA confirmed a 2-way interaction (SDR x AbTx) on both horizontal, $F(1,44) = 11.436$, $p = .01$, and vertical activity $F(1,44) = 7.784$, $p = .01$. *Post hoc* means-comparisons showed that SDR-VEH was significantly less active compared to the other groups, while the SDR-AbTx not only reversed the effects of SDR alone. Interestingly, SDR-AbTx restored both horizontal and vertical activity levels to above baseline over the course of infection. *Post hoc* comparisons also indicated that the SDR-AbTx group was significantly more active in both the horizontal and vertical plane compared to the non-stressed groups.

3.3.3.4. Stride length. A final measure of motoric impairment, stride length, at day 20 pi has previously been shown to be reduced in SDR animals, (Johnson, et al; 2004). Here, stride length at day 20 pi was again assessed. These data are presented in Fig 3.11. An ANOVA confirmed main effects of both SDR, $F(1,39) = 6.918$, $p < .05$ and antibody treatment, $F(1,39) = 12.286$, $p < .01$. A 2-way interaction (SDR x AbTx) was also found, $F(1,39) = 5.335$, $p < .05$. *Post hoc* means comparison showed that the SDR-VEH animals had significantly greater deficits compared to all other groups, indicating that while

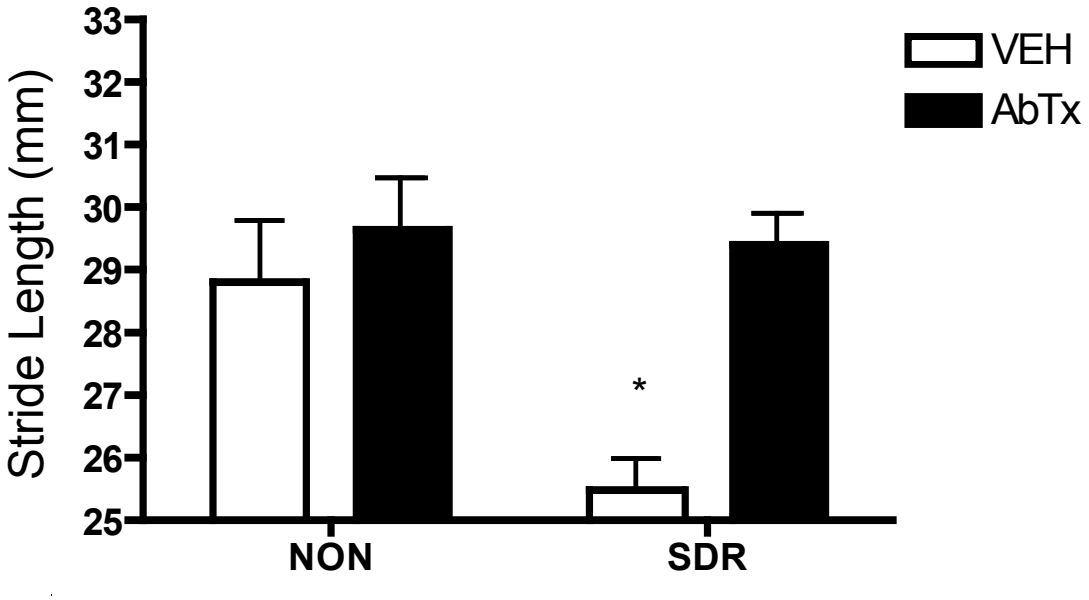


Fig 3.11. Stride Length: Experiment 1.
Stride length will become reduced in the presence of motor impairment. Only the SDR-VEH group developed significant deficits in stride length. Antibody treatment reversed these effects. An asterisk (*) indicates significant differences.

SDR alone induced a deficit, AbTx was able to restore stride length to normal levels due to infection alone. Fore limb data was similar (data not shown).

3.3.4. Glucocorticoid resistance

GCR developed prior to infection (Fig 3.2.C). Resistance was again assessed in the animals sacrificed at day 7 pi. Fig 3.12. depicts the results of this analysis, and indicates that GCR was able to persist in the antibody treated animals. ANOVA analysis confirmed a significant 3-way (SDR x AbTx x Corticosteroid dose) interaction, F (4,32) = 3.876, p < .01. *Post hoc* analysis indicated that while SDR-VEH animals had significant, corticosteroid dose dependent reductions in proliferation, the SDR-AbTx group did not have significant reductions in cell survival, regardless of corticosteroid dose. The non-stressed groups also had significant dose-dependent reductions in cell survival, however, GCR would not be expected in these groups, either prior to infection or at day 7 pi.

3.3.5. Immunological measures

Various measures of the immunological effects of SDR and antibody to IL-6 treatment were collected to examine the physiological effects for this experiment, and these are depicted in the next three graphs. Fig 3.13. depicts spleen and thymus weights. A reduction in weight in these two immunological organs is an indirect indication of reduced immunological function. Viral clearance (Fig 3.14.) in brain and spinal cord was assessed, because a failure to effectively clear the virus in the acute phase is a primary risk factor for

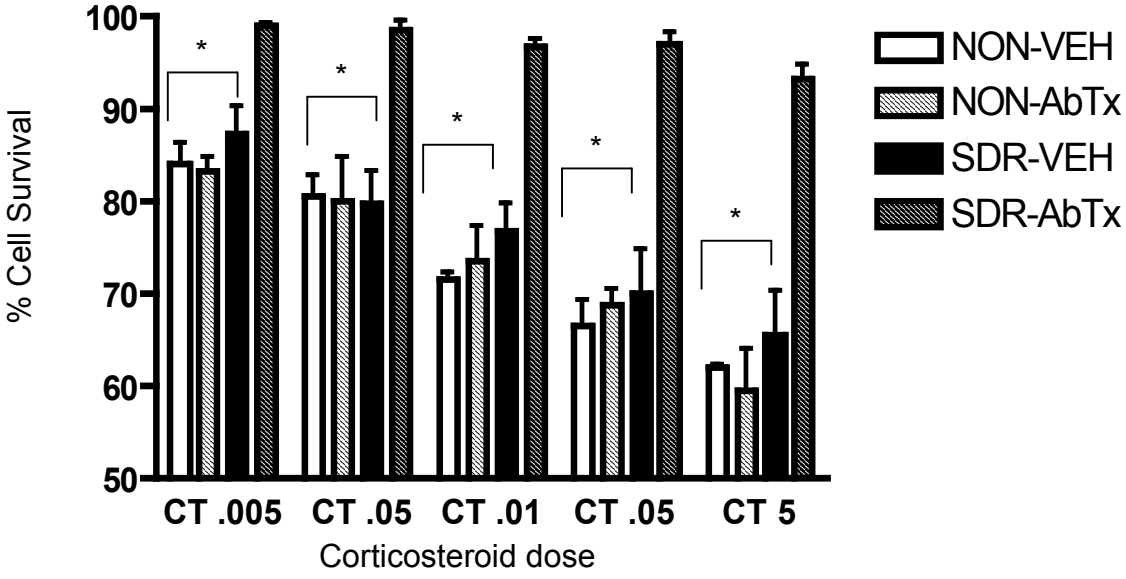


Fig 3.12. Glucocorticoid Resistance at day 7 pi: Experiment 1. GCR development in Experiment 1 at day 7 pi is shown. Both SDR groups developed GCR prior to infection (Fig 4C). However, only the antibody treated animals maintain resistance at day 7 pi. Asterisks (*) indicate significant *post hoc* differences compared to control (LPS-stimulated only) cells.

developing the chronic phase of TMEV infection, a model of MS (Oleszak et al., 2004). Finally, inflammatory cell infiltrate (Fig 3.15.) in both brain and spinal cord was examined because the acute phase of TMEV infection is primarily a CNS-inflammatory process. Excessive inflammation may indicate the severity of the acute phase of TMEV disease. Additionally, greater cytokine activity would also increase inflammation, so elevated inflammation can also be used to infer greater cytokine activation.

3.3.5.1. Organ weights. Spleen and thymus weights were collected at both day 7 and 21 pi. Both days had similar pattern of results, therefore, only day 7 pi are shown. In spleens, ANOVA confirmed a main effect for SDR, $F(1,32) = 39.325$, $p < .0001$, and this is depicted in Fig 3.13.A. *Post hoc* comparisons indicated that SDR lead to increased spleen weights. This SDR induced splenomegoly has been associated with the development of GCR (Avitsur et al., 2001). GCR has typically disappeared by day 7 pi due to infection in our previous studies (Johnson et al., 2004). However, GCR was maintained in the SDR-AbTx animals, but not the SDR-VEH animals or the non-stressed animals (Fig 3.12.).

The thymus is responsible for processing T-cells, and a reduction in thymic weight may indicate a reduction in T-cell efficacy or numbers. Viral clearance in the acute phase of TMEV infection is reliant upon cytolytic T-cells (CD8+); therefore, a reduction in either function or number of T-cells would be detrimental to viral clearance (Borrow et al., 1992). Day 7 pi thymic weights are

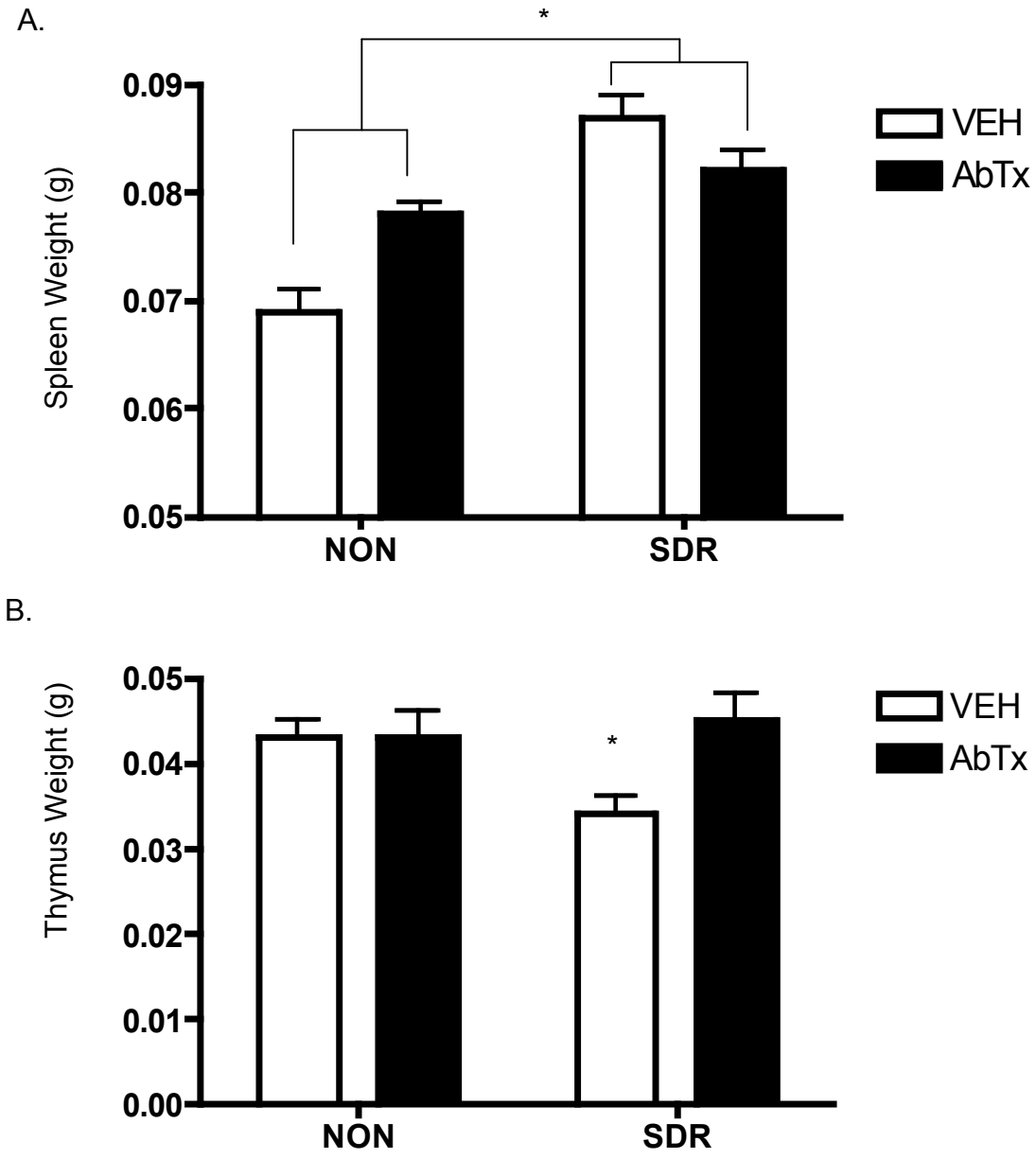


Fig 3.13. Immune Organ Weights: Experiment 1.
Panel A depicts spleen weights. Splenomegoly is associated with SDR induced GCR. Both SDR groups have enlarged spleens compared to the non-stressed groups. Panel B depicts thymus weights. The SDR-VEH group had significantly atrophy of the thymus. Asterisks (*) indicate significant differences.

depicted in Fig 8.1 B. ANOVA confirmed a 2-way interaction (SDR x AbTx) for thymic weights at day 7 pi, F (1,32) = 5.777, p < .05. *Post hoc* comparisons found that SDR alone significantly reduced thymic weights, while SDR-AbTx animals did not differ from the non-stressed groups.

