THE IMPACT OF SOCIAL STRESS ON CENTRAL NERVOUS SYSTEM INFLAMMATION AND T CELL RESPONSE TO THEILER'S VIRUS INFECTION

A Dissertation

by

ELISABETH GOOD VICHAYA

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

May 2011

Major Subject: Psychology

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ABSTRACT

The Impact of Social Stress on Central Nervous System Inflammation and T Cell Response to Theiler's Virus Infection. (May 2011)

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A growing body of evidence suggests that social stress contributes to the pathogenesis of neurodegenerative diseases, such as multiple sclerosis (MS). For example, prior research has shown that social disruption (SDR) stress behaviorally and immunologically exacerbates Theiler's murine encephalomyelitis virus (TMEV) infection. TMEV infection results in acute infection of the central nervous system (CNS) followed by a chronic demyelinating autoimmune disease, similar to that seen in MS. Research suggests that social stress exerts these effects by altering the immune response to infection. More specifically, it is hypothesized that SDR sensitizes the acute inflammatory response to infection and suppresses T cell effector function in the acute phase of disease. It was demonstrated that SDR is sufficient to alter inflammation. Exposure to a single session of SDR increases IL-1β mRNA expression; however, IL-6 mRNA expression, but not IL-1β, is up regulated in response to chronic SDR. Furthermore, chronic SDR prior to infection resulted in increased infection related central IL-6 and IL-1β mRNA expression, and central

administration of IL-6 neutralizing antibody during SDR reverses this increase in neuroinflammation. This suggests that SDR sensitizes infection related CNS inflammation through an up-regulation of IL-6. Chronic SDR prior to infection also resulted in enhanced CNS viral titers and suppression of virus-induced CD4+ and CD8+ T cell IFN-γ release within the CNS. As a whole, this research indicates that SDR exacerbates the disease course of TMEV infection by altering the central innate and adaptive immune response to infection. This research enhances our understanding of the mechanisms by which social stress exacerbates neurodegenerative disease pathogenesis.

DEDICATION

This dissertation is dedicated to my family,
whose enduring love and support
made this possible.

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something I chose for myself, they have been required to endure my absence and distraction for years. Without their understanding, reassurance, and encouragement I would never have been able to complete this journey.

NOMENCLATURE

ACTH	Adrenocorticotropic Hormone
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
BBB	Blood Brain Barrier
BHK	Baby Hamster Kidney
BMEM	Basal Medium Eagle's Medium
cDNA	Complementary Deoxyribonucleic Acid
CRH	Corticotropin Releasing Hormone
CNS	Central Nervous System
CNS-IL	Central Nervous System Infiltrating Lymphocyte
CPE	Cytopathic Effect
CSF	Cerebral Spinal Fluid
DMEM	Dulbecco's Modified Eagle's Medium
DTH	Delayed Type Hypersensitivity
EAE	Experimental Autoimmune Encephalomyelitis
EDDS	Expanded Disability Status Scale
FBS	Fetal Bovine Serum
GC	Glucocorticoid
GCR	Glucocorticoid Resistance
HLA	Human Leukocyte Antigen

HLI	Hindlimb Impairment
HPA	Hypothalamic-Pituitary-Adrenal
HRP	Horseradish Peroxidase
HSV	Herpes Simplex Virus
icv	Intracereboventricularly
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Media
iNOS	Inducible Nitric Oxide Synthase
LPS	Lipopolysaccharide
LSD	Least Squares Difference
KO	Knock Out
MANOVA	Multivariate Analysis of Variance
MBP	Myelin Basic Protein
MHC	Major Histocompatability Complex
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
nAbTx	Neutralizing Antibody Treatment
NK	Natural Killer
PBS	Phosphate Buffered Saline
pfu	Plaque Forming Units

SDR	Social Disruption Stress
SNS	Sympathetic Nervous System
RNA	Ribonucleic Acid
RO	Reverse Osmosis
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
TCID	Tissue Culture Infective Dose
TMEV	Theiler's Murine Encephalomyelitis Virus
Tregs	Regulatory Helper T Cells
TVID	Theiler's Virus Induced Demyelination

TABLE OF CONTENTS

CHAPTER Page

Page

LIST OF FIGURES

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Multiple Sclerosis

Prevalence and Natural History of Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic autoimmune demyelinating disease of the central nervous system (CNS) that affects approximately 2 million people worldwide (Anderson et al., 1992; Hirtz et al., 2007; Noonan et al., 2002; Sospedra and Martin, 2005; WHO and MSIF, 2008). It is characterized by intensive immune cell activation and inflammation within the white matter, resulting in degeneration of the myelin sheath and subsequent sensory (e.g., loss of sensation in limbs and enhanced pain) and motor deficits (e.g., loss of motor control and loss of bladder control). An individual is generally diagnosed in early adulthood and there is a 2:1 preponderance for females.

There is significant variability in disease prognosis after diagnosis. Four categories of disease have been described: (1) relapse-remitting, (2) primary-progressive, (3) secondary-progressive, and (4) progressive-relapsing. Relapse-remitting disease course is most common, with approximately 80-85% of patients presenting with this form. It is characterized by symptom attacks that last days to weeks followed by a gradual, partial to full recovery. This disease course generally

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This dissertation follows the style of Journal of Neuroimmunology.

presents with sensory disturbances, unilateral neuritis, diplopia (double vision, generally due to impairment of eye adduction), Lhermitte's sign (pricking, tingling, and numbness of trunk and limbs evoked by neck flexion), limb weakness, clumsiness, gait ataxia, and neurogenic bladder and bowel symptoms (Noseworthy et al., 2000). Relapses are associated with lesions of the CNS white matter (Neema et al., 2007) and inflammatory events, including microgliosis, activation of macrophages, and the presence of CD4+ and CD8+ T cells (Frohman et al., 2006).

Primary-progressive disease affects 10-15% of MS patients and is characterized by gradual, steady progression of disability without significant periods of unchanged or remission of disease course. This disease course is more likely to be observed in individuals who have a later disease onset and, unlike relapse-remitting disease, is equally common in men and women. The primary early symptom observed is chronic progressive myelopathy, a slow upper-motor-neuron syndrome that leads to increasing stiffness and weakness of the legs (Noseworthy et al., 2000).

The final two disease courses, secondary-progressive and progressive-relapsing, are a merging of these two more common types. Secondary-progressive disease begins as relapse-remitting and eventually evolves into progressive disease. To date, the mechanism underlying this switch is unknown. However, multiple lesions at disease onset are predictive of a more rapid switch (Kantarci and Weinshenker, 2005). Progressive-relapsing is characterized by a steady

progression, like primary progressive, with the acute attacks observed in relapse-remitting disease.

Given the high heterogeneity and unpredictability of disease course, these categories are of limited help in developing a prognosis for an individual. However, generally primary progressive disease course is predictive of poor outcomes, as are an age of onset greater than 40 years, multiple sites of initial involvement, cerebral symptoms, prolonged initial symptoms (> 1 year), and less than six months before first relapse (Weinshenker and Sibley, 1992). Furthermore, for reasons not yet fully understood, males have a more severe disease course than females (Kantarci and Weinshenker, 2005). Factors predictive of a more favorable outcome are isolated optic neuritis and brainstem syndromes (Weinshenker and Sibley, 1992).

Studies provide a wide range of survival statistics; conservative estimates suggest a 50% survival rate at 25 years post-diagnosis, (Phadke, 1987; Riise et al., 1988) while the most optimistic study suggests this rate may be as high as 76.2% (Wynn et al., 1990). Weinshenker et al. (1989) report an average increase of 0.5 +/- 0.02 points per year within the first five years on the Expanded Disability Status Scale (EDSS). The EDSS is a widely utilized ordinal clinical rating scale to assess MS from 0 (normal neurologic examination) to 10 (death due to MS) in half-point increments (Kurtzke, 1983). As the disease progresses, symptom burden increases to include cognitive deficits, sexual dysfunction, paralysis, gait ataxia, incontinence, enhanced pain, depression, and fatigue (Noseworthy et al., 2000).

Diagnosis and Biological Markers

The complexity and variability of MS continues to challenge investigators who seek to explicate the pathogenesis of the disease and prevent its progression. Despite the long history and high incidence of the disease, MS can be difficult to diagnosis. This is primarily due to the lack of symptoms and biological markers that are specific to MS. For example, optic neuritis is a common initial symptom of MS, however, only 38% of patients who present with optic neuritis will go on to be diagnosed with MS (Kantarci and Weinshenker, 2005). Another diagnostic measure is the presence of oligoclonal bands within the cerebral spinal fluid (CSF). As many as 90% of patients with MS have these bands; however, they are also found in a variety of other disorders of the CNS, including, Lyme disease, systemic lupus erythematosus, Syphilis, primary CNS lymphoma, Sjögren's Syndrome, and Guillain-Barre Syndrome.

Given the failure to identify exclusive markers, the diagnosis of MS requires significant time. The protracted nature of this process becomes clear when the marked time between relapses in early disease is considered. A swift diagnostic procedure is important in that it allows for a more rapid start of potentially beneficial treatments. Treatments include agents aimed at symptom management as well as agents intended to delay disease progression. For example, some patients are prescribed drugs that are intended to manage particular symptoms such as spasticity, fatigue, and depression. For the management of acute attacks, glucocorticoids, such as methylprednisolone, prednisone, and dexamethasone, are

commonly used. These agents act by inhibiting CNS inflammatory events that are thought to underlie these attacks. Although they are often effective at resolving the symptom exacerbation induced by the attack, there is no evidence that such treatment results in a delay of disease progression. Treatments aimed at attenuating disease progression include immunosuppressive drugs, such as interferon (IFN) therapy.

The Immunology of Multiple Sclerosis

In 1933 it was first suggested that MS was an autoimmune disease. Autoimmunity develops when the immune system begins to function in an aberrant fashion and attacks the body it is intended to protect. This can occur when the immune system fails to recognize a part of the individual as "self" and attacks as if it were foreign. In multiple sclerosis the immune system attacks the myelin sheaths around nerve axons. Rivers (1933) discovered that injections of spinal cord or brain homogenates into primates resulted in a disease course that resembled MS. He suggested this injection resulted in the development of anti-brain antibodies and, subsequently, the destruction of nervous system tissue. Decades later it was established that protein components of the myelin sheath, such as myelin basic protein (MBP), were the essential component to the induction of this MS-like disease, this disease model is now known as experimental autoimmune encephalomyelitis (EAE) (Einstein et al., 1962; Fritz et al., 1983; Kibler et al., 1977; Laatsch et al., 1962).

It has also been demonstrated that EAE can be induced by transferring myelin-specific CD4+ T cells into a host, however the transfer of myelin-specific antibodies is insufficient (Pettinelli and McFarlin, 1981). This provided evidence that MS is likely a T cell-mediated autoimmune disease. T cells are an important component of the adaptive immune response. There are two major classes of T cells: helper T cells and cytotoxic T cells. Helper T cells become activated when they are presented with antigens by MHC class II molecules on the surface of antigen presenting cells (APCs). These T cells divide and differentiate, based upon the extracellular milieu, and secrete cytokines to recruit and assist with the immune response. They are referred to as CD4+ T cells because they express CD4 protein on their surface. Cytotoxic T cells, also known as CD8+ T cells, attack virus-infected cells. They recognize their target through binding to antigens when presented by a cell on their MHC class I molecules.

Further evidence for the role of T cells in the development of MS comes from the prominence of CD8+ T cells in the CNS of MS patients (Jacobsen et al., 2002; Skulina et al., 2004; Zang et al., 2004). Additionally, it has been demonstrated that T cells from MS patients exhibit heightened activity against MBP (Cabarrocas et al., 2003) and secrete increased levels of chemoattractants for CD4+ myelin specific T cells (Biddison et al., 1998). However, T cells specific to MBP can be found both in MS patients and healthy controls, which suggests that this relationship is complex (Burns et al., 1983).

B cells may also be involved in the pathogenesis of MS. The primary functions of B cells are to produce antibodies (also known as immunoglobulin, [Ig]), present antigen, and become memory B cells. Although antibodies alone are not sufficient to induce MS-like symptoms, they may be involved in the progression of the disease. For example, it has been determined that the CSF from MS patients has increased levels of antibodies and that, in some patients, these increased levels are related to symptom exacerbations (Corcione et al., 2005). Although, these cells do not typically cross the blood-brain barrier, inflammatory process can permit their entry into the CNS. These cells may contribute to the disease process by serving as APCs for autoreactive T cells, recruiting autoreactive T cells to the CNS, producing myelin-specific antibodies, and destroying myelin within plaques (Sospedra and Martin, 2005).

The innate immune system also appears to be important within the immunology of multiple sclerosis. The innate immune response is the body's first line of defense; it responds quickly to contain infection until the adaptive immune system has been recruited. Unlike the adaptive immune response of T cells and B cells, the innate immune system responds nonspecifically to antigens through the development of barriers, mechanical removal, activation of the complement pathway, phagocytosis, and inflammation. Inflammation is a complex cascade of events intended to recruit immune cells to kill potential pathogens and promote tissue repair. The powerful nature of this immune response is essential to survival. However, if the inflammatory response is not properly restrained, damage to

healthy tissue can result. For example, chronic inflammation has been shown to be involved in a variety of diseases processes including MS, Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, atherosclerosis, diabetes, and cancer (Bitsch et al., 2000; Coussens and Werb, 2002; Duncan et al., 2003; Foell and Roth, 2004; Hansson et al., 2006; Liu and Hong, 2003; Minghetti, 2005).

Cytokines are an especially important component of the inflammatory response. The immune system tightly regulates the immune response through the release of proinflammatory (e.g., IL-1β, TNF-α, IFN-γ) and anti-inflammatory (e.g., IL-10, IL-4) cytokines. Other cytokines, such as IL-6, can serve both pro- and anti-inflammatory functions. Proinflammatory cytokines allow leukocytes to exit the blood stream and enter into infected tissue, trigger neutrophils to kill infected cells, and stimulate natural killer (NK) cells and T cells. Cytokines also orchestrate a behavioral response designed to promote healing (Kent et al., 1992; Laye et al., 2000). For example, cytokines induce a febrile response that results in a temperature less suitable for pathogen replication (Hart, 1988; Murray and Murray, 1979). Furthermore, fatigue-like symptoms, such as reduced motivation, increased need to sleep, and social withdrawal, are effective means to conserve energy needed to mount an immune response (Maier and Watkins, 1998).