3.3.5.2. Viral clearance. Examining viral titers in one subset at day 7 pi and in a second subset at day 21 pi assessed viral clearance. Previously, our work has shown that viral clearance was altered by SDR alone (Johnson et al., 2004). Viral clearance in brain (Fig 3.14.A) and spinal cord (Fig 3.14.B) was again impaired by SDR treatment, and restored beyond normal levels in SDR-AbTx animals. ANOVA confirmed a 3-way interaction (SDR x AbTx x Day pi) for viral clearance in both brains, F (1,40) = 22.706, p < .0001, and spinal cords, F (1,40) = 38.022, p < .0001. *Post hoc* comparisons indicate that the non-stressed groups significantly cleared virus from day 7 to day 21 pi, while the SDR-VEH animals had no significant change over time. Finally, the SDR-AbTx group had a significantly reduced viral titer over time, indicating that the AbTx restored viral clearance in the SDR animals.

3.3.5.3. Inflammation. Inflammation (Fig 3.15.) was assessed in brains and spinal cords by measuring the percentage of the perimeter affected by meningitis (microglia and macrophage infiltrate into the meninges) and the percentage of area affected by microgliosis and perivascular cuffing (microglia and macrophage infiltrate in the white and/or grey matter). As in previous

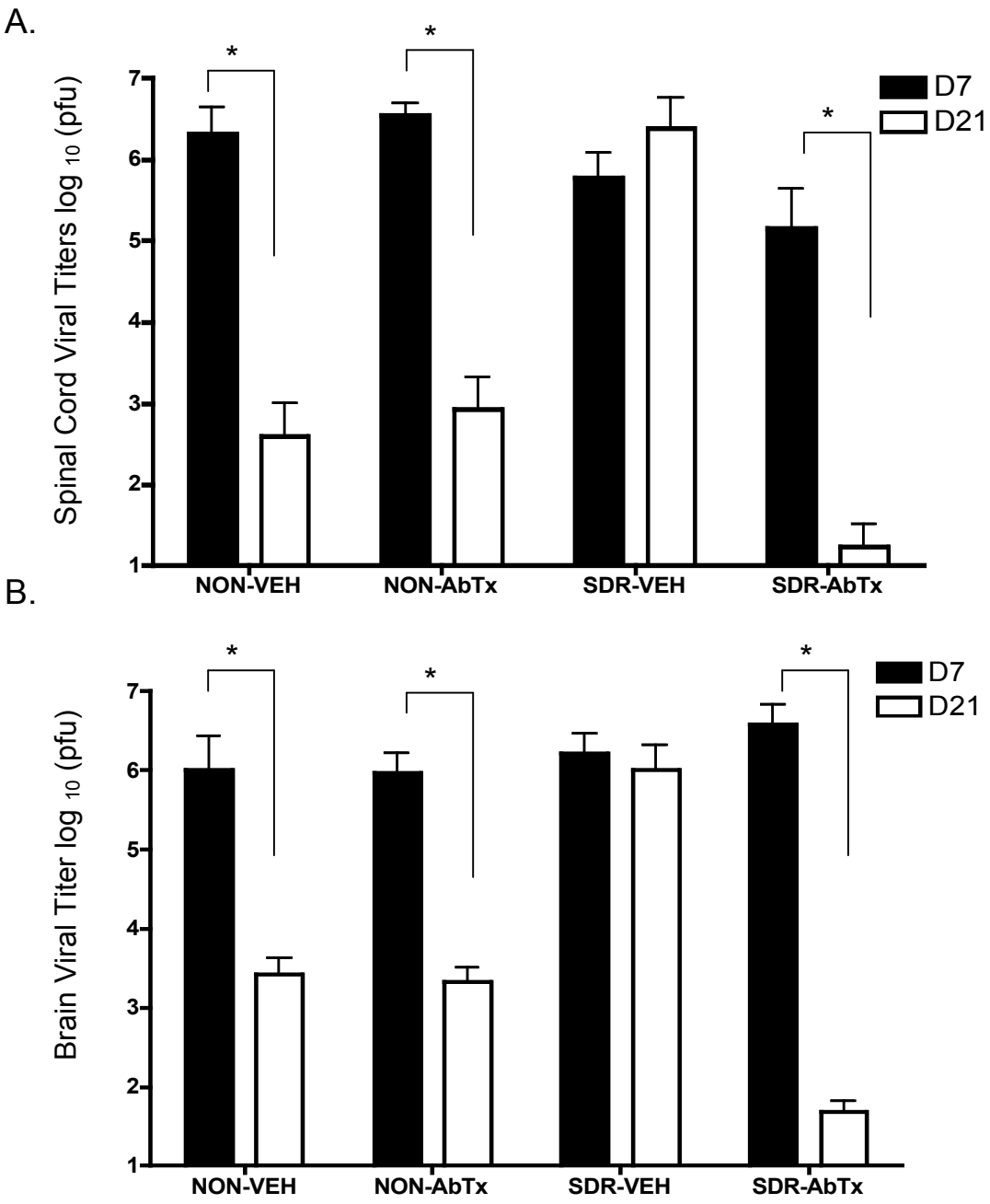
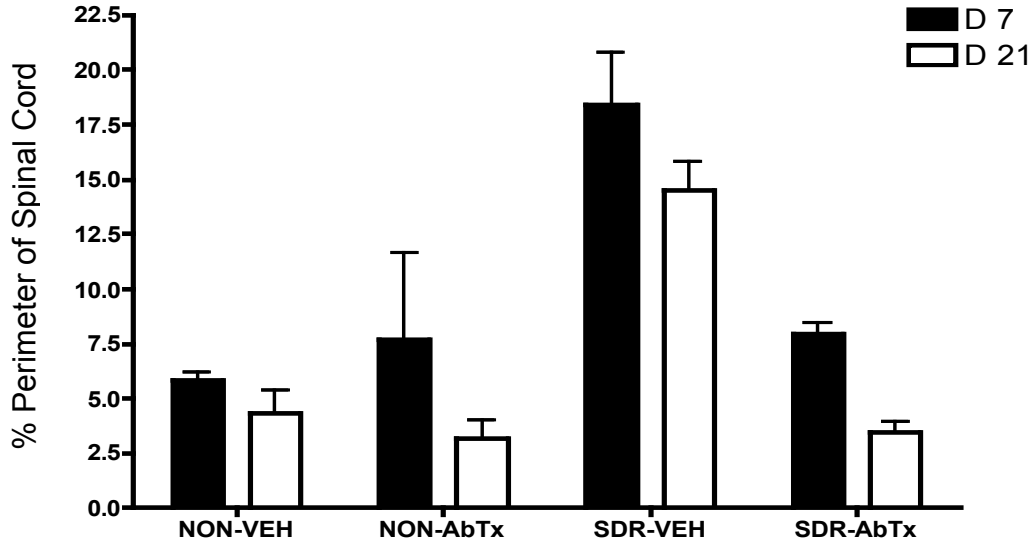


Fig 3.14. Viral Clearance: Experiment 1.
Viral titers were taken from spinal cord (A) and brain (B) at days 7 and 21 pi. A reduction of viral load over time indicates viral clearance. All groups cleared a significant amount of virus over time with the exception of the SDR-VEH group. Significant differences are indicated with asterisks (*).

A.



B.

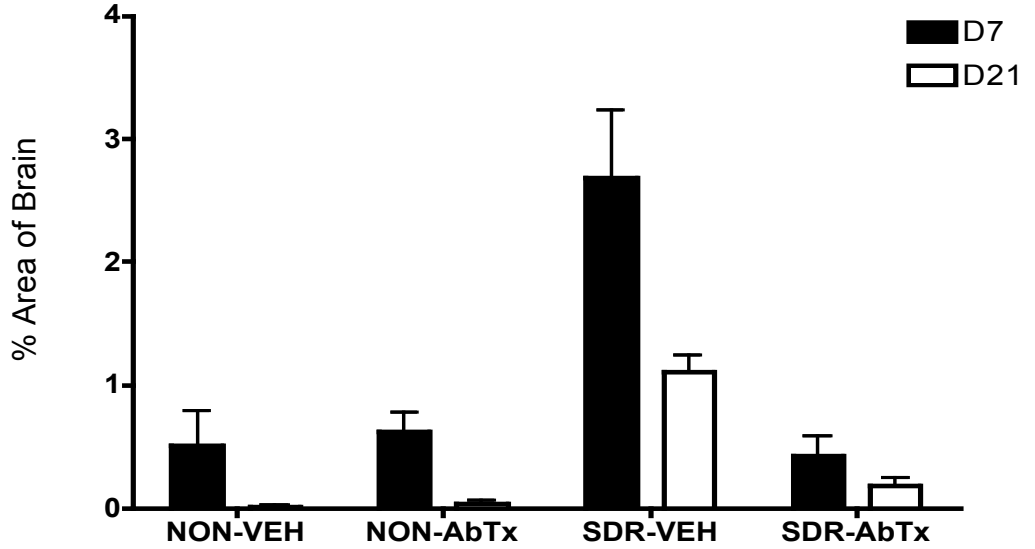


Fig 3.15. Inflammation: Experiment 1.
Inflammation (as a % of perimeter) in the spinal cord (A) and brain (as a % of area) was elevated by SDR alone (SDR-VEH) group. This occurred across both days 7 and 21 pi, in both compartments. Asterisks (*) indicate significance.

studies, SDR caused greater inflammation in both brains and spinal cords. ANOVAs found a 2-way (SDR x AbTx) interaction in brains, F (1,40) = 24.187, p < .0001 and spinal cords, F (1,40) = 19.091, p < .0001. This elevation in inflammation continued to be significant at both day 7 and 21 pi. *Post hoc* comparisons indicate that SDR alone significantly elevated inflammation at both day 7 and 21 compared to the other three groups. In addition, SDR-AbTx had greater inflammation compared to non-stressed groups, at day 7 pi, but not at day 21 pi.

3.3.6. Summary of results

We examined batteries of illness measures, motor impairment measures, and physiological measures. These results are summarized in Table 3.2. Overall, SDR alone (the SDR-VEH group) increased illness measures, motor impairment, and physiological aspects of disease. In contrast, when SDR animals were treated with neutralizing antibody to IL-6, the negative effects of SDR were reversed. In both the illness and motor impairment measures, the SDR-AbTx animals had lower levels of disease indication than the non-stressed groups.

3.4. Discussion

Previous studies found that prior exposure to social stress exacerbates acute and chronic TMEV infection. The aim of Experiment 1 was to determine the necessity of SDR induced IL-6 on acute TMEV infection. Since previous studies also found that SDR causes excessive inflammation and deregulates GC control

Table 3.2. Summary of Results: Experiment 1

	NON-VEH	SDR-VEH	NON-AbTx	SDR-AbTx
Illness Measures	**0**	↑↑	**0**	↓
Body temperature	0	0	0	↓↓
Sucrose preference	0	↑↑↑	0	0
Body weight	↑	↑↑↑	↑↑	0
Horizontal activity	0	↑↑↑	0	↓↓
Mechanical sensitivity	0	↑↑↑	0	↓↓
Motor impairment measures	**0**	↑↑↑	**0**	↓
Hind limb impairment score	0	↑↑↑	↑	↓↓
Mechanical sensitivity	0	↑↑↑	0	0
Horizontal activity	0	↑↑↑	0	↓↓
Vertical activity	0	↑↑↑	0	↓↓
Stride length	0	↑↑↑	0	0
Physiological measures	**0**	↑↑↑	**0**	**0**
Spleen weight	0	↑↑	0	↓↓
Thymus weight	0	↑↑↑	0	0
Viral clearance: Brain	0	↑↑↑	0	↓↓
Viral clearance: Spinal cord	0	↑↑↑	0	↓↓
Inflammation: Brain	0	↑↑↑	0	0
Inflammation: Spinal cord	0	↑↑↑	0	0

Table 3.2. presents a summary of the results found across illness, motor impairment and physiological measures of TMEV infection. The control, non-stressed, vehicle treated groups was assigned 0, as an indicator of normal disease level. When a group exhibited increased disease severity on a given measure, this was indicated by ↑ ($\underline{p} > .05$) or ↑↑ ($\underline{p} > .01$), or ↑↑↑ ($\underline{p} > .0001$). In contrast, when a group exhibited reduced disease severity, this was indicated by ↓ ($\underline{p} > .05$) or ↓↓ ($\underline{p} > .01$). The bold lines are a summary of the results for each set of measures.

of inflammation, the pro-inflammatory cytokine, IL-6 is a likely mechanistic molecule (Johnson et al., 2004). Antibody to IL-6 was used to antagonize IL-6 release due to SDR. Initially, the efficacy of the neutralizing antibody was confirmed, reducing IL-6 activity by 75-to-90% (Fig 3.2.). As in previous studies, the current study found that SDR alone was associated with more severe illness, motor impairment, and physiological measures of TMEV infection. Blocking the SDR-induced IL-6 restored illness, motor impairment, and physiological measures to normal (infected but non-stressed) levels in acute TMEV infection. Thus, IL-6 appears to be necessary for the negative effects of SDR in acute TMEV infection. These findings were internally consistent, as well as convergent with previous SDR studies, however a few exceptions occurred. First, antibody to IL-6 prevented the resolution of GCR by day 7 pi as occurred in previous studies. Second, infection related hypothermia or fever developed differently than expected. Finally, immunological measures were convergent but not consistent with previous studies using SDR in acute TMEV infection.

One of the consistent hallmarks of SDR is the development of GCs resistance. In the past, TMEV infection was associated with the loss of GCR by day 7 pi. In the current study, SDR induced GCR prior to infection, and the development of GCR was not altered by administration of neutralizing antibody to IL-6. In addition, the SDR alone animals also lost GCR by day 7 pi as occurred in past studies. However, at day 7 pi, the antibody treated animals maintained GCR. These data are somewhat difficult to interpret, given that the

signaling pathway for the release of the GCR macrophages from the bone marrow is not well elucidated. One possible explanation may be that blocking IL-6 may also interfere with some signaling pathways for the release of the GCR macrophages. The most likely candidate would be that similar to the HPA-axis, IL-6 can also trigger the SAM-axis, given that norepinephrine may be the signaling molecule that causes the GCR macrophages to traffic to the bone marrow.

Body temperature deregulation is dependent upon several factors in mice, only one of which is IL-6. First, both the type of inflammatory stimuli and dose can alter development of fever or hypothermia. A recent review found that overall, low doses of LPS are associated with hypothermia, while high doses result in fever, although the serotype of LPS used may also be important (Leon, 2002). Sepsis (induced by cecal ligation and puncture) results in hypothermia for the first 24 h, while fever occurred past that point (Leon et al., 1998). Turpentine-induced inflammation caused fever (Horai et al., 1998). Finally, two out of six strains of avian flu induced fatal hypothermia in mice (-4°C), one of the strains induced a milder hypothermia (< 2°C), and the other three strains did not alter body temperature.

These temperature regulation effects may be differentially mediated through IL-6 (as well as other cytokines). Turpentine induced fever is attenuated in both IL-1 and IL-6 knockout mice. In LPS studies, IL-6 knockouts develop mild hypothermia (approximately -.5°C) that persists for at least 12 h at low

doses, while wild type mice developed fever. In contrast, fever developed equally in both IL-6 knockouts and wild type mice at high doses of LPS (Kozak et al., 1998). Given that the majority of studies show hypothermia at the same low dose used in the IL-6 knockout mice, this data is difficult to interpret. If the LPS had induced hypothermia, it is possible that knocking out IL-6 genes would have no effect here as well.

In the current studies, body temperatures were taken 12 h prior to infection and 12 h pi, but only the antibody treated-SDR animals had altered body temperature across the two time points. Prior to infection, the antibody treated- SDR animals had body temperatures that averaged 1- 1.5 ºC *below* the other animals. Thus, blocking IL-6 induced hypothermia in the absence of infection. Post infection, these same animals' body temperatures became elevated by an average of over 1 ºC compared to pre-infection, returning these animals to the same temperature range as the other groups. Interpreting this data is difficult, because antibody treatment appears to have reduced body temperature significantly, while infection overwhelmed this reduction and returned body temperature back to the range of the remaining mice, stressed or not.

One possible explanation may be due to the unique role of IL-6 in HPA-axis activation. As noted earlier, IL-6 also stimulates the HPA-axis, deregulating the negative feedback loop, and increasing the overall levels of corticosteroids (Bethin et al., 2000). By blocking IL-6, the negative feedback loop of the HPA-

axis may be re-instated resulting in an overall reduction in corticosteroids. If corticosteroids are reduced (through the self-regulating negative feedback loop), then the other proinflammatory cytokines (IL-1, TNF-α) would have even less regulation (especially in light of the GCR that is in place at this time). IL-1 is known to mimic LPS, so an increase in this cytokine could possibly induce the pre-infection hypothermia (Leon, 2002). Since the antibody to IL-6 treatment has a half-life of no longer than 10 h (according the manufacturer), the antibody would have been reduced by at least half at 2 h pi. Therefore, infection induced IL-6 at 12 h pi could be stimulating the HPA-axis, allowing GCs to regulate the other cytokines and thus, return body temperature to normal.

Although this is a reasonable explanation, there are a few flaws in the argument. First, antibody treatment in the absence of stress did not induce the pre-infection hypothermia. However, this may be because the other cytokines that might induce hypothermia were not activated in the absence of stress. Second, we know that an important source of both IL-6 and the other proinflammatory cytokines are the macrophages and microglia. Since GCR occurs in the splenic macrophages, the CNS microglia may also be resistant at the time of infection, leading to excessive proinflammatory cytokine release. We also know that in the antibody to IL-6 treated animals that GCR persists through day 7 pi. Thus, the return of cytokines back under GCs control is unlikely at the time these data were collected. Thirdly, SDR was associated with the development of all of the other sickness measures and blocking IL-6 restored

these measures back to normal. Therefore, it would be counter intuitive to deduce that antibody treatment did the opposite only on body temperature. While the data indicates that IL-6 is necessary for development of the majority of sickness behaviors, fever did not converge with the other measures and this contradiction should be explored further.

IL-6 appears necessary not only for the development of exacerbated sickness behaviors due to SDR in acute TMEV infection, but also motor impairment and immunological exacerbations previously noted. However, the immunological data does differ somewhat from our previous work. In past studies, we did not find splenomegoly post-infection due to SDR. Since the increased spleen size was not associated with continued GCR in the SDR alone animals, this finding may be related to a stronger immune response. As a primary immune organ, the spleen in the SDR animals may have been processing a greater number of monocytes and lymphocytes, but not resistant cells.

In addition to enlarged spleen, the current study also found thymic atrophy. In past studies using restraint stress, we have often found mild to extreme thymic atrophy, however we had not seen this in prior social stress studies (Campbell, et al., 2001; Johnson, et al., 2004). The thymic atrophy in the current study was mild, but significant. Considering the essential role of CD8+ T-cells for viral clearance (Oleszak et al., 2004), the thymic atrophy may also help explain the viral clearance findings. In our previous studies, animals

exposed to SDR prior to infection had impaired viral clearance in the spinal cords, but also had lower titers at day 7 pi compared to non-stressed animals. Here, all groups had high titers at day 7 pi. Since the thymic atrophy also occurred in the SDR animals here, perhaps the elevated titers at day 7 pi in the current study are related to thymic function. In addition, in past studies, viral clearance occurred equally in both the SDR and non-stressed animals normally in brain tissue. In the current study, viral clearance did not occur at all in the brains of the SDR group, and the other three groups cleared a smaller percentage compared to previous studies. Thymic atrophy cannot fully account for the less effective viral clearance in the current study compared to past studies, since the atrophy only occurred in the SDR alone animals. Perhaps the additional stress of surgery for cannulation impaired viral clearance for all of these animals, since layering on stressors can alter immune function.

In addition, viral clearance was consistent with previous work, however the relationship between groups was not. In past studies, exposure to SDR prior to infection resulted in spinal viral titers that were reduced approximately 25% at day 7 pi compared to non-stressed animals. These animals failed to develop any further reduction over time. In the current study, the spinal viral titers are not significantly different across groups, although the SDR alone animals once again failed to significantly reduce viral titers over time. In addition, previous work found that viral clearance in the brain tissue occurred in all groups. In contrast, the current study found that SDR alone resulted in a failure to clear a

significant amount of virus over time in both spinal and brain tissue. The failure of the SDR groups to reduce viral titer in either brains or spinal cord tissue, at either day 7 or 21 pi, may be due to the increased inflammation that we also documented in these animals. CD 8+ T-cells and B-cells are needed to clear the virus, but the excessive microglia and/or macrophage activation may prevent the appropriate signaling and trafficking of lymphocytes to the site of infection. This inflammation may be from two sources that act synergistically. First, the cannulation surgery violated the blood-brain barrier, allowing many more macrophages to traffic into the CNS. Second, SDR induced proinflammatory cytokines in microglia and possibly neuronal tissue (that may not be under GC regulatory control). Cannulation alone was not sufficient to cause excessive inflammation. In the absence of additional stimuli, the cytokine release due to cannulation surgery injury resolves within approximately 7 days, 2 weeks prior to the sacrifice in the current study (Holguin et al., 2005). However, previous work has shown that both IL-6 and TNF-α may be elevated due to SDR alone. Therefore, cannulation, SDR, and infection combine to result in elevated inflammation that interferes with appropriate lymphocyte trafficking.

Finally, the effect of SDR on inflammation resulted in greater effect size differences than in previous studies. The effect size for the brain inflammation was .656 (considered a medium effect size) in the current study compared to .112 (no effect) for microgliosis and .26 (small effect) for perivascular cuffing in our previous work (Johnson et al., 2004). For the spinal cord meningitis, the

current study had an effect size of .829 (large), compared to .251 (small) in our previous studies. The difference in effect sizes may be due to several factors, including more sophisticated use of computerized assessment of the area affected by inflammation. It is unlikely to be solely due to the cannulation procedure (because this was equated across groups), however this procedure may have increased the system's vulnerability to inflammation associated with SDR.

While there are differences between the original work and the current studies, the data from Experiment 1 is consistent in that SDR exacerbated acute TMEV infection, and impaired viral clearance. Finally, the illness measures, motoric impairment measures and immunological measures all converge to indicate that blocking IL-6 blocks the exacerbation of TMEV infection due to SDR.

4. EXPERIMENT 2

4.1. Introduction

Experiment 1 resulted in the conclusion that IL-6 is necessary for the negative effects of SDR on acute TMEV infection. In order to fully assess the role of IL-6 in the negative effects of SDR in acute TMEV infection, sufficiency of this cytokine also needed to be determined. Thus, social disruption was replaced with exogenous IL-6, and the procedures from Experiment 1 were replicated.

4.2. Methods

4.2.1. IL-6 substitution

Mouse IL-6 was purchased from R&D Systems (406-ML-005/CF). 5μg was dissolved in 1 mL sterile PBS. This stock solution was diluted by 1:50 in PBS for administration. A volume of 2 μl in 2 min was administered, for a dose of 200 pg per animal, based on the control (NON-SDR) animal IL-6 levels from the efficacy part of Experiment 1. Cytokine was administered at 1600-h daily, while vehicle (sterile saline plus mouse Ig, Santa Cruz Biotechnology, Inc #SC-2025) was administered to control (NON-SDR) animals.

4.2.2. Procedure

Upon arrival, animals were weighed and caged individually. The next morning, cannulation surgeries occurred. Mice were allowed to recover in individual housing for 2 days. On pnd 27, mice were weighed again and

Table 4.1. Procedural Event Table: Experiment 2.