As previously mentioned, MS is not merely an autoimmune disease but also an inflammatory disease of the central nervous system. Increased levels of inflammation appear to accompany disease relapses (Frohman et al., 2006) and agents that decrease rate of relapse also decrease CNS inflammation (Stüve and

Oksenberg, 2010; Warnke et al., 2010). Furthermore, there is evidence that enhancing inflammation, by infection (Buljevac et al., 2002; Panitch, 1994; Sibley et al., 1985) or administration of a proinflammatory cytokine (Panitch et al., 1987)., exacerbates disease. However, this relationship is clearly complex and there is data to suggest that a compromised inflammatory response results in exacerbated disease (Martino et al., 2002; Willenborg et al., 1996). Overall, the data suggests that (1) there is an optimal level of inflammation and deviation in either direction is detrimental and/or (2) different levels of inflammation are needed at different points in the disease progression.

Cytokines are thought to play an important role in the disease process both through the activation of immune cells and their direct damage to myelin. Several different ideas have been proposed concerning the mechanisms through which pro-inflammatory cytokines enhance demyelination. For example, high levels of cytokines may be directly toxic to oligodendrocytes, the cells that produce the myelin sheath. It is also possible that the cytokines may cause macrophages and microglia to phagocytose myelin (Sospedra and Martin, 2005). Macrophages are phagocytic cells that act to engulf and digest cellular debris and pathogens. Despite the protection conferred by the blood brain barrier (BBB), they appear to be able to enter the CNS during times of inflammation. Microglia, enter the brain during fetal development, and serve as resident immune cells within the CNS. Once in the CNS, these two cell types are virtually indistinguishable. Not only are these cells recruited by cytokines, but are also the primary producers of CNS cytokines.

Genetic Risk Factors for Multiple Sclerosis

The etiology of the disease is clearly complex involving both the innate and adaptive immune systems. A single element alone appears insufficient but when genetic and environmental risk factors combine the disease appears to develop. Evidence for a genetic contribution is provided by twin studies that reveal a 20-30% concordance rate among monozygotic twins and only a 3-5% concordance rate in dizygotic twins (Ebers et al., 1986; Hansen et al., 2005; Willer et al., 2003). However, this genetic relationship does not appear to be attributable to a single gene, rather it is thought to result from a combination of many genes. This concept is highlighted by a study showing that at least one locus of all chromosomes except one have been linked to MS (Dyment et al., 2004). One locus that has recently received attention is the human leukocyte antigen (HLA) located on chromosome 6. HLA is associated with the major histocompatability complex (MHC), which is involved in antigen presentation. Having particular alleles at this locus (e.g., DR15 and DQ6) are associated with increased risk of developing MS, while others (e.g., C554 and DRB1*11) are associated with decreased risk of developing MS (Haines et al., 1998; Hauser, 2006). Estimates suggest that the HLA region accounts for 20 to 60% of genetic susceptibility (Haines et al., 1998).

Apart from the HLA region, the non-synonymous coding SNP (T2441) in the IL-7 receptor α chain gene on chromosome 5 represents the most consistently replicated susceptibility gene for MS (Akkad et al., 2009; Gregory et al., 2007; Hafler et al., 2007; Lundmark et al., 2007; O'Doherty et al., 2008; Weber et al., 2008). IL-7

and its receptor are involved in T-cell genesis, survival, expansion, and memory T-cell development. Patients carrying the risk allele are at increased risk of producing high levels of the soluble form of the receptor (Gregory et al., 2007). Another gene showing strong association with MS are two SNPs within the intron 2 of the IL-2 receptor α chain on chromosome 10 (Hafler et al., 2007; Weber et al., 2008; Akkad et al., 2009). These studies have been more complicated to replicate and, consequently, require further evaluation to elucidate.

Environmental Risk Factors for Multiple Sclerosis: Viral Infection and Stress

Given that only 20-30% of disease susceptibility can be explained through genetic variations, much of disease vulnerability must relate to environmental factors. Both viral infection and psychological stress have been linked to the development of MS. Even before the autoimmune and inflammatory nature of MS had been described, researchers and physicians speculated about the role of viruses. This association was primarily considered because the majority of demyelinating diseases observed in humans and animals are of viral origin (Fazakerley and Walker, 2003). For example, demyelinating disease is induced by canine distemper virus in dogs, Maedi-visna in sheep, Theiler's virus in mice, and JC virus in humans (Dal Canto and Rabinowitz, 1982; Fazakerley and Walker, 2003). However, this association alone is insufficient to assume causation.

Epidemiological studies add further support for the role of viruses in multiple sclerosis. Research suggests that viral infection, such as Epstein Barr,

during adolescence is associated with the development of multiple sclerosis (Ascherio and Munger, 2007; Hernan et al., 2001; Jilek et al., 2008; Kurtzke et al., 1995). Furthermore, exacerbations in disease course are often preceded by a viral infection (Buljevac et al., 2002; Panitch, 1994; Perry et al., 2003; Sibley et al., 1985). MS does not, however, appear to be associated with any single infection, rather the data suggest that a variety of viruses may be able to increase susceptibility to and exacerbate multiple sclerosis. Viruses that induce persistent infection appear to be the best candidates. A variety of such viruses have been isolated from the post-mortem brains of MS patients, including measles, mumps, parainfluenza type I, and human herpes virus simplex (Allen and Brankin, 1993). However, these viruses can also be detected in the brains of individuals who do not have MS and the presence of particular viruses associated with MS, such as Epstein Barr, often fail to be detected in the brains of MS patients.

Psychological stress is another environmental factor that has been repeatedly associated with development and exacerbation of multiple sclerosis (Ackerman et al., 2002; Brown et al., 2005; Brown et al., 2006; Grant et al., 1989; Li et al., 2004; Mohr et al., 2004). This relationship is not a new consideration; over 130 years ago Charcot (1877) reported that stressful experiences precipitate the onset of MS. This relationship has been experimentally confirmed; for example a study of bereaved parents demonstrated that the loss of a child results in increased risk for developing MS and those who unexpectedly lost a child were at even higher risk (Li et al., 2004). Not only is stress related to disease onset, but also disease

exacerbation. A meta-analysis of 14 studies evaluating stress and MS, conclude that stressful life events significantly increased the risk of disease exacerbation (Mohr, 2007).

However, the effect of a stressor on disease may depend on the type of stress. While mild to moderate chronic stressors have repeatedly been demonstrated to exacerbate disease (Mohr, 2007), the threat of missile attack during the Persian Gulf War, a severe life-threatening stressor, was found to reduce the rate of relapse (Nisipeanu and Korczyn, 1993). This was the only stressor found to decrease the rate of relapse and its effect only lasted for approximately two months after the war. Furthermore, other larger studies examining war-stress on MS have failed to replicate this finding and instead suggest that war exacerbates disease (Golan et al., 2008). The effects of stress on multiple sclerosis are most likely to occur through stress-induced modification of the immune system.

Due to the complexity of these relationships and the ethical limits of human studies (e.g., generally limited to correlational relationships due to ethics of withholding treatment and limited to easily assessable tissue samples), this area of research benefits from investigation of the role of stress in the pathogenesis of animal models of MS. Animals studies provide allow for true control groups and concrete behavioral and physiological markers. Furthermore, given the abbreviated lifespan of rodent models, they allow the entire lifespan of the disease to be studies in a more rapid time course. This improves the efficiency of identifying underlying mechanisms and testing potential therapeutic strategies.

Theiler's Virus

Theiler's murine encephalomyelitis virus (TMEV) one of the best-characterized models of virus-initiated CNS demyelination in mice. It is a picornavirus and natural pathogen of mice that can be used experimentally to model multiple sclerosis (Theiler, 1934). When contracted, TMEV results in a gastrointestinal infection. However, the virus can cross the blood-brain barrier and results in an infection of the CNS. For experimental purposes, this agent is administered intracranially in order to avoid the uncertainty of CNS infection. There are different stains of the virus, some (e.g., GDVII and FA) are fatal in approximately a week while others lead to a more protracted disease process. For example, infection with the BeAn strain results in a biphasic disease of the CNS in susceptible strains of mice (e.g., SJL and BALB/cJ). The acute phase of TMEV infection is characterized by viral-mediated encephalomyelitis, while an autoimmune-mediated demyelinating disease, similar to MS, characterizes chronic phase disease (Lipton, 1975; Oleszak et al., 2004).

The acute phase involves the virus spreading to infect both neurons and glial cells, which results in microglial proliferation and motor neuron degeneration. This can cause a poliomyelitis-like syndrome, characterized by motor deficits and sickness behavior (Chang et al., 2000; Dal Canto and Lipton, 1982; Meagher et al., 2007; Njenga et al., 1997; Oleszak et al., 1995). The virus spreads through the CNS gray matter and results in microglial proliferation and motor neuron degeneration. This phase generally remits in approximately four weeks, however susceptible

strains of mice fail to completely clear the virus resulting in persistent CNS infection. Approximately three to five months after inoculation with the virus, this persistent infection initiates an inflammatory autoimmune demyelinating disease that is similar to multiple sclerosis (Aubert et al., 1987; Brahic et al., 1981; Campbell et al., 2001; Fiette et al., 1995; McGavern et al., 2000; Rodriguez et al., 1996; Welsh et al., 1987).

Like the EAE model, TMEV infection allows for the study of the pathogenesis of MS; however, it is unique in that it also provides a model of viral persistence within the CNS. Given that CNS viruses have been linked to the development of human multiple sclerosis, this is significant. The initial innate immune response results in the secretion of cytokines that control the rate of viral infection as well as shape the development of the adaptive immune response that ultimately clears the virus (Biron et al., 1998; Olson and Miller, 2009). Although inflammation is generally pathological in the chronic, demyelinating phase of TMEV infection, it is important during the initial response to infection. For example, it has been demonstrated that early release of IFN is necessary for effective viral clearance, such that IFN-KO mice exhibit fatal encephalitis following TMEV infection (Fiette et al., 1995; Jin et al., 2010; Kaminsky et al., 1987). Jin et al. (2010) suggest that this effect is due to IFN's role in mediating CNS immune cell infiltration and shaping local T cell responses.

The early adaptive immune response to TMEV infection determines viral persistence and, consequently, severity of chronic phase demyelinating disease

(Borrow et al., 1992; Borrow et al., 1993; Johnson et al., 2006). Although T cells are implicated in the induction of demyelination during chronic phase disease (Clatch et al., 1987; Rodriguez et al., 1988), as is observed in MS, they are essential during the acute phase for viral clearance. If an inadequate CD8+ and CD4+ T cell response occurs early in the acute phase of the disease, the chronic phase is exacerbated (Borrow et al., 1992; Murray et al., 1998; Welsh et al., 1987).

Data evaluating the relationship between acute and chronic phase symptoms has shown that behavioral measures correlate with both chronic phase disease symptoms and physiological measures (Johnson et al., 2006; Sieve et al., 2004). For example, hindlimb impairment at day seven correlates with chronic phase hindlimb impairment, horizontal movement, and vertical movement as well as antibody to TMEV, MOG33-55, and MBP (Johnson et al., 2006). Furthermore, chronic phase behavioral signs correlates with overall meningitis and perivascular cuffing (Sieve et al., 2004). This suggests that an evaluation of behavior is related useful tool to assessing and predicting disease severity.

Studies of acute phase TMEV infection allows for the examination of factors that contribute to viral persistence and later chronic phase disease exacerbations (Aubert et al., 1987; Brahic et al., 1981; Rodriguez et al., 1996). Therefore, this model allows for the evaluation of the mechanisms by which environmental factors increase CNS viral persistence and demyelination. As is observed with multiple sclerosis, stress generally exacerbates TMEV infection (Johnson et al., 2006; Johnson et al., 2004b; Meagher et al., 2007; Mi et al., 2006b; Sieve et al., 2004;

Steelman et al., 2009). Examining the mechanism by which stress exacerbates TMEV infection has provided some insight into how stress may affect MS, however, much is still unknown.

Stress

Broadly defined, stress is the non-specific response of the body to any demand for change. By this definition stress, encompasses real or perceived psychological and physiological stressors. Psychological stress can be defined as a perceived discrepancy between personal resources and the demands of the situation, whereas physiological stressors are demands on the body that do not require a cognitive appraisal of the event and includes stimuli such as intense physical exertion, injury, or even bacterial or viral infection. These stressors appear to differentially activate stress pathways; based on these differences they have been classified as processive and systemic (Emmert and Herman, 1999; Herman and Cullinan, 1997). Processive, or psychological, stressors are those that have no inherent physical cause and thus require comparison with prior experience to achieve biological significance. Therefore, it is likely that such stressors require stimulus processing in brain regions that regulate memory and emotion. On the other hand, systemic or physiological stressors disrupt internal homeostasis and can activate a stress response without being channeled through limbic and cortical structures. Clearly these types of stressors are not necessarily mutually exclusive.

Both forms of stressors can be acute or chronic. Acute stress is defined as a stress lasting for a period of minutes to hours; it can result from demands and pressures of the recent past or the anticipated future. It can be mild to severe, but passes quickly. Chronic stress, which persists for several hours per day for weeks or months, is the grinding stress that persists and wreaks havoc on the body. It can occur in response to a variety of stimuli including abuse, poverty, family dysfunction, or a dead-end job. Research suggests that acute stressors tend to enhance immunity by increasing immune cell trafficking and enhancing immunological memory (Dhabhar and McEwen, 1999; Dhabhar and Viswanathan, 2005; Viswanathan and Dhabhar, 2005); in contrast, chronic stressors are more likely to suppress immune function (Blecha et al., 1982; Campbell et al., 2001; Campisi and Fleshner, 2003; Dobbs et al., 1993; Johnson et al., 2006; Sheridan et al., 1998; Sieve et al., 2004).