Arrive																
Surgery																
Weigh/ Assign																
Open Field Habituation																
Open Field Baseline																
Illness Behaviors																
IL-6 treatment																
Footprint-Baseline																
INFECT																
Motor -B																
Sacrifice- 7																
Footprint-20																
Sacrifice - 21																
PND	23	24	26	27	29	31	33	35		37						
PI									0		3	5	7	14	20	21

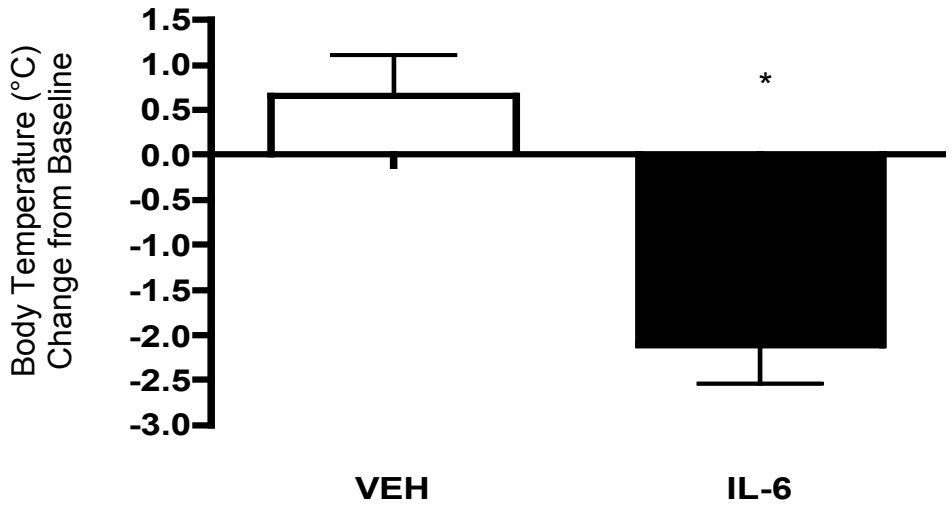
assigned to cages in a counterbalanced manner, based on weight. The Tylenol water was removed and sucrose and plain water were added for each cage. Baseline measures were taken during this time period, with the exception of footprint. On pnd 30-36, exogenous IL-6 or vehicle was administered daily beginning at 1500. On pnd 36 at 2100, all mice were infected. Illness measures were then taken through day 7 pi on all animals. Motor function measures were taken only in those animals sacrificed at 21 pi. Mice were sacrificed at either day 7 pi or day 21 pi, for either viral clearance or histology. Mice sacrificed on day 7 for viral clearance were also tested for GCR. The timeline for all procedures for this experiment is depicted in Table 4.1.

4.3. Results

4.3.1. Illness measures

As in Experiment 1, illness behaviors were assessed by analysis of data either within the first 24 h pi, or comparing data collected in the first 24 h pi to pre-infection data. Measures included body temperature, mechanical hypersensitivity, sucrose preference, body weight, and activity in an open field. Overall, the IL-6 treatment significantly altered body temperature and mechanical sensitivity illness measures in comparison to vehicle treated controls (VEH), and these data are presented in Fig 4.1. The other measures were not altered IL-6, and these are presented in Fig 4.2.

A.



B.

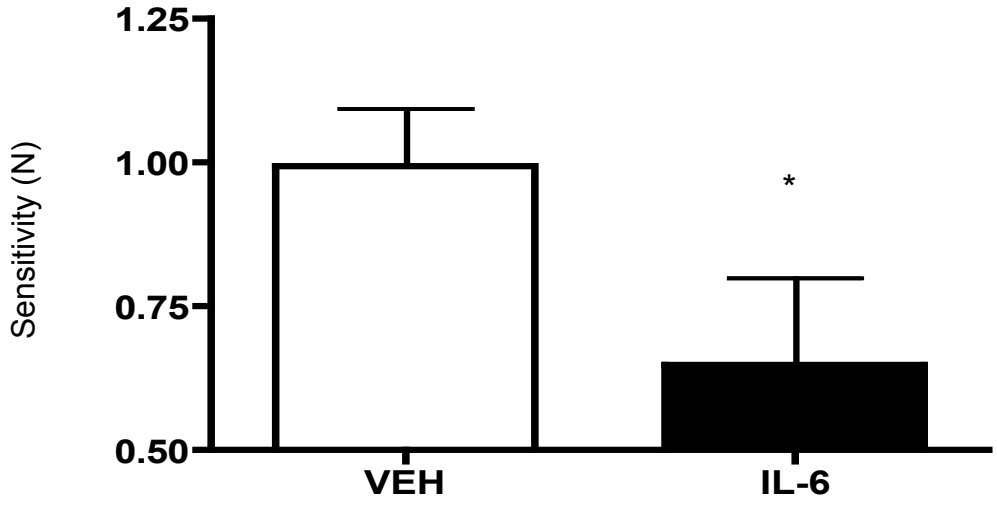


Fig 4.1. Illness Measures Altered by IL-6.
Body temperature (A) and mechanical hypersensitivity (B) were the only measures altered by IL-6 treatment. IL-6 caused significant hypothermia compared to baseline. IL-6 also caused a significantly greater sensitivity to mechanical stimuli to develop post-infection. Significant effects are indicated by asterisks (*).

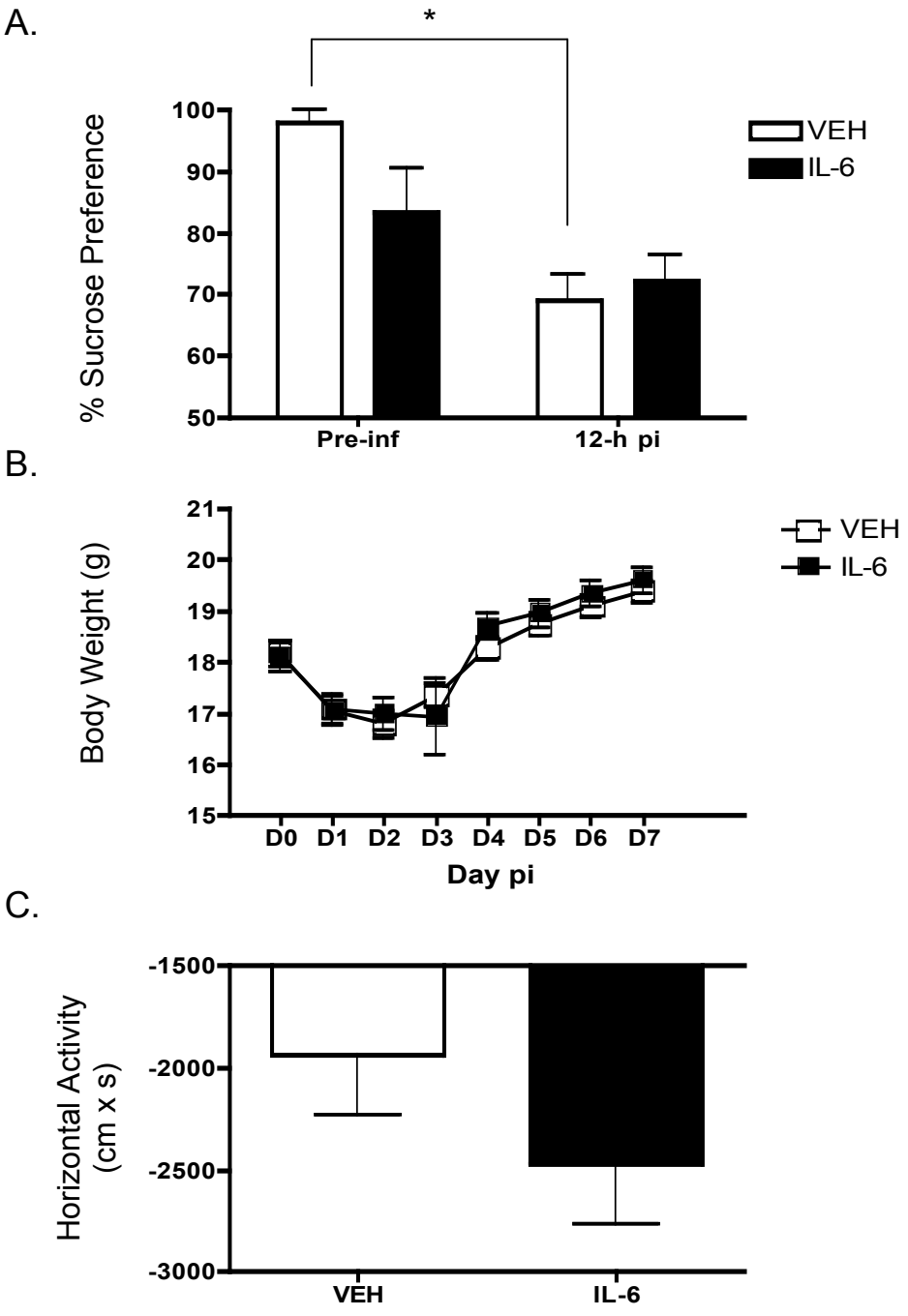


Fig 4.2. Illness Measures Not Altered by IL-6.
Sucrose preference was significantly reduced in the vehicle treated (A), but not in the IL-6 treated mice. An asterisk (*) indicates the significant difference. Neither body weight loss (B) nor horizontal activity (C) were altered by IL- treatment.

Following infection, ANOVA revealed that the IL-6 treated mice had a significant temperature drop, F (1,46) = 20.15, p < .0001, and greater allodynia, F (1, 22) = 8.277, p < .01. *Post hoc* comparisons determined that the IL-6 treatment significantly reduced body temperature and increased mechanical sensitivity. IL-6 treatment failed to alter body weight loss (p = .87) or activity reduction (p = .22). The vehicle treated groups did develop a significant reduction in sucrose preference F (1, 9) = 5.114, p < .05.

4.3.2. Motor impairment measures

As is Experiment 1, HLI, mechanical sensitivity, horizontal activity, vertical activity, and stride length were used to assess motor impairment, and these data are presented in the following four graphs (Fig 4.3.-6.). IL-6 treatment did not significantly alter hind limb impairment, F(1,22) = .6838, p = .68, mechanical sensitivity (p = .16), vertical activity F(1,22) = .002, p = .9, or stride length (p = .9). In addition, ANOVA revealed a trend toward a reduction in horizontal activity in the IL-6 treated animals, F(1,22) = 3.864, p = .06. Thus, IL-6 alone had no significant effects on motor impairment that occurs during acute TMEV infection.

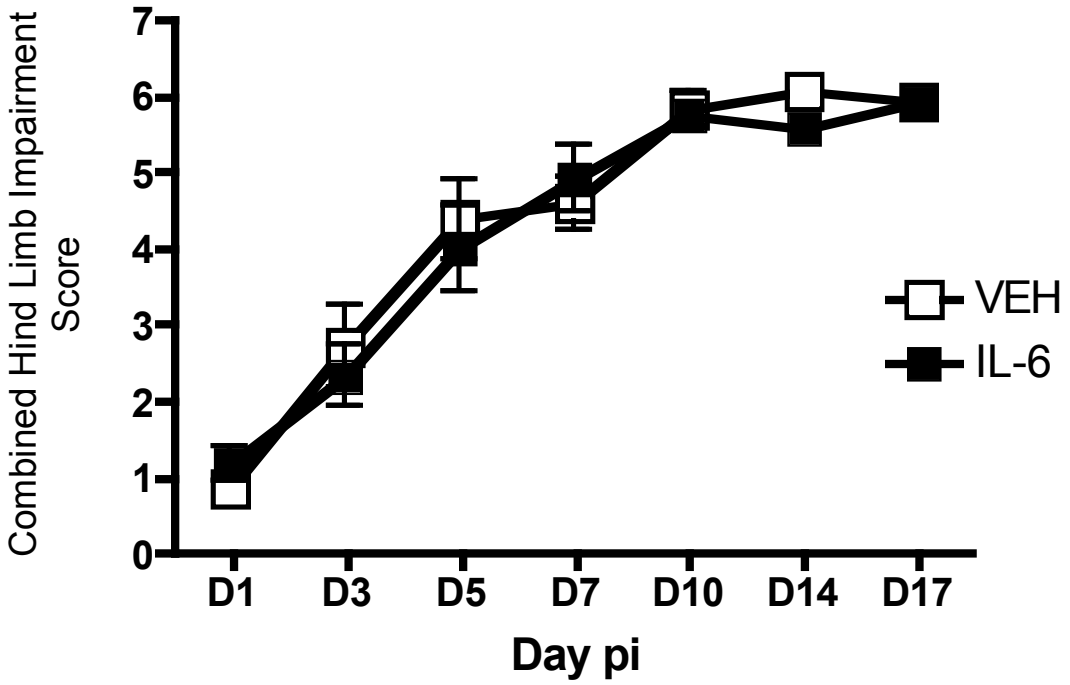


Fig 4.3. Combined Hind Limb Impairment: Experiment 2. Hind limb impairment was not altered throughout the course of the acute phase by IL-6 treatment