It is suggested that stressors exert profound physiological responses intended to maintain an individual's internal balance or homeostasis (McEwen, 2000). Most notably the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) occur in response to stress. The limbic system, of which the hypothalamus is part, is a complex set of brain structures that are highly responsive to emotional stimuli, such as psychological or processive stress. Direct activation by systemic stress or activation via cortico-limbic system structures by processive stress of the paraventricular nucleus of the hypothalamus results in the synthesis and secretion of corticotropin releasing

hormone (CRH). The CRH acts on the pituitary gland causing adrenocorticotropic hormone (ACTH) to be released into the bloodstream. In response to the ACTH, the adrenal cortex produces glucocorticoids (GCs), such as cortisol in humans and corticosteroids in rodents. GCs have widespread effects on the body because almost all cells express glucocorticoid receptors. One of their functions is to mediate metabolic processes, acting to stimulate glucose release in the liver and fat break down in adipose tissue. Tight regulation of their release is important due to the powerful effects they can exert. Therefore, secretion of GCs is suppressed by a negative feedback loop; hypothalamus secretion of CRH is inhibited by GCs (see Fig. 1).

Activation of the sympathetic branch of the autonomic nervous system results in release of norepinephrine from sympathetic neurons and triggers the adrenal medulla to synthesize and secrete epinephrine and norepinephrine into circulation. This results in the activation of the fight-or-flight response, first described by Walter Cannon (1914) who suggests that states the SNS activation is designed to prepare an animal facing danger to engage (fight) or avoid (flight) the threatening stimuli. Epinephrine release affects almost all organ systems; to mention a few, it increases heart rate, contracts blood vessels, and dilates air passages. Furthermore, immune cells can respond to hormones released by the SNS. For example, it has been suggested that norepinephrine can trigger microglia to release proinflammatory cytokines (Blandino et al., 2006).

Stress has been shown to suppress immune function and, thereby, increase susceptibility to infections and cancer. Paradoxically, stress can also exacerbate diseases characterized by exaggerated immune activation, such as allergic, autoimmune, and inflammatory diseases. This suggests that stress may also be capable of enhancing immune function. These effects appear to be mediated by stress-induced hormones (Dhabhar and McEwen, 1999). Research suggests that stress hormones affect the efficacy of both the innate and adaptive immune response. It is through this mechanism that stress is likely to exert its effects on the pathogenesis of MS and Theiler's Virus induced demyelination (TVID).

Glucocorticoids and Immune Function

It has been well demonstrated that GCs affect immune system responses. It is generally accepted that GCs act to suppress immune function, such that exogenous glucocorticoids (e.g., methylprednisolone, prednisone, and dexamethasone) are commonly used to treat diseases caused by over-activation of immune functions, including autoimmune and inflammatory diseases like MS. This effect appears to occur through the inhibition of gene transcription. For example, suppressing proinflammatory pathways, such as NF-κβ and c-Jun, within macrophages and microglia leads to a reduction in inflammation (see Fig. 2).

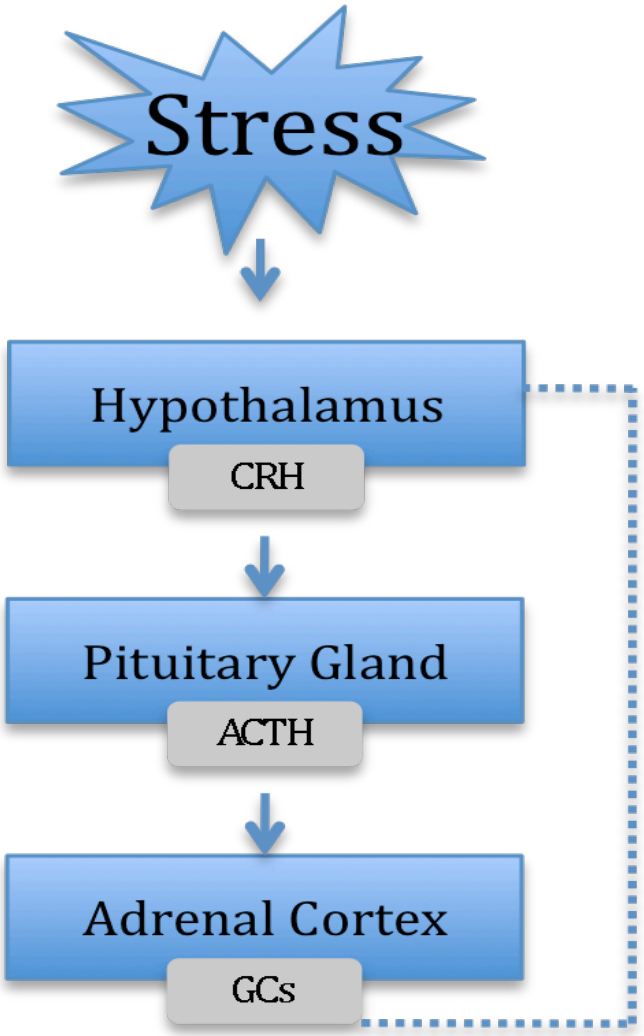


Fig. 1. The Hypothalamic-Pituitary-Adrenal axis. When a stressor, physiological or psychological, is assessed the hypothalamus, within the brain, releases corticotrophin releasing hormone (CRH), which acts on the pituitary gland. The pituitary gland releases adrenocorticotropic hormone (ACTH) to be released into the bloodstream, Once in the blood stream ACTH is able to act peripherally and cause the adrenal cortex to release glucocorticoids (GCs), cortisol in humans and corticosterone in rodents. Importantly, GCs form a negative feedback loop that acts centrally to inhibit hypothalamic release of CRH.

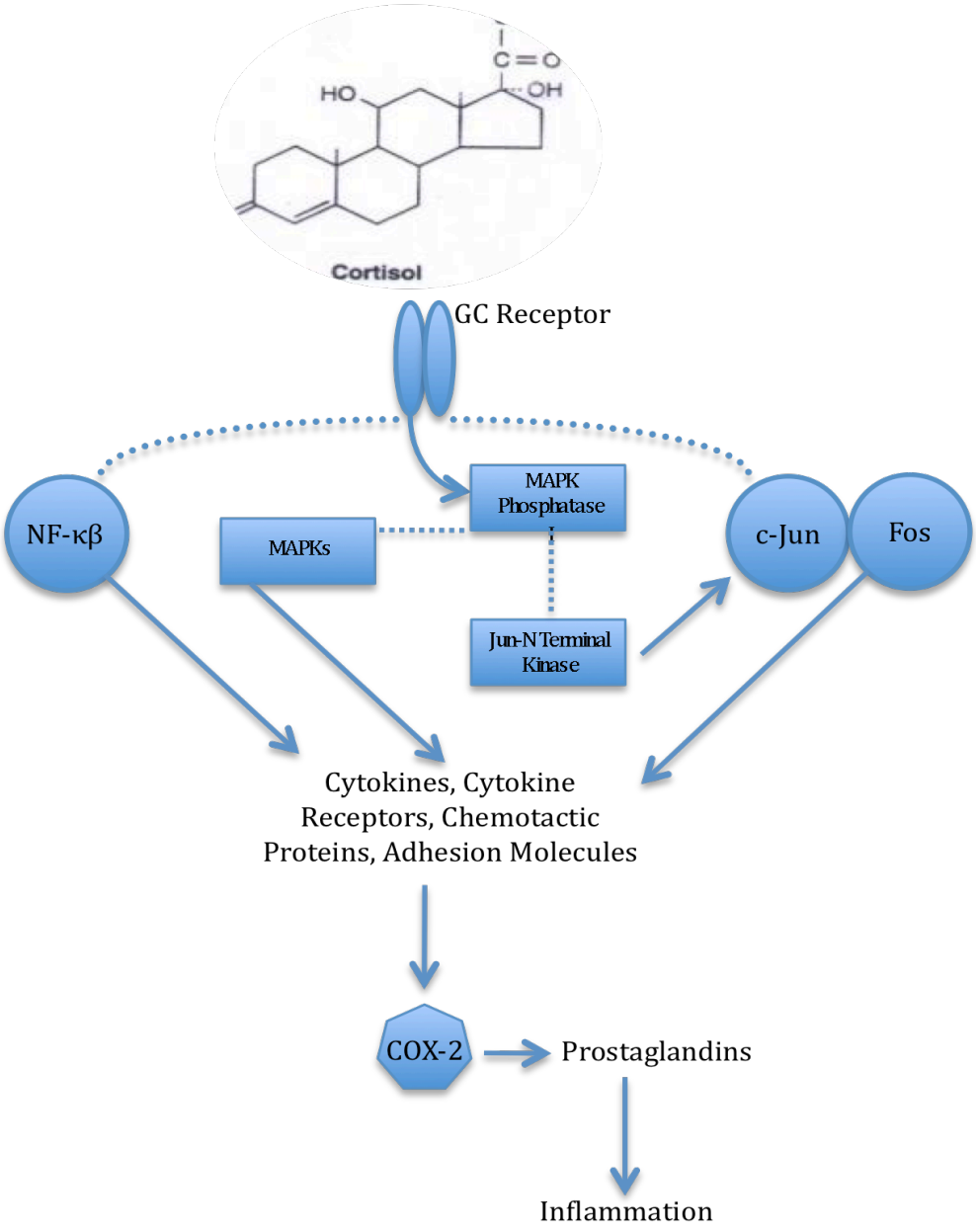


Fig. 2. The impact of GCs on inflammation. GCs, such as cortisol, act on GC receptors, which are G-protein coupled receptor, that initiates a variety of intracellular processes leading to the inhibition of inflammation. In this figure solid lines are activational pathways, while doted lines are inhibitory pathways. For example, cortisol inhibits the NF-κβ and c-Jun inflammatory pathways. Furthermore, stimulation of the GC receptor results in the activation of MAPK phophatase, which results in an inhibition of c-Jun and MAPKs.

Furthermore, GCs can simultaneously antagonize these pathways by inducing release of MAPK phosphatase I, which inhibits MAP and Jun N-terminal kinases and, consequently, reduces inflammation (Rhen and Cidlowski, 2005). However, it has been demonstrated that in some cases GCs can act to enhance inflammation. For example, GCs facilitate prostaglandin and leukotriene sythesis (Garcia-Fernandez et al., 2000), are necessary for HSV to cause brain inflammation (Ben-Hur et al., 2001), and increase infiltration of inflammatory cells in the hippocampus following excitotoxic seizures (Dinkel et al., 2003).

Not only do GCs affect the inflammatory response, they can also modify adaptive immune responses. These effects appear to occur through direct and indirect routes. Given that T cells have GC receptors they may lead to a direct suppression or even induce apoptosis of T cells (Ashwell et al., 2000; Gold et al., 2001). They may also functionally impair T cells through disrupting natural killer cell priming of T cells (Elftman et al., 2010) or by affecting the selection and development of T cells within the thymus (Stojic-Vukanic et al., 2009). Furthermore, it has been demonstrated that stress can alter the CD4+ T cell differentiation (e.g., a Th1/Th2 shift)(Cannarile et al., 2006).

The immunosuppressive actions of GCs can result in the activation latent or persistent viruses (Cacioppo et al., 2002; Guliani et al., 1999; Mayer et al., 2006; Noisakran et al., 1998; Pastoret et al., 1980). Given that it is hypothesized that multiple sclerosis is associated with the activation of latent or persistent viruses within the CNS, this may be one mechanism by which stress is associated with the

onset of MS. However, immunosuppression after disease onset (e.g., administration of exogenous GCs) may be protective because it suppresses the action of autoreactive T cells. This may also explain why severe stressors, which result in high levels of glucocorticoids, may reduce disease relapses in MS patients (Nisipeanu and Korczyn, 1993).

However, the body can develop resistance to the immunosuppressive effects of glucocorticoids, a phenomenon known as GC resistance (GCR). This effect appears to occur in response to social processive stressors (i.e., social disruption stress), not non-social stressors (i.e., restraint stress), and has been demonstrated in various species (Avitsur et al., 2001; Cohen et al., 1997; Miller et al., 2002; Quan et al., 2001; Raison and Miller, 2003). With chronic high-level exposure to GCs, immune cells compensate by down-regulating the expression and function of GC receptors, thereby making cells more resistant to the anti-inflammatory effects of GCs. This resistance has been demonstrated to develop in the immune cells of the blood, spleen, and brain in response to SDR (Avitsur et al., 2001; Johnson et al., 2004b; Meagher et al., 2007; Miller and Chen, 2006; Quan et al., 2001). For example, Quan et al. (2001) demonstrated that SDR results in a reduction of glucocorticoid receptor mRNA in the spleen and brain as well as induce functional GCR in splenocytes. Although there may be circumstances when GCR is adaptive, it appears that it may be one mechanism by which social stress increases vulnerability to inflammatory diseases, such as MS.

Catecholamines and Immune Function

In addition to mediating the fight or flight response, catecholamines affect the immune response. They are particularly important in the regulation of pro-inflammatory cytokines. Epinephrine and norepinephrine exert their effects by acting on β_2 adrenergic receptors spread widely throughout the body. These receptors are located on immune cells as well, providing an avenue by which SNS activation might influence immune function. For example, they are expressed in high levels on macrophages. Activation of the β_2 adrenergic receptor on macrophages results in the production of proinflammatory cytokines, such as IL-6 and IL-1β (Blandino et al., 2009; Gornikiewicz et al., 2000; Mohamed-Ali et al., 2001; Nguyen et al., 1998; Steptoe et al., 2001; Takaki et al., 1994; Tan et al., 2007).

Furthermore, it has been suggested that microglia become activated by stress (Frank et al., 2007; Nair and Bonneau, 2006; Sugama et al., 2007), presumably through activation of the β_2 adrenergic receptor. Microglia are the resident macrophages of the CNS and their activation results in the release a variety of proinflammatory factors. Unlike the all-or-nothing activational profile of neurons, microglia have a graded activational profile. In their basal state they are highly branched and constantly sample from the extracellular environment. When microglia encounter an activational signal (e.g., tissue damage, virus, bacteria, proinflammatory cytokines), they undergo morphological and phenotypic changes designed to address the situation. These changes usually include the release of pro-inflammatory cytokines, and in their highest activational state microglia, they

become like macrophages, having an amoeboid morphology and engaging in phagocytosis and antigen presentation.

Cytokines and the Stress Response

The relationship between cytokines and stress is complex. Not only can stress alter systemic and CNS proinflammatory cytokine levels (Bartolomucci et al., 2003; Blandino et al., 2006; Deak et al., 2005a; Johnson et al., 2004a; Johnson et al., 2006; Meagher et al., 2007; Merlot et al., 2003; Merlot et al., 2004a; Mi et al., 2006a; Minami et al., 1991; Miyahara et al., 2000; Nguyen et al., 1998; Nguyen et al., 2000; O'Connor et al., 2003b; Quan et al., 2001; Shintani et al., 1995a; Shintani et al., 1995b; Stark et al., 2002), cytokine (e.g., TNF-α, IL-1, IL-6, and IL-12) release can also initiate the release of stress hormones (Maier and Watkins, 1998; Watkins and Maier, 2000). It has been demonstrated that stress and cytokines have significant bi-directional communication. Proinflammatory cytokines within the CNS, released in response to immune system activation, can activate the HPA axis (Bethin et al., 2000; Turnbull and Rivier, 1999). This is a potential mechanism by which a systemic stressor activates stress pathways. Furthermore, cytokine release results in stereotyped sickness behaviors, including anorexia, anhedonia, reduced general activity and social activity, increased pain behavior, and learning and memory impairments (Barrientos et al., 2002; Maier and Watkins, 1998; Pugh et al., 1999).

Furthermore, proinflammatory cytokine release can be sensitized* by stress- and immune-induced proinflammatory cytokines (Cunningham et al., 2005; Frank et al., 2007; Johnson et al., 2002; Johnson et al., 2004a; Perry et al., 2003; Quan et al., 2001). For example, in a model of prion disease, a challenge with lipopolysaccharide (LPS; a cell wall component of gram negative bacteria) results in a dramatic increase in IL-1β expression, as well as increased neutrophil infiltration and inducible nitric oxide synthase (iNOS) expression, in the brain parenchyma of prion disease infected mice compared to non-infected mice (Cunningham et al., 2005). Furthermore, administration of IL-6 sensitizes stress-induced expression of IL-6 within the hypothalamus (Matsumoto et al., 2006). Increases in inflammation also results in increases in sickness behavior (Dantzer, 2006; Watkins et al., 1994). This suggests that a shared neural substrate may be primed by either stress or immune activation. Given that microglia are the primary producers of CNS inflammation, they may be the underlying mechanism (Cunningham et al., 2005; Frank et al., 2007; Nair and Bonneau, 2006; Perry et al., 2003; Sugama et al., 2007).

Given that CNS inflammation is associated with sickness behavior (Dantzer, 2006; Watkins et al., 1994) and MS is associated with exaggerated levels of proinflammatory cytokines within the CNS (Hauser and Oksenberg, 2006; Martino et al., 2002), it is not unexpected that patients commonly report fatigue, depression,

* Within the field of psychology, sensitization refers to an experience dependent enhancement in response, as observed in non-associative learning. This is in contrast to its immunological definition, which refers to the acquired ability of the immune system to respond to a foreign substance upon re-exposure. Throughout this dissertation "sensitization" will be used by its psychological definition.

pain, and cognitive dysfunction (Braley and Chervin, 2010; Chiaravalloti and DeLuca, 2008; Kargiotis et al., 2010; Krupp et al., 2010; Nurmikko et al., 2010). It is also no surprise that an environmental stimulus that exacerbates inflammation, such as psychological stress or viral infection, would be sufficient to exacerbate disease processes. However, the exact mechanism underlying this effect is not fully understood.

This complex relationship is simpler to disentangle in animal models of MS, such as TMEV infection. As previously suggested, the nature of a stressful event determines the physiological response to the stressor. For example, social stress, but not restraint stress, reduces the sensitivity of immune cells to the anti-inflammatory effects of glucocorticoids (Avitsur et al., 2001; Miller et al., 2002; Quan et al., 2001; Sheridan et al., 2000; Stark et al., 2002). This SDR-induced glucocorticoid resistance (GCR) diminishes the ability of stress-induced glucocorticoids to inhibit proinflammatory cytokines. Consequently, chronic social stress may be more likely to enhance inflammation than a chronic physical stressor, such as restraint stress.

Restraint Stress

Restraint is a common procedure for studying the effect of stress in laboratory animals and involves placing mice in well-ventilated tubes overnight (Pare and Glavin, 1986). Acute restraint stress has been shown to enhance leukocyte (e.g., neutrophil, macrophage, NK cell, and T cell) infiltration in response

to surgery or immune cell activation (Viswanathan and Dhabhar, 2005). Furthermore, it has been demonstrated that two to five hours of acute stress result in an enhancement of antigen-specific, cell-mediated immune responses, known as a delayed type hypersensitivy (DTH) response (Dhabhar and McEwen, 1997). In contrast, when this stressor was administered chronically (6 h for 3 to 5 weeks) it resulted in a suppression of the DTH response.

Similarly, chronic restraint stress has been shown to suppress the immune response to TMEV infection (Campbell et al., 2001; Mi et al., 2006a; Steelman et al., 2009; Welsh et al., 2004). In this paradigm restraint was administered nightly beginning the night prior to infection and continuing until day eight post-infection. This stressor has been shown to enhance glucocorticoid secretion without loss of GC sensitivity, exacerbate sickness, decrease viral clearance, and increase mortality following TMEV infection (Campbell et al., 2001). It has also been shown to decrease innate immune function, as measured by a reduction in NK cell activity (Welsh et al., 2004) as well as lower cytokine expression in the spleen and CNS (Mi et al., 2006a). Recently it has been demonstrated that restraint also suppresses virus specific CD4+ and CD8+ T cell function (Steelman et al., 2009).

Social Disruption Stress

Although restraint stress provides insight into how the activation of the HPA axis influences disease course, its translational value may be limited. It is unclear what human stressor may be comparable to restraint stress in mice. Given that

stressors do not necessarily exert uniform change, it is important to establish models that are more readily translated to human conditions. Due to the propensity and ubiquitous nature of social stress (Blanchard et al., 2001), it is seems to be important to evaluate the immunological consequences of social stress in animal models.

Social disruption (SDR) stress is an animal model of social stress that consists of introducing a dominant male mouse into the home cage of younger male mice. The intruder displays dominance (e.g., tail shaking, chasing, mounting, or biting) and requires the other mice in the cage to display submissive behavior (e.g., standing on hindlimbs with ventral body surface exposed and forelegs raised). This disrupts the established hierarchy and results in glucocorticoid release (Johnson et al., 2004b; Quan et al., 2001). When the stressor is administered chronically (six 2-hour sessions within one week), GCR develops (Avitsur et al., 2001; Johnson et al., 2004b). Furthermore, subjects exposed to SDR prior to an immune challenge demonstrated an enhanced inflammatory response (Quan et al., 2001). Similarly, it has been demonstrated that SDR results in enhanced serum levels of IL-6 in response to TMEV infection (Johnson et al., 2006), contrary to what has been observed in response to restraint stress (Mi et al., 2006a). However, this increase in inflammation is consistent with reports that circulating levels of IL-6 are elevated in humans with major depression and chronic stress (Kiecolt-Glaser et al., 2003; Maes et al., 1999; Maes et al., 1998).

IL-6 is a pleiotropic cytokine produced by a panoply of cells, including, but not limited to, macrophages, microglia, astrocytes, T cells, and endothelial cells. Furthermore, it can be released due to a variety of stimuli, including cytokines (e.g, IL-1, TNF-α), bacterial and viral infection, disease (e.g., MS, cancer, heart disease), and stress. Upon release, it can act as both pro- and anti-inflammatory. IL-6 plays a key role in inflammation through induction of factors such as C-reactive protein. Furthermore, IL-6 influences acquired immune function through induction of T cell growth and cytotoxic T cell differentiation. An over production of IL-6 is generally related to inflammatory-mediated disease and is accompanied by an increase in IL-1β. Despite the negative impact of high levels of IL-6, blocking this cytokine can also have pro-inflammatory effects suggesting anti-inflammatory actions of IL-6 as well. Research reveals that it serves as negative feedback for proinflammatory cytokines.

Previous research has also demonstrated that exposure to chronic SDR prior to TMEV infection exacerbates both acute and chronic phases of the disease (Johnson et al., 2006; Johnson et al., 2004b; Meagher et al., 2007). It results in increased inflammatory cell infiltration in the spinal cord and brain, an exacerbation of motor impairment, enhanced sickness behaviors, and a disruption of viral clearance from CNS. Furthermore, it results in increased levels of circulating antibodies to Theiler's virus and myelin during the chronic phase. This suggests that social stress alters both the immune response and the autoimmune response to infection. Moreover, it has been demonstrated that central administration of a neutralizing antibody to IL-6 during the stress exposure period can reverse the

adverse effects of stress on acute phase disease (Meagher et al., 2007). Although these findings suggest that IL-6 partially mediates the deleterious effects of SDR on sickness/motor behaviors and immunity during acute TMEV infection, the exact mechanism remains unknown. Based on this data, this dissertation tested the hypothesis that stress-induced enhancement of IL-6 release results in an altered inflammatory response to infection and, subsequently, disrupts the adaptive immune response, which would exacerbate TMEV pathogenesis.

Experimental Hypotheses

The central hypothesis of this dissertation was that prior exposure to social disruption stress exacerbates TMEV infection by dysregulating the innate and adaptive immune response to acute infection. Specifically, I hypothesized that prior exposure to chronic social stress results in virus-induced enhancement of inflammatory cytokine expression and suppression of the T cells production within the CNS during the acute phase of TMEV infection.

The dysregulation of the innate immune response is addressed in Chapter III. Prior data suggest that stress-induced IL-6 mediates the adverse effects of SDR on TMEV disease course (Meagher et al., 2007). Therefore, this series of experiments sought to determine if stress results in an enhancement of CNS inflammation, a priming of TMEV infection induced inflammation, and if the protection conferred by neutralizing IL-6 during the stress exposure period is through the inhibition of this enhancement in virus-induced inflammation. Prior

research demonstrated that exposure to stress can potentiate or enhance the release of proinflammatory cytokines following an immune challenge (Johnson et al., 2002; Johnson et al., 2006; Merlot et al., 2004b; Quan et al., 2001). Given that prior research has shown that IL-6 neutralizing antibody treatment during the stress exposure period reverses the adverse effects of stress on TMEV infection, it seems plausible that SDR-induced increases in central IL-6 production may be one mechanism by which stress exacerbates acute and chronic TMEV infection.

Furthermore, given that it has been demonstrated that stress can lead to the activation of microglia (Frank et al., 2007; Nair and Bonneau, 2006; Sugama et al., 2007), these experiments indirectly assessed the potential role of microglia in mediating the enhancement in neuroinflammation by examining the expression of CD11b, a marker of microglia activation. These experiments began by seeking to replicate prior data showing that social disruption stress can exacerbate sickness behavior in response to Theiler's virus infection (Chapter III, experiment one [Experiment 3.1]). Experiment 3.2 evaluated SDR-induced changes in levels of inflammatory cytokine expression and CD11b within the hippocampus. It was hypothesized that stress will lead to an increase in proinflammatory cytokines and microglia activation within the hippocampus. The hippocampus was selected based upon its role in regulating stress responses. Finally, Chapter III examined if stress, prior to infection, enhances the central and peripheral inflammatory response to viral infection and if the protective effects conferred by IL-6 neutralizing antibody treatment during the stress exposure period acts by

reversing this enhancement in inflammatory cytokine expression (Experiment 3.3).

The dysregulation of the adaptive immune response is examined in Chapter IV. Although prior data demonstrated that restraint stress results in a suppression of virus specific CD4+ and CD8+ T cells (Steelman et al., 2009), it is important to determine if a SDR similarly altered T cell expression. Despite the differences in stressor types, it was hypothesized that social disruption stress would also result in a suppression of virus specific T cells. This hypothesis is based upon the reduction in viral clearance observed in response to SDR. Although prior work on the role of SDR in TMEV infection was carried out in BALB/cJ mice (Johnson et al., 2006; Johnson et al., 2004b; Meagher et al., 2007; Skulina et al., 2004), this series of experiments was carried out in SJL/J mice. This change was based primarily upon the fact that no suitable viral peptides exist for the assessment of Theiler's virus-specific T cell activity in BALB/cJ mice. Furthermore, this allows for the generalizablity of the SDR effect to be examined.

My dissertation began by determining if that SDR prior to infection disrupts viral clearance (Experiment 4.1) and enhances sickness behavior (Experiment 4.2 and 4.3) in SJL/J mice. Next, it examined the expression of CD4+ and CD8+ T cells within the cortex via RT-PCR to determine if infection increases expression and if SDR results in a blunting of this increase (Experiment 4.2). Finally, Experiment 4.3 sought to determine if infection increases Theiler's virus-specific CD4+ and CD8+ T cell activity within the CNS and periphery and if SDR attenuates this effect.

CHAPTER II

GENERAL METHODS

Subjects

Male BALB/cJ and SJL/J mice (22-24-days-old; Jackson Labs, Bar Harbor, ME) were maintained on a 12-h light/dark cycle (lights off at 17:00 h) with continuous white noise (64 dB) and *ad libitum* access to food and water. Animals were individually housed until they recovered from cannulation surgery, at which time they were group housed, two to three per cage. Intruder mice were retired male breeders of the same strains (Jackson Labs, Bar Harbor, ME). All animal care protocols were in accordance with the Texas A&M University Laboratory Animal Care and Use Committee (ULACC).

Cannulation and IL-6 Neutralization

For studies in Chapter III, mice underwent cannulation surgery the day after they arrived. They were placed into an induction chamber and exposed to 5% isoflurane gas. After induction they were moved to a stereotaxic apparatus and maintained on 2% isoflurane. A 33-gauge guide cannula (PlasticsOne, Roanoke, VA, C315GS-2/SPC) was stereotaxically implanted into the left lateral ventricle (+1.0 mm lateral and -0.4 mm rostral to bregma, and 1.75 mm from the top of the skull). They were provided with softened food and acetaminophen treated water (162.5 mg/L) for at least 48 h prior to being group housed.