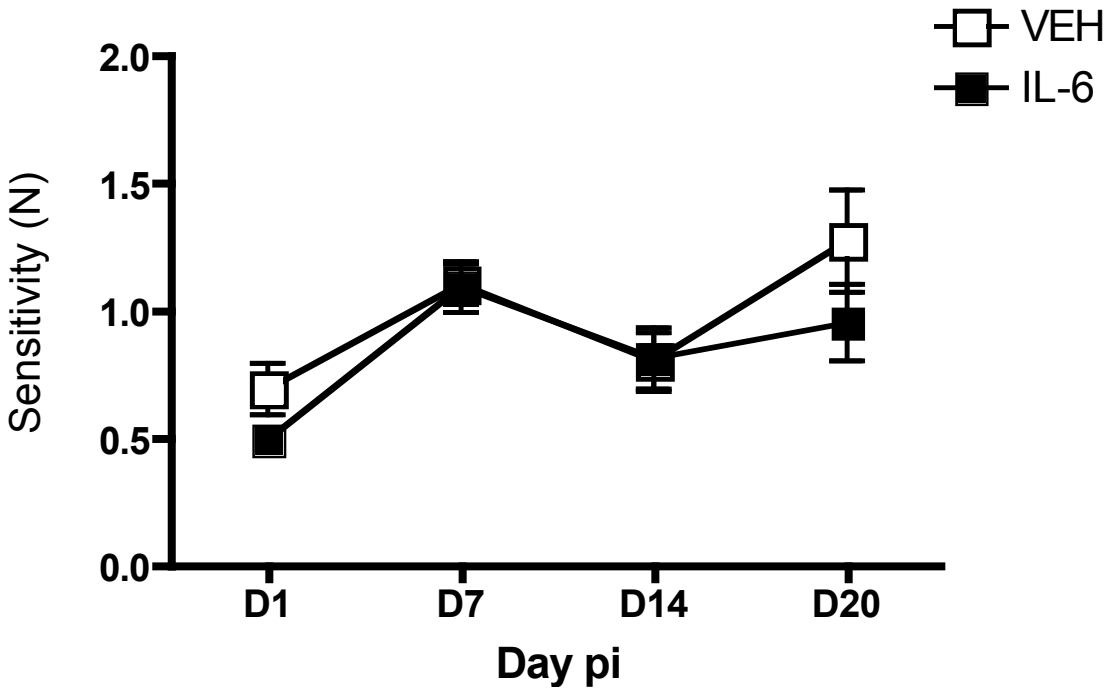
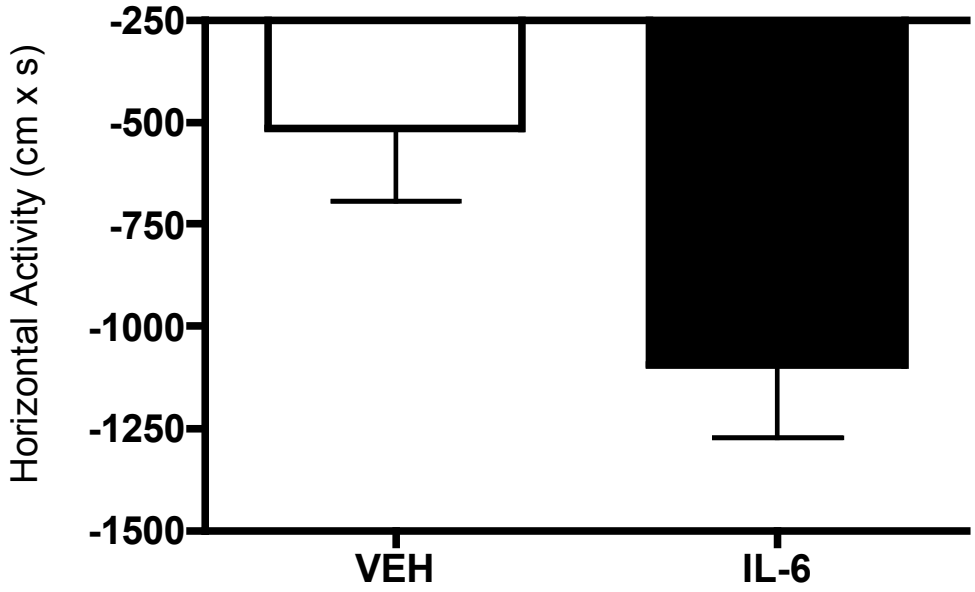


Fig. 4.4. Mechanical Sensitivity: Impairment Related, Experiment 2. IL-6 treatment did not alter mechanical sensitivity throughout the course of infection

A.



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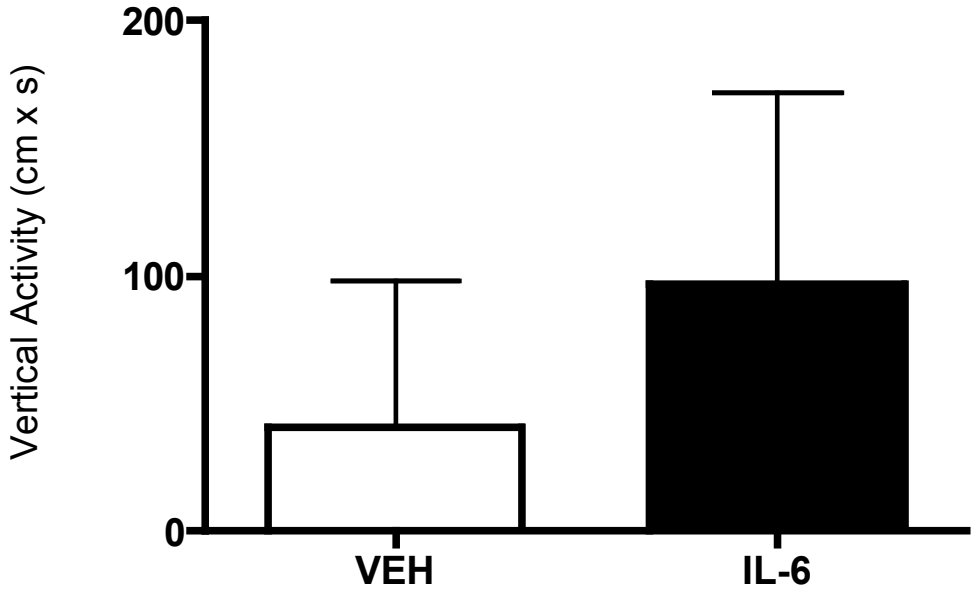


Fig 4.5. Activity Measures: Experiment 2.
Horizontal (A) and vertical (B) activity are shown. IL-6 treatment did not significantly alter either activity measure.

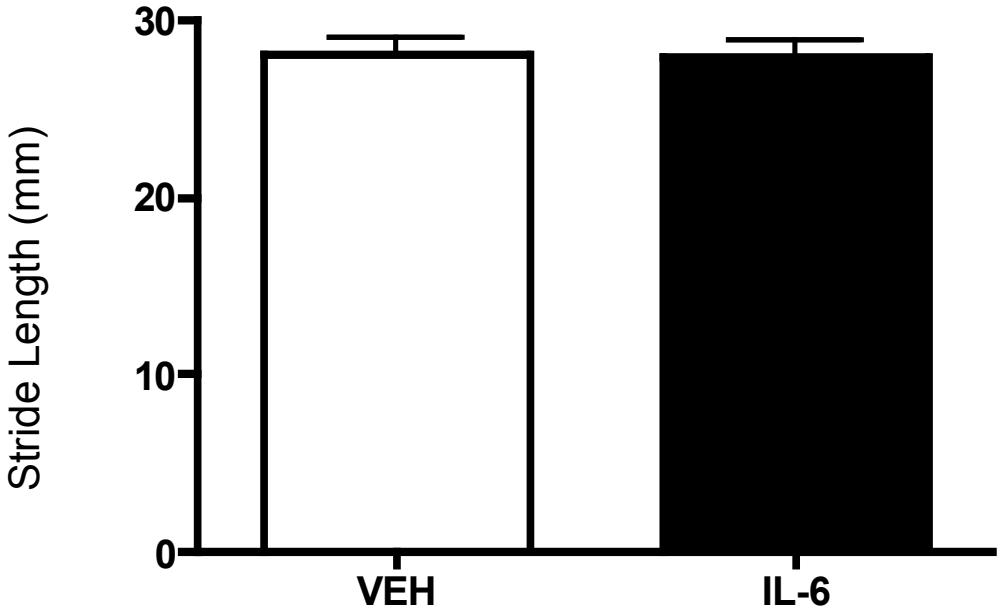


Fig 4.6. Stride Length: Experiment 2.
Footprint stride length was examined. IL-6 treatment did not alter stride length.

4.3.3. Immunological measures

As in Experiment 1, spleen and thymus weights, viral titers over time (for viral clearance) in brain and spinal cord and inflammation in brain and spinal cord were examined as measures of the efficacy of IL-6 in mimicking SDR in acute TMEV infection. These data are presented in the following three graphs (Fig 4.7.-9.). As in the motor impairment measures, IL-6 treatment did not result in significant changes from controls (VEH). ANOVA found no effect for IL-6 treatment on spleen ANOVA found a main effect for time in brains, F (1,20) = 5.62, $p < .0001$, and spinal cords, F (1,20) = 7.97, $p < .0001$, indicating that the virus was effectively cleared in both groups there was no difference between groups ($p = .55$). ANOVA also found no significant differences between groups in inflammation in either brain or spinal tissue ($p = .38$).

4.3.4. Summary of results

We examined batteries of illness measures, motor impairment measures, and physiological measures in order to assess the efficacy of IL-6 in mimicking the effects of SDR. These results are summarized in Table 4.2. Overall, IL-6 increased disease indications of body temperature and mechanical sensitivity, illness measures. However, there were no significant differences between the vehicle and IL-6 treated groups on the motor impairment and physiological aspects of disease.

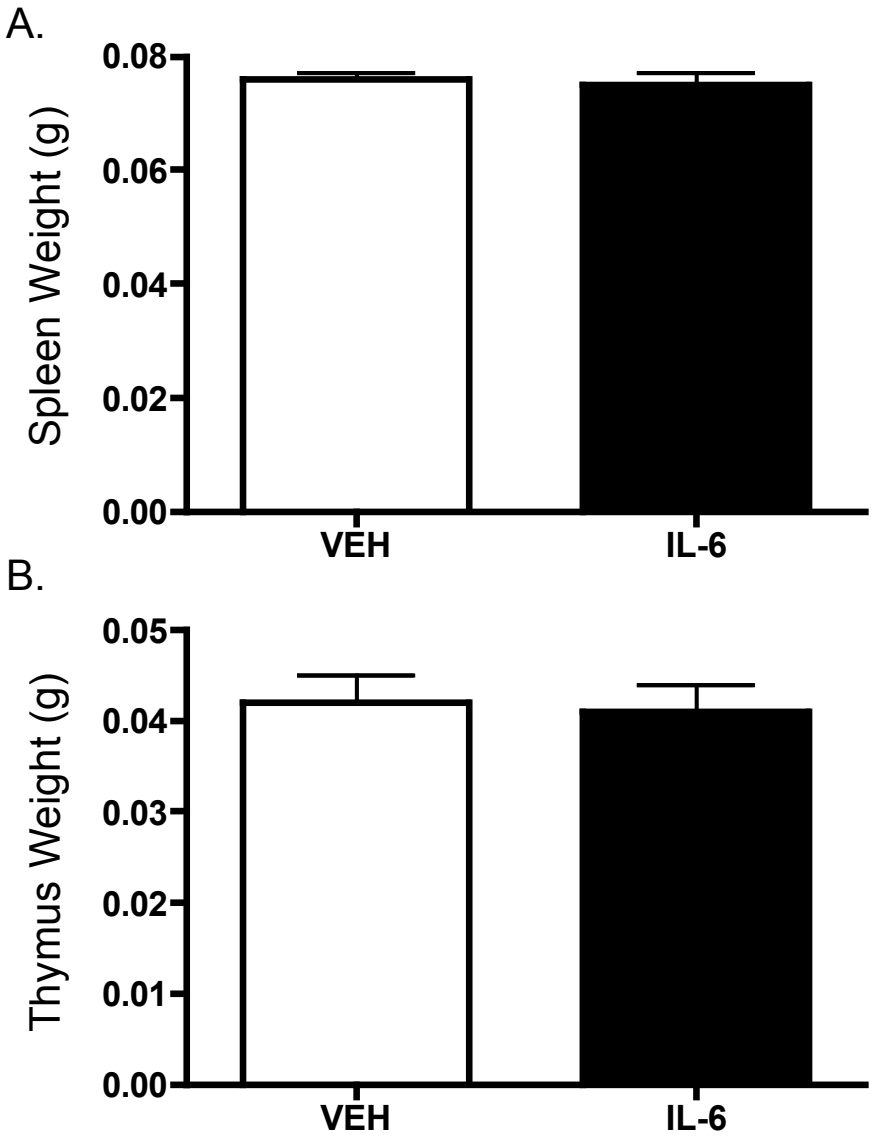
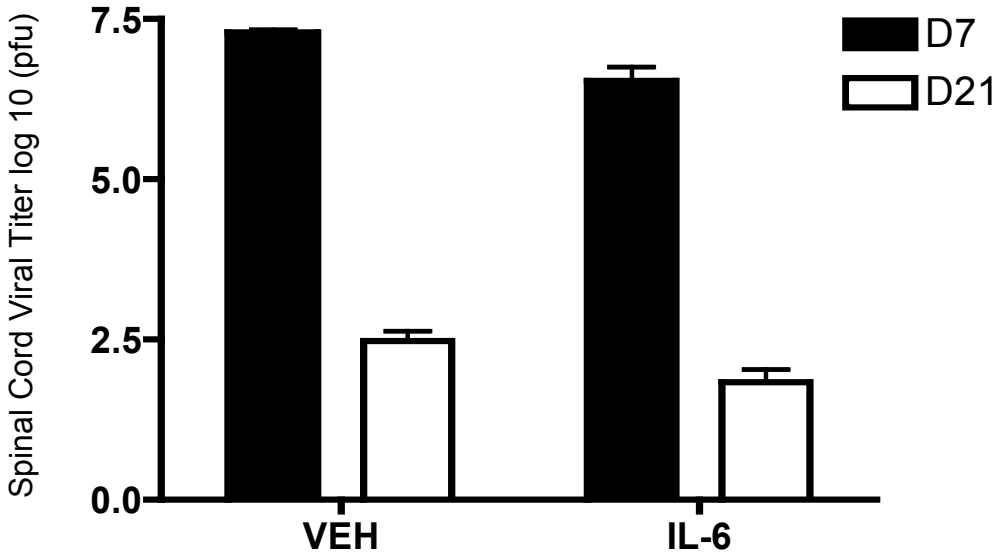