To block the effects of stress-induced IL-6 in the brain, IL-6 neutralizing antibody was administered intracereboventricularly (icv) four hours prior to each session of SDR. This method has been used previously to reverse the effects of specific cytokines in the brains of mice and rats (Jean Harry et al., 2003; Meagher et al., 2007; Nadeau and Rivest, 2003). IL-6 neutralizing antibody (polyclonal goat anti-mouse; 10 ng; R&D Systems, Madison, WI; AF-406-NA) or vehicle (goat Ig; Santa Cruz Biotechnology, Inc #SC-2342) was infused in a 2 μL volume at a rate of 1 μL/min via the implanted guide cannula and a microinjection pump.

Social Disruption Stress

Cages were randomly assigned to either the control or SDR condition. SDR began at the onset of the dark cycle (17:00 h). During the SDR procedure control mice remained undisturbed in their home cage, while SDR mice were moved to another room and an aggressive male intruder was introduced to the cage for two hours. This occurred for a total of six sessions, three consecutive nights, then one off, followed by three consecutive nights (Avitsur et al., 2001; Johnson et al., 2006; Johnson et al., 2004b; Stark et al., 2001). To minimize habituation to social defeat, a new intruder was used for each session. The sessions were monitored by video camera to ensure that the intruder demonstrated dominant behavior and the residents demonstrated submissive behavior. If an intruder failed to attack within 10 min, they were replaced and the session was continued for the remaining two hours.

To systematically assess the degree of wounding induced by SDR, we used a measure adapted from Merlot et al. (2003). Prior to the onset of SDR all subjects were assigned a fur score from one to five, as follows: 1 (well groomed and polished), 1.5 (fur not well polished, looking a bit ruffled or dirty), 2 (bristling of the fur), 2.5 (one small bite), 3 (more than one fur marks with bristling of the fur), and 4 (one or more visible wounds where the fur was obviously disrupted). Immediately following each session of SDR mice were examined under a red light for gross wounding.

Virus and Infection

The BeAn strain of Theiler's virus was kindly provided by Dr. H.L. Lipton (Department of Microbiology-Immunology, University of Illinois, Chicago, IL) and was propagated in L2 cells (Welsh et al., 1987). Mice were anesthetized with isoflurane (Vecco Inc., St Joseph, MS) and inoculated with $5x10^4$ pfu of TMEV in a 20 μL volume into the right mid-parietal cortex (Campbell et al., 2001; McGavern et al., 2000; McGavern et al., 1999; Theil et al., 2000). Non-infected mice were sham-infected with an equal volume of sterile PBS. Inoculation occurred two hours following the final session of SDR (21:00 h).

Behavioral Assessment

Theiler's virus infection results in acute microglia proliferation and motor neuron degeneration (Lipton, 1975). Although acute phase TMEV has previously described as asymptomatic, a more detailed analysis reports a variety of sickness behaviors in BALB/cJ mice in response to acute Theiler's virus infection, including acute weight loss, reduced activity, increased mechanical sensitivity, hindlimb impairment, and encephalitis which can be enhanced by prior exposure to SDR (Johnson et al., 2006; Johnson et al., 2004b; Meagher et al., 2007). These behavioral changes are noteworthy given they are predictive of exacerbated chronic phase (Johnson et al., 2006; Sieve et al., 2004). In this study, behavioral data were collected on BALB/cJ mice to verify that it could be replicate prior research and as a manipulation check.

Furthermore, to determine if prior exposure to SDR also exacerbates sickness in SJL/J, who generally exhibit a less pronounced acute phase disease, these measures were assessed in the SJL/J mice as well. This strain was evaluated because viral peptides to evaluate effector T cell function have been established for SJL/J mice (Kang et al., 2002; Steelman et al., 2009), but are not currently available for BALB/cJ mice.

Weight Loss

A drop in body weight and food consumption is generally observed immediately following infection with TMEV. Furthermore, it has been shown that initial weight loss is associated with some chronic phase behaviors (Sieve et al., 2004). Therefore, we assessed body weight and food weight daily at 09:00 h using a scale sensitive to 0.01g (Scout® Pro Portable Balance, Ohaus, Pine Brook, NJ).

Anhedonia

Anhedonia, a loss of interest in pleasure seeking, was assessed by evaluating preference for a sucrose solution (Maier and Watkins, 1998; Pollak et al., 2000). Anhedonia is thought to be mediated by increased CNS inflammatory cytokines (Dantzer et al., 1999; Dantzer et al., 2001) and has previously been shown to occur in TMEV infected animals previously exposed to stress (Meagher et al., 2007). Throughout experiments mice were provided with two-bottle choice, one bottle contained a 2% sucrose solution and the other contained tap water (bottle position was alternated in order to prevent the development of a place preference). Sucrose preference was defined as a 60% or greater preference for the sucrose solution, calculated by dividing the intake of sucrose solution by total fluid intake and multiplying by 100. Only cages that exhibited a sucrose preference prior to infection were included in data analysis.

Mechanical Sensitivity

Prior research has demonstrated that stress exposure can result in enhancements of virus-induced sensitivity to typically non-noxious stimuli, also known as allodynia (Bardin et al., 2009; Chung et al., 2007; Khasar et al., 2005; Young et al., in preparation). Specifically, it has been shown that SDR exacerbates TMEV-induced allodynia (Meagher et al., 2007). Although the exact mechanism mediating this effect is unknown, research suggests that an upregulation of CNS cytokines and microglia mediate this effect (Bradesi et al., 2009; Sweitzer et al., 1999; Watkins and Maier, 2005; Wen et al., 2009).

In this study mechanical allodynia was assessed prior to infection and on day one and seven post-infection by placing the mice in transparent circular chambers positioned on a raised mesh screen (2 mm gauge), and nylon filaments (von Frey monofilaments; Stoelting, Wooddale, IL; 0.008 – 2.0 grams) were applied to the hindpaw to determine their withdrawal threshold. Starting with the smallest filament, each filament was applied to the left and right hindpaws in an ABBA manner. Mice received three trials: ascending, descending, and ascending (Dixon, 1980). Mice were habituated to the apparatus for 20 min prior to each test.

Open Field Activity

Given that another consequence of enhanced CNS proinflammatory cytokines is reduced activity, this study also evaluated subject's activity in an open field (Dantzer, 2006). SDR prior to infection has previously been shown to suppress

horizontal activity in an open field (Meagher et al., 2007). Therefore, open field horizontal activity was assessed using six optical beam activity monitors (Model RXYZCM-16). Each equipped with two banks of eight photocells per wall. The boxes are interfaced with digital-multiplexors and Versamax software (Model DCM-4, Omnitech Electronics, Columbus, OH). Mice were habituated to the chambers for 60 min prior to baseline data collection. Test sessions were conducted in the dark beginning at 15:00 h for 30 min with white noise (64 dB) to mask extraneous disturbances.

Hindlimb Impairment and Clinical Scores

Furthermore, it has previously been shown that SDR prior to infection results in increased hindlimb impairment (HLI) and clinical scores (Meagher et al., 2007). Therefore, these measures were assessed at baseline and on day's one, four, and seven post-infection by a rater blind to experimental condition. Details on these scales have been presented elsewhere (Johnson et al., 2004b). Briefly, the hindlimb impairment scale was developed to quantify the behavior of BALB/cJ mice after infection with TMEV. This scale assesses weakness and paresis by observing alterations in locomotor function on an inverted grid. A separate score was given to each hindlimb (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). The two scores were summed to achieve the HLI score.

Clinical scores were given on a 0-4 scale that considers the degree of hunched posture and piloerection, which are non-specific signs of sickness. Hunching was scored on a scale of zero (no hunching) to six (having a sharp, high bump between the shoulder blades with the rear hind quarters abnormally dropped to the ground). Piloerection, or ruffling, was also scored on a scale of zero to six, with 0 being smooth fur and 6 being oily, clumped fur covering the entire body.

Analysis of Changes in Gene Expression

Tissue Collection

At the termination of the study the experimental subject was injected with 50-mg/kg pentobarbital and perfused via the left ventricle with cold RNase-Free water. Serum, brain, and spleen were collected. Tissue was flash frozen and stored at -80°C until analysis. A mouse brain matrix (PlasticsOne, Roanoke, VA) was used to assist with the microdissection of the hippocampus in Experiment 3.1.

Real Time RT-PCR

For real time RT-PCR analysis total RNA from CNS tissue and spleen were homogenized in QIAzol. RNA was isolated and purified through the use of Qiagen lysis reagent and RNeasy kit (Qiagen) with on-column DNase digestion according to manufacturer's instructions. cDNA was generated through the use of an RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's

instructions. RT-PCR was run to assess mRNA expression levels on markers of inflammation, including IL-6, IL-1β, IL-10, TNF-α, CD11b, and IFN-γ, and for T cell expression, CD4+ and CD8+. TaqMan probes and primers from Applied Biosystems were used, and β-actin was used as a control. The $2^{(-\Delta\Delta CT)}$ method was used to determine fold difference in expression.

Serum Cytokine Levels

Serum was collected at the termination of Experiment 3.3 in Chapter III for analysis of IL-6 and IL-1β protein levels. Commercially available ELISA Kits were purchased (R&D Systems, Minneapolis, MN; M6000B & MLB00B) and run per the manufacturer's instructions.

Viral Titer Assay

Viral load peaks within one to two weeks after infection, and a reduced rate of viral clearance is associated with exacerbated disease course (Welsh et al., 1987). Therefore, viral titers were assessed at day eight post-infection. Subjects were injected with a lethal dose of Beuthanasia special 150-mg/kg (Schering-Plough Animal Health) (Welsh et al., 2004). Mice were perfused through the left ventricle. Brain and spinal cords were collected, homogenized in Dulbecco's modified Eagles' medium DMEM (Gibco BRL, Grand Island, NY), and centrifuged. Supernatant was collected and used for this assay. BHK-21 cells were grown in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Irvine

Scientific, Irvine, CA) and maintained in BMEM with 1% FBS post inoculation. The titration of viruses was performed in BHK-21 cells by cytopathic effect (CPE).

Cells were seeded in 96-well plates at a concentration of 10^5 cells per well. When the cells reached confluency, each well was washed once with serum-free Iscove's Modified Dulbecco's Media (IMDM) and then inoculated in quadruplicate with 0.05 µL virus suspension per well in tenfold dilutions in DMEM. After viral adsorption the cells were washed in serum-free IMDM and incubated with 1% FBS-containing IMDM. The CPE was assessed daily and the TCID-50 was calculated by the Reed and Muench formula (Reed and Muench, 1938).

Determination of Virus-Specific T Cell Responses

Viral Peptides

The immunodominant CD4+ T cell peptide QEAFSHIRIPLPH corresponding to TMEV $VP_{274\text{-}86}$ was used to determine CD4+ cell specific responses (Gerety et al., 1991; Gerety et al., 1994). Immunodominant CD8+ T cell peptide FNFTAPFI corresponding to $VP3_{159\text{-}166}$ was used to determine CD8+ T cell specific responses to TMEV (Kang et al., 2005). The non-specific peptide sequence RLNRITKDSYPNS was used as a control peptide to determine non-specific immune responses. All peptides were purchased from Genemed Synthesis (San Antonio, TX).

Preparation of Feeder Cells

Spleens were aseptically removed from aged-matched unstressed/uninfected controls. Feeder cells were irradiated with 3000 rads (CO^{60} source, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University).

Tissue Isolation

On day eight post-infection all subjects were injected with a lethal dose of Beuthanasia special 150 mg/kg (Schering-Plough Animal Health) (Welsh et al., 2004). Mice were perfused through the left ventricle with cold Hanks Balanced Salts solution containing heparin (10 U/mL) buffered at pH7.2. After perfusion, spleens, brains and spinal cords were aseptically removed. Single cell suspensions were prepared as described previously (Steelman et al., 2009; Welsh et al., 2004). CNS infiltrating lymphocytes (CNS-ILs) were prepared from CNS tissue using nylon mesh and incubating in complete RPMI (RPMI-1640 containing 250 mg/mL collagenase Type IV, 10% FBS, and 15 mL each of L-glutamine and penicillin/streptomycin cocktail) for 45 minutes at 37°C and 5% CO_2 (Kang et al., 2002). Following incubation, CNS-ILs were isolated by 35/70% percoll gradient centrifugation and re-suspended in complete RPMI-1640.

ELISPOT Assay

The effects of social disruption stress on T cell effector function to TMEV were assessed, in part, by the ability to CNS-ILs to generate IFN-γ in response to either the immunodominant CD4+ or CD8+ T cell specific peptide (VP_{274-86} or $VP_{3159-166}$, respectively). In this assay, 96-well filtration plates (MAIPS4510) containing PVDF membranes (Millipore, Bedfirdm NA) were coated with 1.0 mg (in 100 µL sterile PBS) of anti-mouse IFN-γ capture antibody (AN-18; eBioscience) overnight at 4°C. The plates were blocked with 200 µL complete RPMI-1640 for two h at room temperature. Then 2.0 x 10^4 CNS-ILs were mixed with 1 x 10^6 irradiated feeder cells, in 150 µL of complete RPMI-1640 with a final concentration of 2.0 mM peptide (CD4+, CD8+, or non-specific) and then added to the plate. Following an incubation at 37°C and 5.0% CO_2 for 24 h, the plates were washed with PBS containing 0.05% Tween-20 and rinsed once with water purified by reverse osmosis (RO H_2O). One-hundred µL assay diluent (PBS with 10% FBS) containing 0.1 mg of the biotin labeled anti-INF-γ detection antibody (R4-6A2; eBioscience) was added to each well, and the plates were incubated at room temperature for two h. After the incubation, the plates were washed six times with wash buffer (PBS with 0.05% Tween-20). Next, 100 µL of avidin-HRP (horseradish peroxidase) (eBioscience) diluted 1/1000 in assay diluent was added to each well and the plates were incubated for 30 mins at room temperature. Plates were washed six times with wash buffer, spots were developed using 100 µL of 3-amino-9-ethyl-carbazole (AEC) substrate solution (1.0mL AEC, 1.0mL dimethylformamide,

14 mL 0.1M citrate-phosphate buffer pH 5.0, and 10.0 µL H_2O_2). Plates were rinsed three times with 200 µL of RO H_2O and read with an ELISPOT plate reader (AID EliSpot Reader System, Straberg, Germany).