Fig 4.7. Immune Organ Weights: Experiment 2.
Immune organ weights were taken as a measure of immune function. IL-6 treatment did not alter spleen (A) or thymus (B) weights.

A.



B.

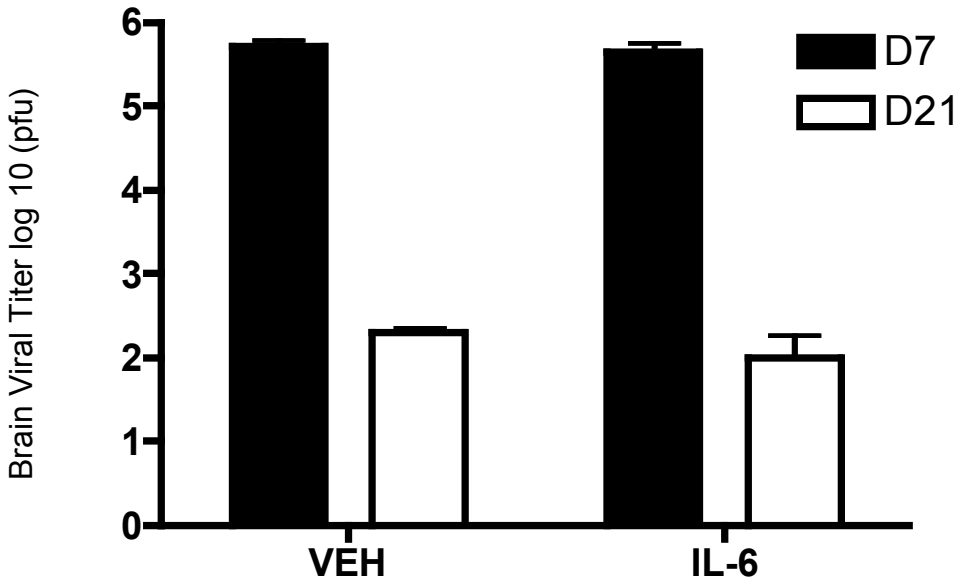
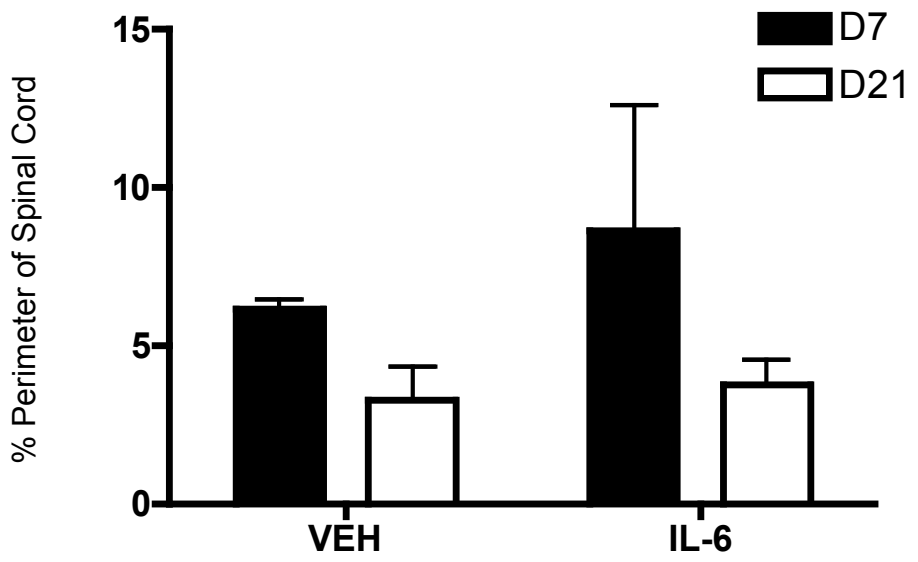


Fig 4.8. Viral Clearance: Experiment 2.
Viral clearance was determined by examining viral load at day 7 pi compared to day 21 pi. Viral titers over time in the spinal cord (A) and brain (B) were not altered by IL-6 treatment. Both groups had significantly reduced viral loads over time in both compartments.

A.



B.

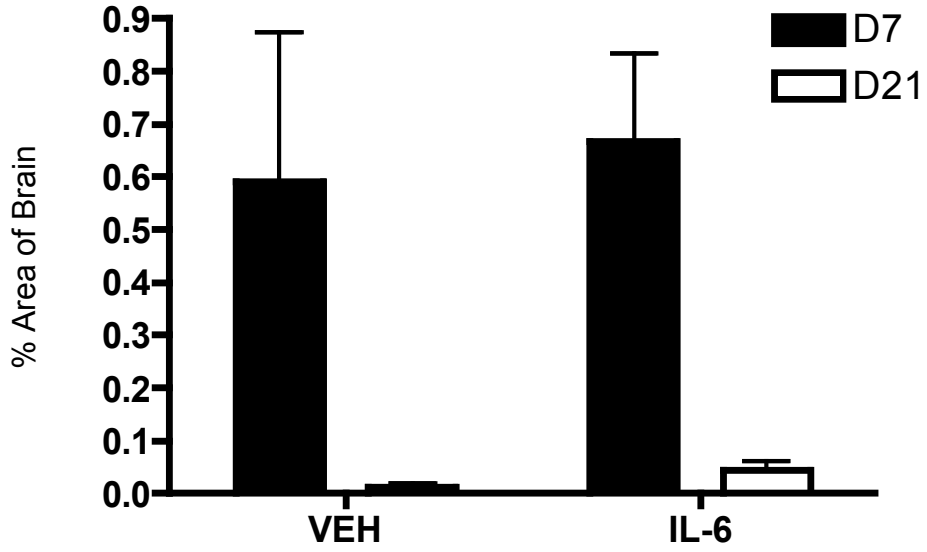


Fig 4.9. Inflammation: Experiment 2.
Meningitis was examined in the spinal cord (A), while perivascular cuffing and microgliosis were more common in the brain (B). Although a great deal of the inflammatory infiltrate was reduced over time, IL-6 treatment did not alter inflammation in either compartment at either day 7 or 21 pi.

Table 4.2. Summary of Results, Experiment 2.

	VEH	IL-6
Illness measures	**0**	↑
Body temperature	0	↑↑
Sucrose preference	0	0
Body weight	0	0
Horizontal activity	0	↑?
Mechanical sensitivity	0	↑
Motor impairment measures	**0**	**0**
Hind limb impairment score	0	0
Mechanical sensitivity	0	0
Horizontal activity	0	↑?
Vertical activity	0	0
Stride length	0	0
Physiological measures	**0**	**0**
Spleen weight	0	0
Thymus weight	0	0
Viral clearance: Brain	0	0
Viral clearance: Spinal cord	0	0
Inflammation: Brain	0	0
Inflammation: Spinal cord	0	0

Table 4.2. presents a summary of the results found across illness, motor impairment and physiological measures of TMEV infection. The control, vehicle treated groups was assigned 0, as an indicator of normal disease level. When the IL-6 treated group exhibited increased disease severity on a given measure, this was indicated by ↑ ($\underline{p} > .05$) or ↑↑ ($\underline{p} > .01$), if the increased severity was marginal, this was indicated by ↑? ($\underline{p} < .05$).

4.4. Discussion

In order to fully examine the role of IL-6 in the deleterious effect of SDR in acute TMEV infection, Experiment 2 aimed to elucidate the sufficiency of IL-6 in replicating those effects. IL-6 treatment was able to somewhat increase sickness behaviors. IL-6 decreased body temperature, and increased mechanical sensitivity to von Frey stimuli. However, IL-6 treatment did not increase body weight loss due to infection, nor did it significantly alter activity levels compared to vehicle treated controls. Previous work on cytokine sickness syndrome indicated that IL-6 can independently induce body temperature changes but is not known to alter the other behaviors in the absence of IL-1β or TNF-α (Bluthe et al., 2000; Lenczowski et al., 1999). Research examining allodynia (or increase sensitivity to non-noxious stimuli) also indicated that IL-6 often induces hypersensitivity to mechanical and cold stimuli (Anderson and Rao, 2001; DeLeo et al., 1996; Vissers et al., 2005). Taken as a whole, IL-6 alone induced a milder sickness syndrome compared to SDR, but significantly greater than vehicle treated animals overall. These data were consistent with previous work examining both IL-6 related cytokine sickness behavior and allodynia.

IL-6 treatment prior to infection resulted in mild post-infection hypothermia. This is consistent with many low-dose LPS and sepsis studies, that have also found hypothermia in mice (Leon, 2002). Interestingly, one report has shown that IL-6 knockout mice also develop hypothermia in response to low

dose LPS (Kozak et al., 1998). This conflict requires further investigation, however the nature of the infection (LPS vs. TMEV), or the route of infection (LPS is usually administered intraperitoneally, while TMEV is administered ic), or the cannulation surgery in the current study may be plausible factors. Any combination of these factors may also result in the conflict in IL-6/hypothermia data found here and/or those found by Kozak and colleagues (1998).

Cytokine mediated sickness behavior has been shown to be mediated by both IL-1β, and to a lesser degree, TNF-α (Espinosa and Bermudez-Rattoni, 2001). In contrast, IL-6 generally exacerbates sickness behaviors induced by other cytokines (Bluthe et al., 2000). IL-6 has only been administered to rats in previous systematic sickness behavior studies, and these studies found that IL-6 induced fever, but no other sickness behaviors independently (Lenczowski et al., 1999). Since LPS at low doses also induces fever in rats, but hypothermia in mice, these findings are consistent with the current findings (Leon, 2002). Although Lenczowski and colleagues (1999) did not find mechanical hypersensitivity or sucrose preference reduction, others have found that IL-6 elevations (in response to infectious or stress stimuli) results in hypersensitivity to various non-noxious stimuli in both rats and mice (Anderson and Rao, 2001; DeLeo et al., 1996; Vissers et al., 2005) and IL-6 elevations in mice that fail to develop sucrose preference (Ballok et al., 2003; Sakic et al., 1997). Taken together, these data indicate that sickness behaviors found in the current study

are consistent with those found in previous studies, although there is a paucity of data on the direct effects of IL-6 administration in mice.

In contrast to illness behaviors, IL-6 treatment was not sufficient to alter motor impairment development or the physiological development of acute TMEV infection compared to vehicle treated controls. Thus, IL-6 was not shown to be sufficient to mimic the negative effects that SDR has on acute TMEV infection. However, the dose used here was based on our endogenous data from Experiment 1, and thus exploratory. It is therefore possible that a higher dose would be more effective in mimicking SDR effects in TMEV infection.

The failure of IL-6 to mimic the effects of SDR is not surprising. The effects of SDR are most likely multi-factorial. While each factor may be necessary (such as IL-6), individually they are not sufficient. SDR is associated with GCR, and GCR may be mediated by norepinephrine signally to the bone marrow, triggering the trafficking of the GCR macrophages to the spleen (Engler, et al., 2004). Additionally, other cytokines may also be mediating the effects of SDR as well. Therefore, at least GCs, catecholamines, and cytokines are possible factors in the effects of SDR on acute TMEV infection. At this point, we know that IL-6 is necessary, but probably not sufficient. In addition, IL-6, as well as other stress-induced cytokines may also influence the development of GCR, and through GCR alter TMEV infection. Both the current work and previous studies have shown that removing IL-6 (by blocking IL-6 with neutralizing antibodies or IL-6 knockouts) does not alter GCR development (Stark, et al.,

2002). Although IL-6 may not be necessary, it may contribute to GCR or infection processes, if available within the organism. However, other factors (such as norepinephrine) may be more essential. Further investigation of the other suspect substances (i.e. other cytokines, catecholamines, corticosteroids) will continue to elucidate both the direct role of SDR on TMEV infection, as well as on GCR.

5. GENERAL DISCUSSION

The current study sought to delineate the role of IL-6 in the negative outcomes associated with SDR in acute TMEV infection. Past studies have demonstrated that exposure to SDR prior to infection led to elevated motor impairment, elevated inflammation, and altered viral clearance (Johnson et al., 2004, Johnson et al., in submission). In addition, GCR was present at the time of infection when SDR was administered prior to infection. GCR results in deregulation in the production of proinflammatory cytokines, allowing inflammatory stimuli to persist. The *a priori* hypothesis of this study was that inflammation, mediated through SDR induced GCR, may be responsible for the exacerbation of acute TMEV infection caused by SDR. The proinflammatory cytokine, IL-6, was known to be elevated due to SDR, and was a likely biochemical mediator. Fig 5.1. presents a theoretical model of possible pathways for SDR to alter TMEV infection.

In the current study, SDR induced GCR, and the SDR alone animals in Experiment 1 had elevated sickness behaviors. These animals developed increased mechanical sensitivity, anhedonia, and decreased motor behavior beyond that of the non-stressed animals. In previous studies, GCR developed only in animals exposed to SDR prior to infection. If SDR is administered coincident infection, GCR does not develop, and TMEV outcomes are less severe than in control (NON-SDR) animals (Johnson, et al., 2004). Therefore, the development of GCR may be an essential mechanism for SDR to

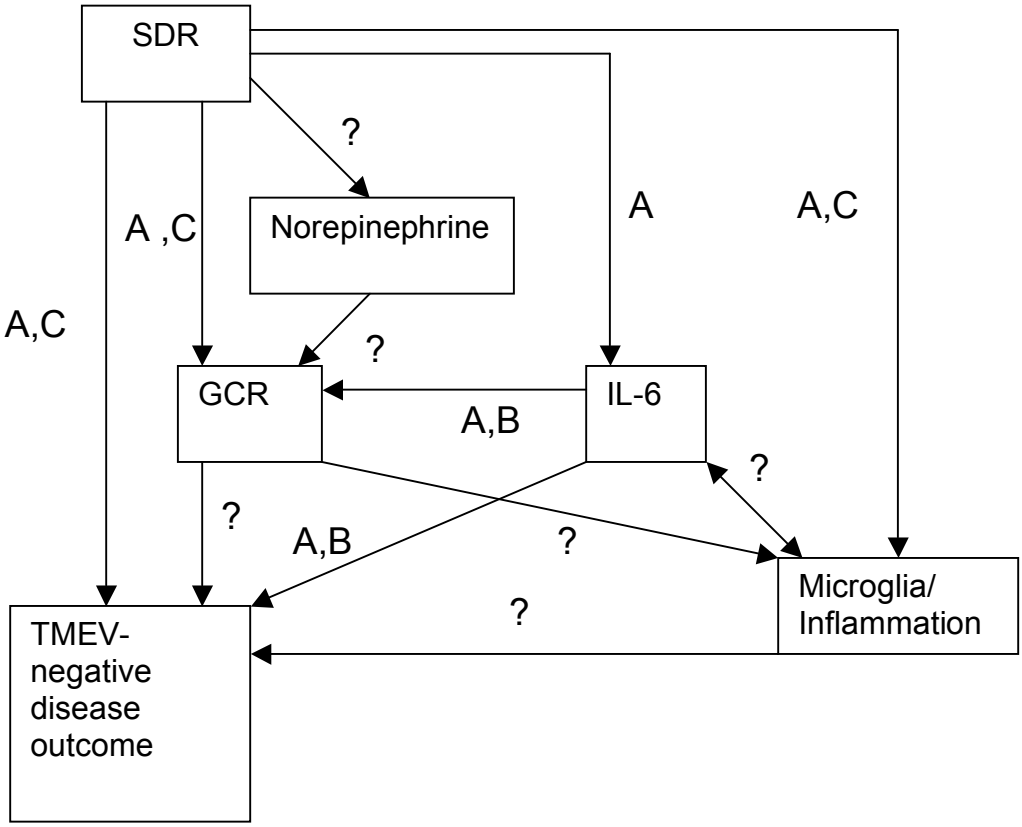


Figure 5.1. Theoretical Pathways for Effects of SDR on TMEV Infection. This diagram depicts the relationships of possible pathways through which SDR acts to exacerbate TMEV infection. "A" represents pathways that are known based on Experiment 1. "B" represents pathways that are known based on Experiment 2. "C" represents pathways that are known based on Johnson, et al., 2004. "?" indicate pathways that need elucidation.

exacerbate TMEV infection. The role of GCR in TMEV exacerbation would be highly clinically relevant for MS (and possibly other inflammatory neurodegenerative diseases). MS is associated with multiple pathologies, and is highly variable (Lublin and Reingold, 1996). The vast majority of MS patients are defined as relapsing-remitting (80%), and these patients are primarily inflammatory in pathology (Noseworthy et al., 2000; Sospedra and Martin, 2005). Many patients with relapsing-remitting MS are also hyper-responsive to stress, resulting in a higher than expected release of cortisol (Fassbender et al., 1998; Mohr and Pelletier, 2005). Interestingly, chronic stress is associated with GCR in MS (Mohr and Pelletier, 2005). Therefore, many MS patients have an inflammatory pathology combined with excessive cortisol release, and as stress often precedes MS exacerbations, they may also have GCR. The GCs are known to be anti-inflammatory, through down regulation of the release of proinflammatory cytokines (Adcock, et al., 2004). This leads to one theory of how stress might be associated with exacerbations of MS symptoms. Once the stress resolves, cortisol release may return to baseline or even decline to below normal levels until the body can recover homeostasis. During this refractory period, inflammation would be able to increase, increasing inflammatory mediated symptoms (Mohr and Pelletier, 2005). If these patients have developed GCR, then stress will result in excessive release of GCs, but they would be less effective in suppressing inflammation. Since the refractory period

would still occur, those cells that still responded to GC regulation would lose those immunosuppressive effects as well.

Furthermore, exogenous GCs are often used pharmacologically to help ameliorate inflammatory MS symptoms. For those patients that are resistant, GC treatment would be significantly less effective, and may even have negative effects. Excessive exposure to GCs can cause excitotoxic neuronal damage to corticosteroid sensitive cells in the hippocampus that are part of the negative feedback loop for HPA-axis regulation. Stress can also cause fat to relocate to the visceral fat pad, increasing risk for heart disease and diabetes (Barnes et al., 1998; Chrousos, 2000a; Chrousos, 2000b). Finally, excessive exposure to GCs is known to lead to metabolic syndrome, and increase risk for cardiovascular disease and obesity (Rosmond, 2005).

MS is also commonly associated with various mood disorders, including depression (Arnett, 2005; Brown et al., 2005; Dalton and Heinrichs, 2005; Ehde and Bombardier, 2005; Galeazzi et al., 2005; Hart et al., 2005; Heesen et al., 2005; Iwasaki et al., 2005; Rickards, 2005; Siegert and Abernethy, 2005). Depressive symptoms include weight loss, anhedonia (loss of interest in pleasurable activities), hyperalgesia and/ or allodynia, decreased activity, and alterations in sleep patterns and quality of sleep. Interestingly, cytokine-mediated sickness syndrome includes many of these symptoms as well (Charlton, 2000; Elenkov et al., 2005). Depression is also commonly associated with elevated pro-inflammatory cytokines (Heesen et al., 2005). However, one

of the most compelling theories of depression is that GCR is the underlying cause of depression, through dysregulation of the pro-inflammatory response (Caamano et al., 2001; Hill and Gorzalka, 2005; Wang et al., 2005).

Although IL-6 may contribute to the development of GCR, SDR induced GCR is also responsible for the overall increase in pro-inflammatory cytokine (IL-6) levels, through the deregulation of cytokine production from the macrophages. In animals with elevated IL-6 (SDR-VEH), TMEV infection resulted in elevated sickness behaviors, including body weight loss, anhedonia, allodynia, and reduced activity. By blocking the activity of IL-6, these effects were also blocked. Those animals with elevated sickness behaviors also had exacerbated motoric impairment, impaired viral clearance and excessive inflammation. Therefore, SDR induced exacerbations of TMEV infection parallels the effects of stress in MS in several ways, including the development of depression and motor impairment. Although the acute phase of TMEV is not generally considered the important period in relation to MS, it is CNS inflammatory, providing a model for the inflammatory periods in MS and other CNS inflammatory diseases.

In the future, the role of GCR in the development of both depressive symptoms and motor impairment in TMEV infection should be examined, separate from stress. By separating stress and GCR, we can increase understanding of whether stress directly alters TMEV infection development, primarily acts through GCR, or some other pathway. Currently, in animal

models of stress, only social stressors effectively induce GCR, while other types (i.e. restraint) fail to do so (Avitsur et al., 2003a; Avitsur et al., 2003b; Avitsur et al., 2002; Bailey et al., 2004). Using other social stress models, or other immune activation models would not allow the investigation into the isolated effects of GCR in this model. One other option is available, casein could be used to examine the effects of GCR in the absence of stress. The milk protein, casein, induces GCR as well (Carbone et al., 1990; Chen et al., 1998; Dmitrieva et al., 1990; Kay et al., 1981; Kehoe and Shoemaker, 1991; Rosene et al., 2004; Thibault and Roberge, 1988; Thurmond and Brown, 1984). Thus, casein administration could be used to examine the effects of GCR alone, in the absence of social stress, on acute TMEV infection, as well as depression-like sickness behaviors.

While the broader systemic role of GCR is interesting, it would also be important for future studies to examine GCR in the CNS inflammatory responses detected by the current study. In the current study, SDR alone was associated with significantly greater levels of inflammation, primarily in the hippocampal and penduncular regions of the brain. In contrast, IL-6 treatment was not associated with elevation in inflammation, but blocking IL-6 with neutralizing antibodies blocked SDR-related inflammation. Therefore, once again, GCR may be important.

SDR-induced GCR is known to occur in the macrophages in the spleen (Avitsur et al., 2002). Development of GCR directly in the CNS would have

important implications. Inflammation in the CNS is primarily due to the presence and action of microglia and macrophages. In addition, functional GCs are essential in protecting CNS tissue during inflammatory responses (Nadeau and Rivest, 2003). Thus, developing an assay that would assess GCR in brain and/or spinal tissue and further assessment of what cell types are responsible for the GCR would be informative.