The effects of SDR on splenic T cell effector function were determined using the methods described above. However, for these assays, 1.0 X 10^6 isolated spleen cells were used in the absence of feeder cells. All samples were run in triplicate. The generation of spots to the non-specific peptide was used as a measure of background and was subtracted from CD4+ and CD8+ T cell virus-specific peptide responses.

Statistical Analysis

Data are presented as mean+/-SEM. Analysis of variance (ANOVA) for studies with more than two groups or student's t-tests were used to determine if statistically significant difference emerged across conditions. Repeated measures ANOVA and multivariate ANOVA (MANOVA) were used as appropriate. These analyses were followed by *post hoc* analysis with Fisher's LSD, where appropriate. A *p*-value less than 0.05 was considered significant for all studies.

CHAPTER III

SOCIAL DISRUPTION SENSITIZES CNS INFLAMMATORY RESPONSE TO THEILER'S VIRUS INFECTION

Introduction

Prior research suggests that SDR exacerbates acute and chronic TMEV infection (Johnson et al., 2006; Johnson et al., 2004b; Meagher et al., 2007). However, the mechanism(s) by which stress exerts these effects has yet to be fully elucidated. It has been demonstrated that SDR leads to an increase in serum IL-6 post-infection (Johnson et al., 2006) and neutralization of IL-6 during SDR can reverse the adverse effects of stress (Meagher et al., 2007). This suggests that SDR may alter disease course by enhancing inflammation. Although the immunosuppressive properties of stress are better understood, recent studies have demonstrated that stress is capable of augmenting inflammation (Badowska-Szalewska et al., 2009; Blandino et al., 2009; Curry et al., 2010; Deak et al., 2005b; Johnson et al., 2003; Minami et al., 1991; Muhling et al., 2001; New et al., 2001; Nguyen et al., 1998; Nguyen et al., 2000; O'Connor et al., 2003a; O'Connor et al., 2003b; Quan et al., 2001; Shintani et al., 1995a; Shintani et al., 1995b; Steptoe et al., 2001).

Based upon these data, it was hypothesized that the adverse effects of SDR on TMEV infection are mediated by the sensitization of virus-induced cytokine expression. More specifically, it was hypothesized that social stress enhances levels

of CNS inflammation at the time of infection resulting in an exaggerated inflammatory response to viral infection. Furthermore, given that microglia are the primary producers of CNS inflammation and that stress exposure has been shown to activate microglia (Frank et al., 2007; Nair and Bonneau, 2006; Sugama et al., 2007), it is hypothesized that stress-induced potentiation of virus-induced inflammation occurs through the priming of microglia.

Experiment 3.1: Social Disruption Exacerbates Sickness Behavior in BALB/cJ Mice Infected with Theiler's Virus

Experiment 3.1 sought to replicate previously observed behavioral findings that suggest that SDR exacerbates the behavioral manifestations of sickness in response to TMEV infection. Although it has been shown that SDR prior to infection induces anhedonia, increases motor impairment, enhances allodynia, and decreases activity (Johnson et al., 2006; Johnson et al., 2004b; Meagher et al., 2007), the ability to replicate these findings at a different time point, by another investigator, and under different conditions further establishes this relationship. A failure to replicate previous finding would suggests that these behavioral changes were coincidental or could be explained by other factors (e.g., seasonal variation or extraneous stressors increasing the magnitude of sickness behavior).

Procedure

Subjects were assigned to either the SDR or non-SDR condition counterbalanced by weight (N=22). Two hours following the final SDR session, all subjects were infected intracranially with TMEV (Welsh et al., 1987). Behavioral measures previously demonstrated to be sensitive to the effects of SDR (e.g., sucrose preference, mechanical sensitivity, open field activity, and hindlimb impairment) were collected prior to infection and for seven days post-infection.

Results

As previously described, SDR significantly enhances infection-realted sickness behavior. Repeated measures ANOVAs revealed significant main effects of SDR on mechanical sensitivity, clinical score, hind limb impairment, and open field horizontal activity, all Fs > 4.687, ps < 0.05. Furthermore, significant day by SDR interactions were observed for open field activity and mechanical sensitivity (data not shown). *Post hoc* analyses confirmed that on day one and four post-infection mice pre-exposed to SDR exhibited enhanced mechanical sensitivity, $p<0.05$ (Fig. 3A). Furthermore, on day four post-infection a reduction in open field horizontal activity $p<0.001$, (Fig. 3B) and increased hind limb impairment $p<0.05$, (Fig. 3C) and clinical scores, $p<0.05$ (Fig. 3D) were observed in subject pre-exposed to SDR. The effects of stress on hindlimb impairment, clinical score, and horizontal activity persisted though day seven post-infection, all ps < 0.05.

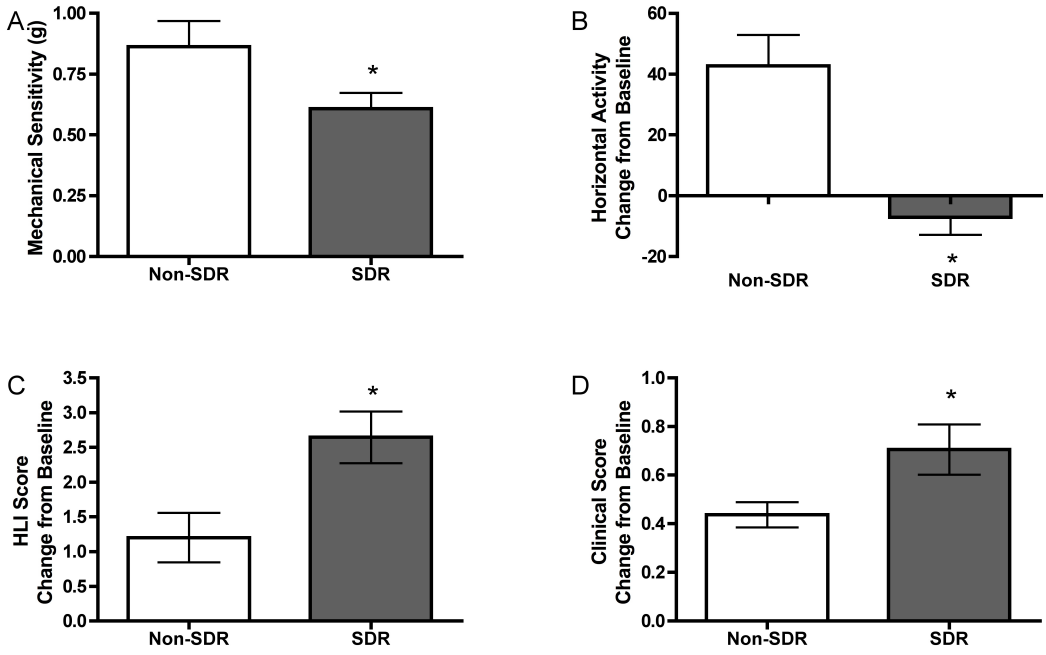


Fig. 3. SDR exacerbates sickness behaviors during acute TMEV infection. SDR resulted in increased mechanical sensitivity (A), decreased open field horizontal activity (B), and increased hindlimb impairment (C) and clinical score (D). Asterisks indicate significant differences between groups.

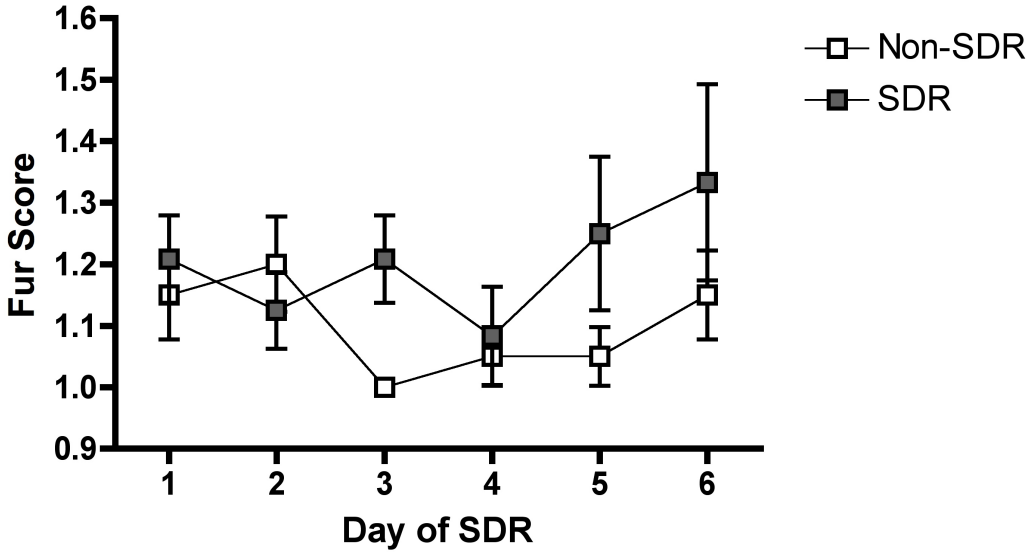


Fig. 4. Fur score in response to SDR. There were no significant effect of SDR on fur score, suggesting that SDR in BALB./cJ mice results in less wounding than more aggressive mouse strains (Avitsur et al., 2001; Merlot et al., 2003).

Contrary to previous data, the effect of sucrose preference failed to reach significance (p>0.05). However, this may be due to the significantly smaller group size than used in previous studies. The current study had less than half the subjects per group than did Meagher et al. (2007). The effect of sucrose preference may be subtle, such that group size in this study may have been insufficient to pull out the effect. In addition to increasing group size, another method that may allow this effect to be statistically resolved would be through the addition of a concurrent SDR group. Prior studies by Johnson et al. (2004; 2006) have demonstrated that when SDR is administered concurrent to infection, rather than prior to infection, a benefit is observed such that concurrent SDR improves disease course. This group adds statistical power to the analysis and, if added, may allow this effect to be resolved.

In addition to the replication of most previously observed findings, wounding and fur score data were evaluated in this experiment. Prior data suggest that wounding may be necessary for the adverse effects of SDR to be observed (Avitsur et al., 2001; Merlot et al., 2003). However, personal observations suggest that little wounding is observed during SDR in BALB/cJ mice. This was confirmed by daily analysis of fur scores (see Fig. 4), which showed a non-significant effect of SDR (p>0.05). Furthermore, although ruffling of fur was noted immediately following the sessions of SDR, only one tail bite was observed over the course of SDR. This level of wounding is significantly less than that observed in other laboratories and suggests that the effect of SDR on TMEV infection is not dependent upon wounding.

Experiment 3.2: Social Disruption Alters Central Inflammatory Cytokine Expression

Prior research has demonstrated that IL-6 is elevated in serum and brain in response to SDR in BALB/cJ mice (Meagher et al., 2007). Furthermore, central administration of IL-6 neutralizing antibody during the stress exposure period reverses the adverse effect of SDR on sickness/motor behaviors, CNS inflammation, and viral clearance during acute TMEV infection (Meagher et al., 2007). This suggests a role for IL-6 in mediating the deleterious effect of SDR, but the exact mechanisms remain undetermined. One possibility is that SDR-induced increases in CNS inflammation sensitize the neuroinflammatory response to TMEV infection, resulting in a dysregulation of innate and acquired immune responses to infection. Specifically, it is hypothesized that chronic SDR will result in an elevation of IL-6 mRNA expression within the hippocampus. To test this hypothesis, the present experiment examined whether SDR increases markers of inflammation within the hippocampus of BALB/cJ mice during the stress exposure period. The hippocampus is a component of the limbic system involved in the assessment of processive stressors. It was targeted in this study because it has been shown to be highly sensitive to the effects of stress (Alonso et al., 2004; Badowska-Szalewska et al., 2009). Furthermore, it is essential in the regulation of stress-induced neuroinflammation due to its high levels of glucocorticoid receptors (De Kloet et al., 1998; Frank et al., 2007).

Procedure

The levels of inflammatory markers were compared between acute stress (SDR-1), chronic stress (SDR-6), and home cage controls (N=54). Subjects were sacrificed immediately following SDR (19:00 h), two hours following SDR (21:00h), or 12 hours post-SDR (07:00 h). The hippocampus was microdissected and analyzed for IL-6, IL-1β, and CD11b mRNA expression. For analysis all groups were compared to the 0-hour home cage control condition.

Results

The data demonstrate that IL-6 is sensitized by SDR, while IL-1β was only increased following acute SDR. An ANOVA revealed a significant effect of SDR condition on IL-6 mRNA expression levels, $F(2,50)=20.487$, $p < .001$ (Fig. 5A). *Post hoc* analyses reveal that the chronic SDR condition was exhibited significantly more IL-6 mRNA than the home cage control mice. Further *post hoc* analyses indicated that at 0-hours chronic SDR results in a significant increase in IL-6 mRNA compared to the non-SDR and acute SDR conditions. Furthermore, the chronic SDR condition had elevated IL-6 mRNA expression levels, compared to home cage controls, at 12 h post-SDR in the chronic SDR condition. There were no significant changes in IL-6 mRNA expression following a single session of SDR. This suggests that the IL-6 response to SDR was only enhanced by repeated exposure sessions and remained elevated for at least 12 h following the last session of chronic SDR.

A significant effect of time, $F(2,49)=11.063$ ($p < .001$), and a significant time by SDR condition interaction, $F(4,49)=1.838$, ($p<.001$), was observed for IL-1β mRNA levels (Fig. 5B). *Post hoc* analysis indicated that there was a significant elevation in IL–1β 12 hours after a single session of SDR compared to all other groups at all other time points. No significant changes in IL-1β mRNA expression were observed following chronic SDR. Thus, while SDR induced an increase in the IL-1β response after one session of SDR, the IL-1β response appears to habituate by the sixth session of SDR.

Furthermore, an ANOVA revealed a significant effect of time, $F(2,49)=6.447$ ($p<.01$) on CD11b, a marker associated with the activation of microglia and macrophages. This time effect indicates that higher levels of CD11b were detected at 2 and 12 hours post-SDR. The time by SDR condition interaction failed to reach significance ($p=.16$), however, the trend suggested that repeated exposure to SDR results in an increase in CD11b mRNA expression, a marker of microglia activation (Fig. 5C).

Stress did not result in significant differences on body weight, mechanical sensitivity, food consumption, or sucrose preference. A repeated measures ANOVA looking at sucrose preference suggested a potential trend ($p=0.22$) toward a subtle reduction in sucrose preference due to SDR (Fig. 6). It is possible that increases in the size of these groups would allow this effect to become significant.

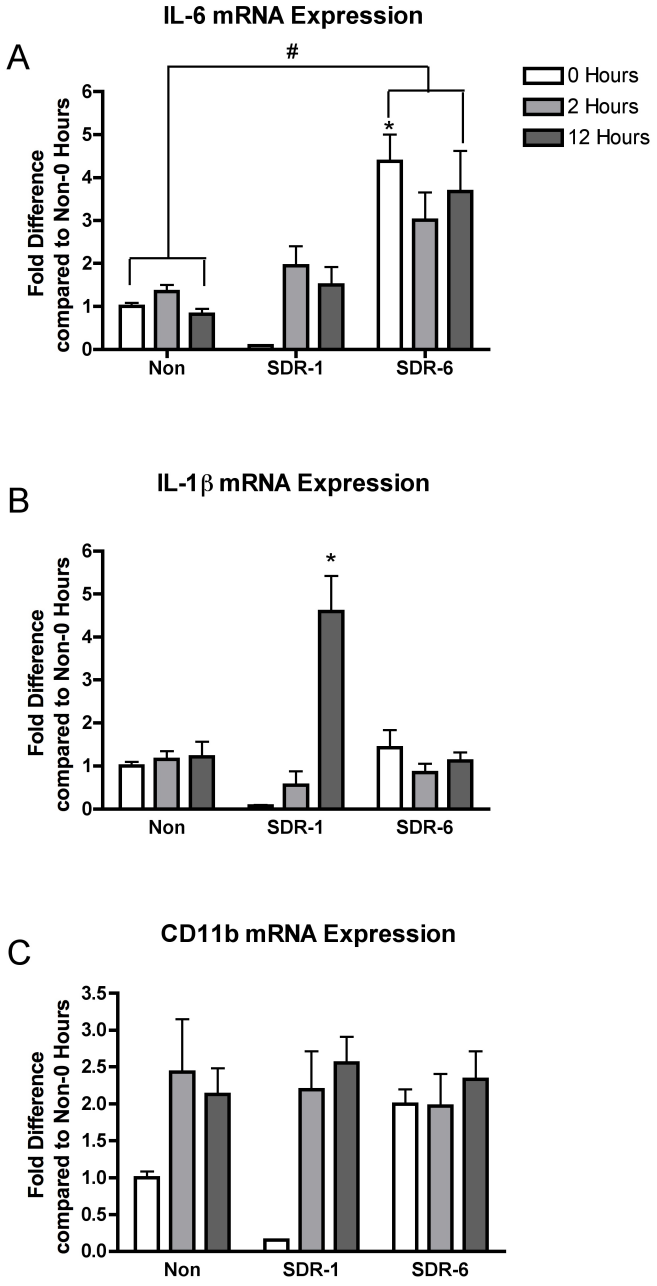


Fig. 5. SDR alters CNS inflammatory cytokine expression. IL-6 is elevated by exposure to chronic SDR (SDR-6) (A), while a single session of SDR (SDR-1) resulted in an elevation of IL-1β (B). No significant change in CD11b, a marker associated with microglia/macrophage activation, was observed (C). Asterisks indicate significant differences between groups.

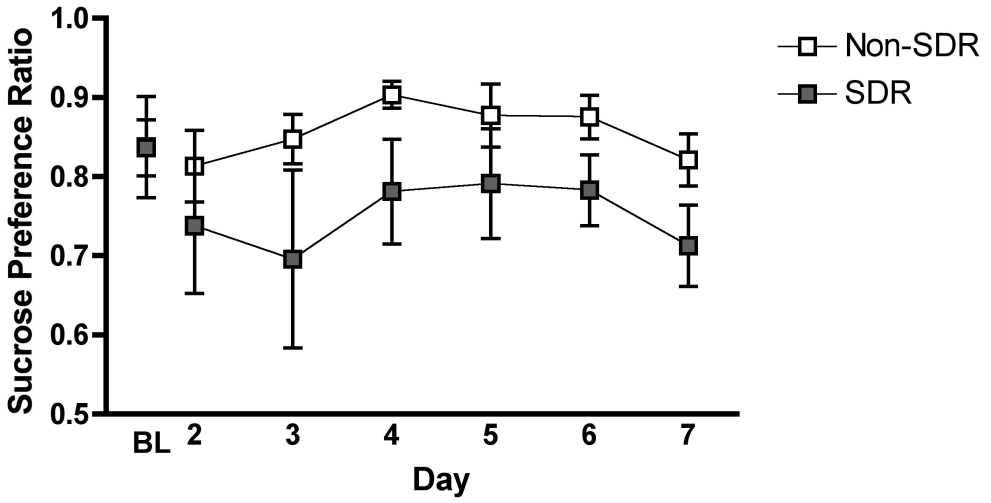


Fig. 6. SDR does not significantly alter sucrose preference. Although a trend was observed, SDR did not result in a significant reduction in sucrose preference. It is possible that larger group size would allow this effect to reach significance.

Experiment 3.3: Social Disruption-Induced Sensitization of the CNS Inflammatory Response to Theiler's Virus Infection Depends Upon Stress-Induced IL-6 Release

Prior data suggests that central cytokine release contributes to the deleterious effects of social stress on TMEV disease progression (Johnson et al., 2004a; Johnson et al., 2006; Meagher et al., 2007). Social stress increases CNS inflammatory lesions, including microgliosis, perivascular cuffing, and meningitis (Johnson et al., 2004a; Meagher et al., 2007). Furthermore, it has been demonstrated that SDR increases central and peripheral IL-6 expression during the stress exposure period and this exacerbation can be reversed through IL-6 neutralizing antibody administration (Meagher et al., 2007). Furthermore, IL-6 neutralizing antibody during the stress-exposure period can also reverse SDR-induced exacerbation of TMEV infection (Meagher et al., 2007). This suggests that SDR-induced increases in inflammation mediate the adverse effects of SDR on disease progression. Therefore, this experiment was designed to test the hypothesis that SDR-exacerbates disease by sensitization of virus-induced proinflammatory cytokine expression and the protective effects of IL-6 neutralizing antibody occur by reversing this sensitization.

Procedure

A two (SDR and Non-SDR) by two (nAbTx and Vehicle) experimental design was used (N=48). Subjects received microinjections of either IL-6 neutralizing antibody or vehicle four hours prior to each SDR session. Two hours after the final session of SDR all subjects were intracranially infected with Theiler's virus (Welsh et al., 1987). Clinical score and hindlimb impairment were collected as readouts of the effects of SDR and antibody treatment on infection. Subjects were sacrificed on day eight post-infection and brain, spleen, and plasma were collected for analysis of inflammatory markers.

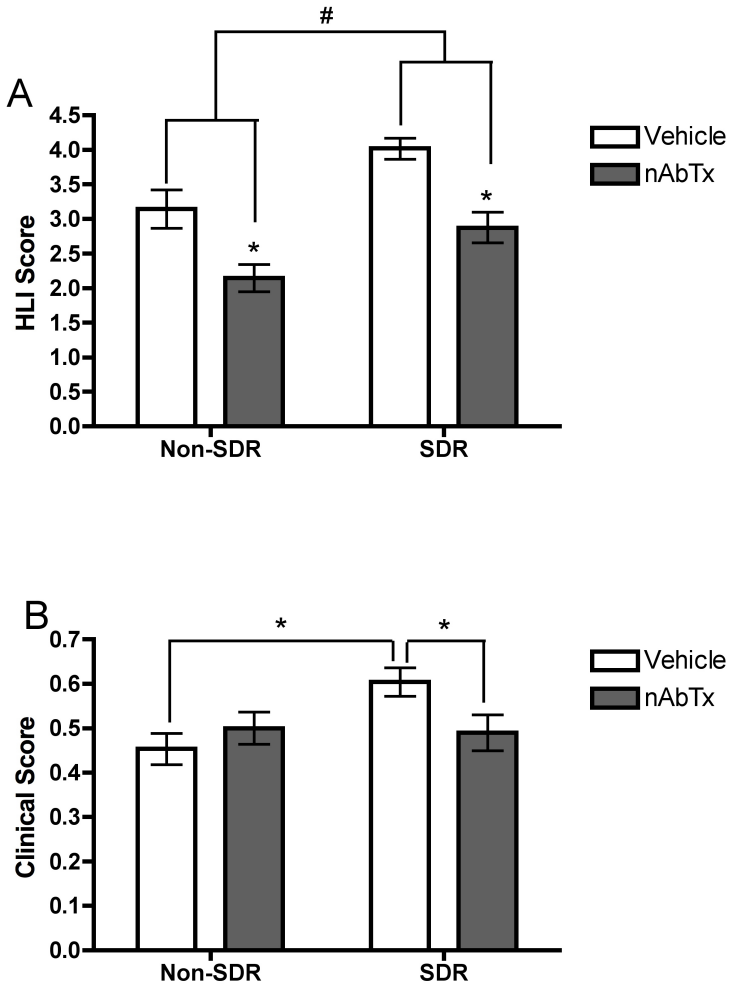
Results

Behavior. As anticipated, a one-sample t-test indicated a significant drop in body weight on day one post-infection, t=-8.457, p<.001 (data not shown). However, no significant differences between groups in weight loss amount were observed. Also consistent with prior data, SDR enhanced clinical score and hindlimb impairment on day four post-infection and IL-6 neutralizing antibody treatment reversed these effects (Fig. 7). A repeated measures ANOVA on hindlimb impairment revealed a significant main effect of time, neutralizing antibody treatment, and SDR, all Fs > 4.8, ps< .01, and a significant time by neutralizing antibody treatment condition interaction, $F(2,88) = 4.817$, $p \leq .01$. For clinical score, a repeated measures ANOVA revealed significant main effects of time and SDR condition, Fs > 6.1, ps <.05, and a significant interaction between SDR condition and

neutralizing antibody condition, $F(1,44)=7.488$, $p < .01$. These results confirm prior data indicating that SDR exacerbates disease and IL-6 neutralizing antibody treatment is protective.

Central Cytokine Expression. An ANOVA conducted on IL-6 mRNA expression levels in the brain revealed a significant interaction between SDR and neutralizing antibody condition, $F(1,43)=4.135$, $p<.05$ (Fig. 8A). *Post hoc* analyses revealed that the SDR-Vehicle group has a significantly higher level of IL-6 mRNA expression than all other groups. Similarly, analysis of IL-1β mRNA expression levels in the brain revealed a significant SDR condition by neutralizing antibody condition interaction, $F(1,42)=7.586$, $p<.01$ (Fig 8B). *Post hoc* analyses reveal that the SDR-vehicle group had significantly higher levels of IL-1β mRNA than the Non SDR-vehicle group and the SDR-neutralizing antibody group. This suggests that administration of neutralizing antibody to IL-6 during the stress exposure period reverses the sensitizing effect of stress on the proinflammatory cytokines, IL-6 and IL-1β.

Analysis of the CD11b mRNA expression levels in the brain revealed no significant main effects or interactions ($p>.05$), however the mRNA expression pattern is similar to that of IL-6 and IL-1β, with an elevation in the SDR-vehicle group compared to all other groups (Fig. 8C). Brain TNF-α, INF-γ, and IL-10 mRNA levels were also examined, however, no significant main effects or interactions were observed.



Fig, 7. Neutralizing antibody to IL-6 (nAbTx) prevents the adverse effects of SDR on acute TMEV infection. SDR exacerbates both hindlimb impairment (A) and clinical score (B) and administration of IL-6 neutralizing antibody reversed these adverse effects. Asterisks indicate significant differences between groups.

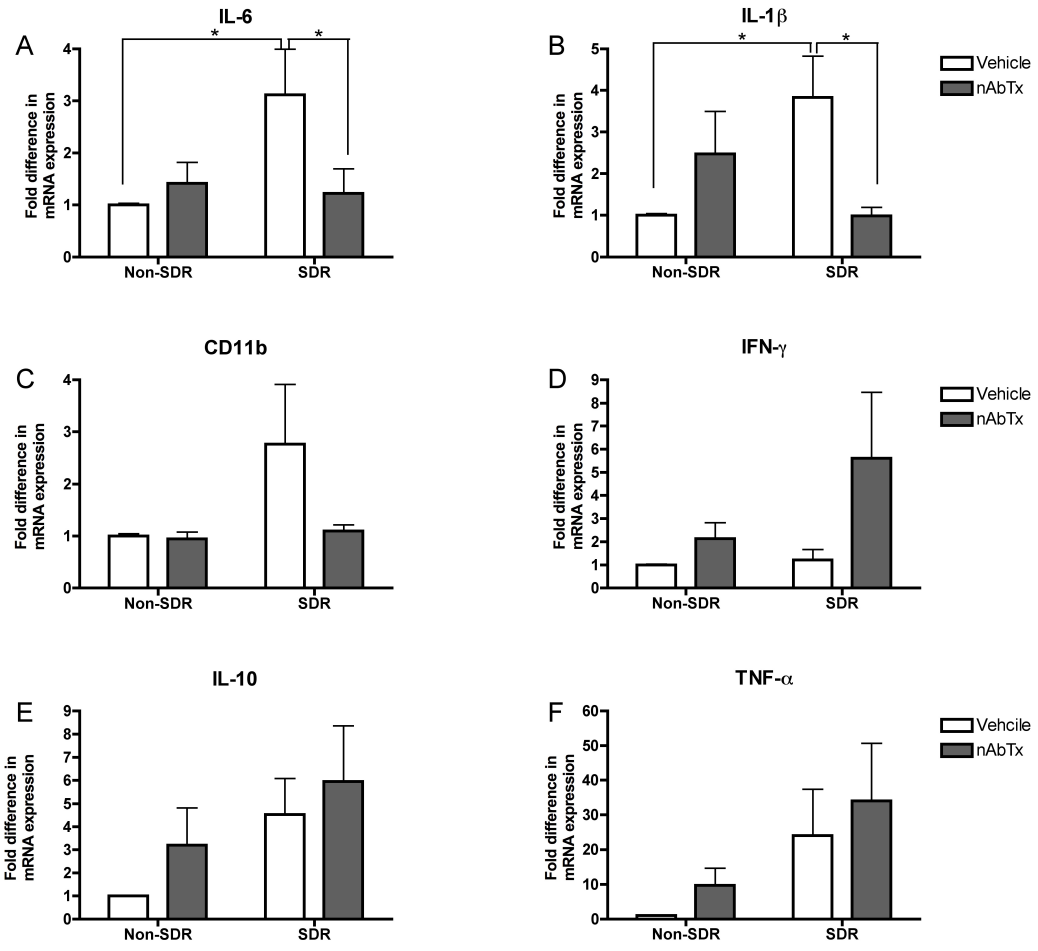


Fig. 8. Neutralizing antibody to IL-6 (nAbTx) prevents the SDR enhancement of virus-induced CNS inflammation. On day eight post-infection real time RT-PCR analyses demonstrated that SDR increases CNS IL-6 (A) and IL-1 β (B) mRNA expression and that these effects were reversed by IL-6 nAbTx. A similar, but non-significant pattern was observed for CD11b mRNA expression (C), a marker of microglia activation. No significant main effects or interactions were observed for IFN-γ (D), IL-10 (E), and TNF-α (F). Asterisks indicate significant differences between groups.