In the current study, GCR was associated with IL-6 elevation. However, as in previous studies, IL-6 was not necessary nor sufficient for the development of GCR (Stark et al., 2002). Thus, it seems plausible that the GCR may contribute to the deregulation macrophage production, which in turn may lead to the elevation of IL-6. However, it is also possible that IL-6 may influence GCR through other co-factors. For example, it has been established that elevated proinflammatory cytokines are correlated with increased expression of the beta subtype of the GC receptor. Glucocorticoid resistance is also associated with elevated levels of the beta subtype of the GC receptor (Necela and Cidlowski, 2004). Therefore, it may be possible for elevated IL-6 to increase expression of the beta subtype GCR receptor, and this may lead to the development of GCR. IL-6 plays an important role in both SDR and acute TMEV infection. In TMEV infection, IL-6 is essential for microglia to become efficient antigen presenting cells (Mack et al., 2003). This can have both positive and negative outcomes. First, antigen presentation is an essential step in the development of the humoral and cellular adaptive immune responses. If the antigen is not properly

presented, then the appropriate cells will not traffic to the area of insult and neutralize the pathogen, or lyse the infected cell. However, TMEV infection results in an autoimmune mediated demyelinating process. One important vector for the development of the autoimmune response is through molecular mimicry. This occurs when T-cells and B-cells first develop responses to the virus. Then, if the viral molecular structure is similar to that of myelin, then the responses that develop will also attack and kill myelin (Fujinami and Zurbriggen, 1989). The portion of the virus that is presented by the antigen-presenting cell is an important factor in this development of molecular mimicry. Without antigen presentation, the virus would not be able to cause the development of auto-reactive T-cells and B-cells. Without antigen presentation the virus may take over the organism and directly kill the host, but antigen presentation may also be the cause of later autoimmune reactivity.

The current data indicated that IL-6 was not sufficient to induce the negative effects of SDR. However, in order to fully examine this issue, additional experiments should be considered. As mentioned previously, a higher dose may be needed. IL-6 may have an essential role that was not accessed by the current study. We have a sub-threshold dose of IL-6 used here. This dose should be combined with a sub-threshold dose of SDR (i.e. fails to develop GCR, and/or fails to alter acute TMEV infection). This experiment would allow a more nuanced investigation of the role of IL-6. Perhaps it is the IL-6 released due to the higher levels of SDR that results in the negative effects in acute

TMEV infection. Thus, the effects of SDR and IL-6 may be additive on TMEV infection.

Although the current data and other sources demonstrate that excessive IL-6 is associated with the negative outcomes in TMEV infection, IL-6 can also have protective effects. In one recent study, IL-6-/- mice with the appropriate MHC haplotype (so the mice were susceptible to TMEV infection), developed greater mortality and spinal anterior horn demyelination compared to IL-6+/+ mice (Pavelko et al., 2003). Thus, a complete lack of IL-6 is also damaging to neurological tissue. An additional study has also found central administration of human recombinant IL-6 can be protective in TMEV infection (Rodriguez et al., 1994).

The current data indicates that fewer microglia are activated when IL-6 is blocked. Therefore, microglia activation may be important in SDR-TMEV infection interactions. While IL-6 may be important in activating and aiding in proliferation of microglia and/or trafficking of macrophages, the microglia may have independent effects on TMEV infection. In addition, future studies should block microglia activation during SDR. The drug, minocycline inhibits microglia activation and proliferation (Ledeboer et al., 2005; Tikka et al., 2001), and could be used to examine the separate effects of SDR, GCR and microglia on TMEV infection. Minocycline could be administered during SDR to block SDR induced microglia activity (and probably IL-6, although IL-6 may be sourced from other neuronal or astrocytic tissue). Minocycline could also be administered during

infection to examine the specific role of microglia in TMEV separately from SDR. Administration of minocycline could also be used in conjunction with casein administration to examine GCR related microglia activation in the absence of SDR, examining both GCR and microglia activity in acute TMEV infection. TMEV up regulates many of the pro-inflammatory cytokines as early as 1 h post infection in cell culture including IL-1, IL-6, and TNF-α (Olson et al., 2001; Palma et al., 2003). Microglia cultures became efficient antigen-presenting cells of myelin proteins following infection with TMEV, indicating that microglia may be important in developing the autoimmune regulated demyelination in the chronic phase of TMEV infection (Olson et al., 2001). In vitro, these cytokines are also known to be elevated in sera (Chang et al., 2000). Thus, blocking microglia would block many of the probable mechanisms that exacerbate the infection process.

SDR-induced GCR has one additional mechanism that also needs to be elucidated, separate from the effects on acute TMEV infection. GCR occurs in splenic macrophages, not due to a morphological change, but though release of more mature macrophages from the bone marrow that then traffic to the spleen (Engler et al., 2004). GCs are unlikely to have this action, in part because GC receptors are not common in bone marrow cells. In addition, previous studies from our laboratory used exogenous corticosteroids, and mimicked restraint stress, and restraint stress does not induce GCR (Avitsur et al., 2001; Satterlee et al., 2001). Norepinephrine is a more likely candidate, as there is direct

sympathetic innervation of the bone marrow (Felten et al., 1998). Norepinephrine (NE) blockade through drugs such as nadalol would be a good first step to examine the necessity of NE. Another option would be to sympathectomize the mice with 6-ODHA. This procedure has many other negative side effects, including that the mice become very delicate, making subsequent exposure to SDR difficult. Because these mice are usually sickly, the dominant is unlikely to attack them, and if the dominant does attack, the mice are more likely to die. With these limitations, osmotic pumps or pellet implants of nadalol or other similar drugs would be the most likely to result in interpretable data.

The role of stress is also important to consider, separate from the physiological pathways discussed. For instance, in studies from other laboratories, wounding is required in SDR for the development of GCR (Avitsur et al., 2005; Avitsur et al., 2003a; Avitsur et al., 2003b; Avitsur et al., 2002; Avitsur et al., 2001; Stark et al., 2002; Stark et al., 2001). The studies from other laboratories all use the mouse strain, C57BL/6J, whereas the current study used Balb/cJ. C57BL/6J mice are not susceptible to TMEV infection. Having the genetic haplotype(s) for susceptibility is required for the development of both TMEV infection in mice (Oleszak et al., 2004), and MS in humans (Sospedra and Martin, 2005). Thus, in order to examine the role of SDR in TMEV infection, only susceptible strains of mice, such as the Balb/cJ strain, can be used. The Balb/cJ mice developed

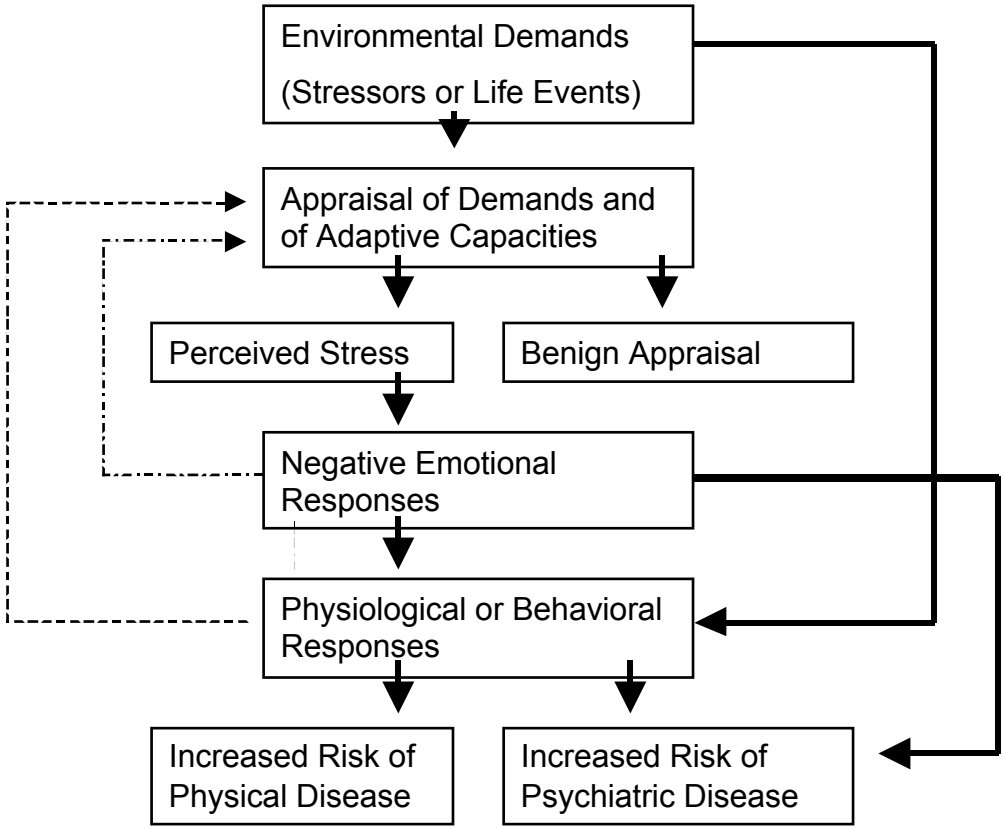


Fig 5.2. Diagram of the Integrated Model of Stress. Conceptualization of stress across environmental, psychological, and biological perspectives (Cohen et al., 1995).

GCR in the absence of wounding. Therefore, the Balb/cJ mice differ from the C57BL/6J mice in ways other than having TMEV susceptibility. One study has shown that Balb/cJ mice are more stress hyper-responsive than the C57BL/6J mice (Shanks et al., 1990). In contrast, the C57BL/6J mice may require more antagonistic interactions, resulting in wounding in order for GCR to develop compared to the Balb/cJ strain.

C57BL/6J mice may not assess threat unless wounding occurs. In contrast, Balb/cJ mice may perceive the dominant's presence, and/or attacks as being more threatening than the C57BL/6J mice (without wounding). This explanation fits nicely with Cohen's model of stress response (Fig 5.2.). Cohen's model includes an important role for threat assessment. Once a potential environmental demand occurs, the organism then appraises the demand as well as the organism's coping skill, resulting in either a benign or threatening assessment (Cohen et al., 1995). Some evidence to support this theory has previously been documented, showing that under similar basal or stress conditions, Balb/cJ mice have greater corticosteroid responses compared to C57BL/6J mice (Shanks et al., 1990; Tang et al., 2002).

The stress-responsivity may effect both the development of GCR, as well as have direct effects on disease development. One possible way to examine this issue physiologically would be to administer anti-anxiolytics, in order to reduce the Balb/cJ stress response. By altering the stress response, this treatment may alter either the development of GCR, the release of IL-6, or more

directly the development of TMEV infection. In humans (e.g. in MS patients) both psychotropic drugs, such as anti-anxiolytics, could be used, but also less invasive options such as cognitive-behavioral stress management.

Cognitive behavioral stress management has been shown to help maintain T-cell counts and improve quality of life in AIDS patients (Antoni et al., 1991). MS is also mediated through auto-reactive CD4+ T-cells (Sospedra and Martin, 2005). While increasing CD4+ cells or function in MS would be counter-productive, cognitive behavioral therapy may still be helpful to improve quality of life. As previously noted, depressive symptoms common to MS are likely to improve with such interventions.

The current study demonstrated the necessity, but not sufficiency, of IL-6 in mediating the effects of SDR in exacerbating acute TMEV infection. These findings implicate several other important physiological pathways in the development of TMEV infection that are also relevant to MS. This study also further demonstrates the importance of the TMEV model in investigating the effects of social stress in MS. By pursuing the pathways implicated in the current study, a great deal of understanding of stress and MS would result. Finally, further elucidation of SDR on acute TMEV may also aid in understanding other CNS inflammatory neurodegenerative disorders (Alzheimer's disease, Parkinson's disease, AIDS) as well as depression. Thus, SDR-TMEV infection interaction studies will continue to benefit a broad range of public health issues.

6. CONCLUSION

The current study examined the role of IL-6 in the negative effects of SDR on acute TMEV infection. Past work had shown that SDR applied prior to infection exacerbated disease course in both the acute and chronic phase of TMEV infection. The working hypothesis was that IL-6 would be necessary, but not sufficient, in mediating the effects of SDR on acute TMEV infection. In order to examine the development of the disease, a battery of illness, motor impairment, and physiological measures were collected.

The objective of Experiment 1 was to examine the necessity of IL-6 in the effects of SDR in the acute phase of TMEV infection. IL-6 was blocked with a neutralizing polyclonal antibody against murine IL-6. When IL-6 was blocked, the exacerbating effects of SDR were reversed in the illness, motor impairment and physiological measures. These data indicated that IL-6 was in fact necessary, confirming our original hypothesis.

The objective of Experiment 2 was to examine the sufficiency of IL-6 in mimicking the effects of SDR in acute TMEV infection. SDR was replaced with a dose of recombinant murine IL-6 that was similar to that found in SDR animals in the current study, as well as others (Stark et al., 2002). IL-6 alone induced hypothermia and increased mechanical sensitivity in the first 24-h of infection, indicating that this dose of IL-6 was sufficient to induce some illness effects, somewhat similar to SDR. However, IL-6 was not sufficient to induce the remainder of illness effects. In addition, this dose of IL-6 was not sufficient to

replicate SDR effects on motor impairment or physiological measures. These data also confirm the original hypothesis that IL-6 alone is not sufficient to mimic the effects of SDR. However, this conclusion should not be considered concrete, as a higher dose of IL-6 may be more effective.

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