Peripheral Cytokine Expression. These same markers were evaluated in the spleen. Contrary to the interactions observed with IL-6 and IL-1β in the brain, no significant interactions between SDR and neutralizing antibody were observed in the periphery, suggesting that the protective effect of IL-6 neutralizing antibody treatment is centrally mediated. However, a significant main effect of SDR on IL-1β, $F(1,40)=8.663$, $p<.01$, and TNF-α, $F(1,38)=8.83$, $p<.01$, was observed indicating that SDR prior to infection results in increased mRNA expression of these cytokines. Furthermore, there was a significant main effect of IL-6 neutralizing antibody on INF-γ mRNA expression levels, $F(1,38)=5.242$, $p<.05$, such that IL-6 neutralizing antibody resulted in increased levels (data summarized in Table 1). ELISAs were performed to measure serum level of IL-6 and IL-1β (Fig. 9). No significant main effects or interactions were observed at eight days post-infection.

Discussion

The present findings replicate prior work showing that SDR exacerbates the behavioral response to TMEV infection. Furthermore, it demonstrates that these effects are based on the psychological/processive stress of SDR rather than the physical/systemic stress of wounding. These results may differ from those of Avistur et al. (2001) due to the strain differences (BALB/cJ vs. C57BL/6). BALB/c

Table 1. Summary of main effects and interactions of SDR and IL-6 neutralizing antibody treatment (nAbTx) on TMEV infection-induced cytokines at day eight post-infection. In general, the data indicates that SDR exacerbates both central and peripheral inflammation, but IL-6 nAbTx only reverses these central increases. ⇧ = significant increase, ⇩= significant decrease, and -- = no significant change in mRNA expression.

	Brain			**Spleen**		
	SDR	nAbTx	SDR x nAbTx	SDR	nAbTx	SDR x nAbTx
IL-6	--	--	⇧⇩	--	--	--
IL-1β	--	--	⇧⇩	⇧	--	--
CD11b	--	--	--	--	--	--
INF-γ	--	--	--	--	⇧	--
IL-10	--	--	--	--	--	--
TNF-α	--	--	--	⇧	--	--

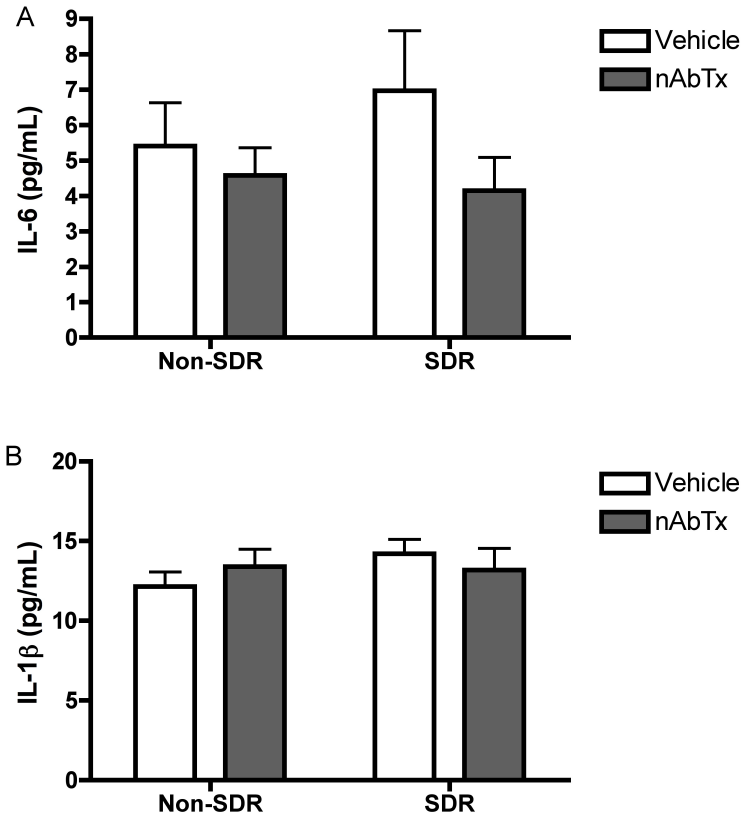


Fig. 9. No significant difference in serum levels of proinflammatory cytokines was observed on day 8 post-infection. The level of IL-6 (A) and IL-1β (B) in serum was determined by ELISA

mice generally exhibit a more hyper-reactive phenotype (Belzung and Griebel, 2001; Millstein et al., 2006; Willott et al., 2003) and, therefore, they may exhibit deficits at lower levels of stress.

Furthermore, SDR enhanced levels of proinflammatory cytokines within the brain. This provides further support for the hypothesis that social stress exacerbates TMEV infection by priming the inflammatory response to the virus. These effects are subtle, such that they were insufficient to exert statistically significant effects on behavior. The nature of this change in cytokine expression evolves across the sessions of SDR. Following a single session of SDR a significant increase in IL-1β mRNA expression was observed. However, by the sixth session no measurable increase was detected. Conversely, following a single session of SDR there is no change in IL-6 mRNA expression, however, following the sixth session there was a significant increase that lasted for at least 12 hours post SDR.

Given that infection occurs two hours following the final session of SDR, these data suggest that IL-6 mRNA is significantly up regulated at the time of infection in subjects pre-exposed to social disruption stress. These results, along with prior data showing that administration of IL-6 neutralizing antibody during the stress exposure period reverses the adverse effects of SDR on TMEV infection (Meagher et al., 2007), supports the hypothesis that SDR may exacerbate disease through the up-regulation of virus-induced inflammation. Further support for this comes from Experiment 3.3, which demonstrated that SDR prior to TMEV infection enhances the inflammatory cytokine response to infection by increasing the mRNA

expression of IL-6 and IL-1β in the brain and IL-1β and TNF-α in the spleen. Furthermore, IL-6 neutralizing antibody treatment reverses this enhancement in inflammation in the CNS but not in the spleen. Given that IL-6 neutralizing antibody can protect against the adverse effects of SDR on TMEV infection, a central enhancement in proinflammatory cytokines may drive the adverse effects of SDR on TMEV infection.

CHAPTER IV

SOCIAL DISRUPTION RESULTS IN A SUPPRESSION OF THE ADAPTIVE IMMUNE RESPONSE TO ACUTE THEILER'S VIRUS INFECTION

Introduction

TMEV infection is capable of inducing a demyelinating disease similar to multiple sclerosis in susceptible strains of mice (e.g. BALB/cJ and SJL/J). Susceptible strains are unable to effectively clear TMEV from the CNS during early infection. This results in CNS viral persistence and, eventually, immune-mediated demyelination (Clatch et al., 1990; Theiler, 1937; Zheng et al., 2001). Effective clearance of TMEV appears to depend on mounting an effective adaptive immune response. For example, it has been shown that depletion of CD8+ T cell populations or altering the functionality of these cells increases disease susceptibility (Borrow et al., 1992). The CD4+ T cells response has also been implicated in the in pathogenesis of TMEV. It has been demonstrated that mice with reduced CD4+ T cell populations show more severe inflammation, demyelination and axonal degeneration (Lin et al., 2004; Murray et al., 1998) as well as increased mortality (Welsh et al., 1987).

Further evidence for the role of the adaptive immune response in the pathogenesis of Theiler's virus induced demyelination (TVID) includes genetic factors. Susceptibility to disease appears to be associated with the MHC class I locus, H-2D (Lipton and Melvold, 1984; Lipton et al., 1995; Rodriguez and David,

1985). Mutations of the H-2D gene are associated with poorer viral clearance during the acute phase of TMEV infection and increased viral persistence (Lipton et al., 1995). Given that MHC class I is involved in antigen presentation to CD8+ T cells, this suggests a role for CD8+ T cells in the clearance of TMEV from the CNS.

Furthermore, recent research has demonstrated that restraint stress, which exacerbates disease pathogenesis, results in a suppression of virus specific T cells in the spleen and CNS of SJL/J mice (Steelman et al., 2009). In this study lymphocytes from the brain and spleen were stimulated with virus-specific CD4+ or CD8+ peptides. These peptides cause T cells to release IFN-γ; thus the number of cells producing IFN-γ can be used to determine T cell effector function. Steelman et al. (2009) demonstrated that infection increases both CD4+ and CD8+ T cell effector function and that restraint stress results in a suppression of virus specific CD4+ and CD8+ T cells in the spleen and CD8+ T cells in the CNS.

Therefore, this dissertation sought to determine if SDR prior to infection also disrupts T cell function. Given that SDR inhibits viral clearance (Johnson et al., 2006; Johnson et al., 2004b; Meagher et al., 2007), it is hypothesized that SDR will suppress virus specific CD4+ and CD8+ T cells. In contrast to the anti-inflammatory effects of restraint stress, SDR is pro-inflammatory. Prior data suggests that an enhanced innate immune response is capable of increasing (Pullen et al., 1994; Turrin, 2008) or decreasing (Olson and Miller, 2009) vulnerability to TMEV infection. Thus it is also possible that SDR may inhibit or enhance T cell expression. It is also possible that no change in T cells would be observed, suggesting that SDR

exacerbates TMEV disease course by a different pathway than restraint stress. Therefore, the impact of SDR on adaptive immune function is evaluated in this chapter.

These experiments use SJL/J mice instead of BALB/cJ mice. There are several reasons underlying this transition. The primary advantage is that virus specific peptide, such as those used by Steelman et al. (2009), are available for SJL/J mice, but not for BALB/cJ mice. Furthermore, this transition allows for the generality of the adverse effects of SDR on TMEV infection to be examined. Prior work focused on BALB/cJ mice due to their pronounced acute phase symptoms. However, SJL/J mice develop more pronounced TVID at a more rapid time course; SJL/J mice develop chronic phase symptoms in 2-3 months (McGavern et at al., 1999) in about half the time it takes BALB/cJ mice to express symptoms (approximately 4-5 months) (Johnson et al., 2006). Therefore, if their disease course is also exacerbated by SDR exposure, as is observed in BALB/cJ mice, they would provide a more efficient model of SDR-induced exacerbation of chronic phase, demyelinating disease.

The subsequent experiments evaluated if SDR inhibits viral clearance in SJL/J mice. Furthermore, it sought to determine if SDR prior to TMEV infection enhances acute phase sickness behavior. Then the effect of stress on infection-related central T cell mRNA expression was evaluated. Finally, the levels of virus-specific T cells were evaluated within the brain and spleen.

Experiment 4.1: Social Disruption Prior to Theiler's Virus Infection Impairs CNS Viral Clearance in SJL/J Mice

Effective viral clearance during the acute phase is related to chronic phase disease course. Prior studies have demonstrated that viral titers in the CNS peak one to two weeks post-infection and by three to four weeks it is cleared to non-detectable levels (Welsh et al., 1987). Subjects that fail to completely clear the virus from the CNS during the acute phase later develop chronic phase, autoimmune-mediated TVID (Aubert et al., 1987). Prior research has demonstrated that prior exposure to SDR inhibits viral clearance within the CNS in BALB/cJ mice, such that at day 21 post-infection animals pre-exposed to SDR had viral titers equal to their day 7 levels (Johnson et al., 2006; Johnson et al., 2004b; Meagher et al., 2007). This experiment was designed to determine if exposure to SDR prior to infection also impairs viral clearance in SJL/J mice.

Procedure

Subjects were exposed to either SDR or remained undisturbed in their home cage (N=12). All mice were infected two hours following the final session of SDR. On day eight post-infection, brain and spinal cord samples were collected for the determination of viral titer by TCID-50.

Results

A Student's t-test revealed significantly higher CNS viral titers at day eight post-infection in subjects exposed to SDR prior to infection, $t = 4.589$, $p \leq 0.01$ (Fig. 10). This demonstrates that SDR also inhibits viral clearance in SJL/J mice. Furthermore, it provides further evidence SDR modifying the adaptive immune response.

Experiment 4.2: Social Disruption Exacerbates Sickness Behavior and Decreases Cortical T Cell mRNA Expression in Response to Theiler's Virus Infection

Viral clearance occurs through the action of the adaptive immune response, primarily the actions of helper (CD4+) and cytotoxic (CD8+) T cells. The poorer viral clearance observed in mice exposed to SDR prior to TMEV infection suggests that SDR is disrupting T cell function. This hypothesis is supported by Steelman et al. (2009) who demonstrate that restraint stress attenuates virus specific CD4+ and CD8+ T cells within the spleen and CD8+ T cells within the CNS at day eight post-infection. To begin to evaluate the impact of SDR on adaptive immune function the impact of SDR prior to infection on overall cortical T cell mRNA expression was assessed.

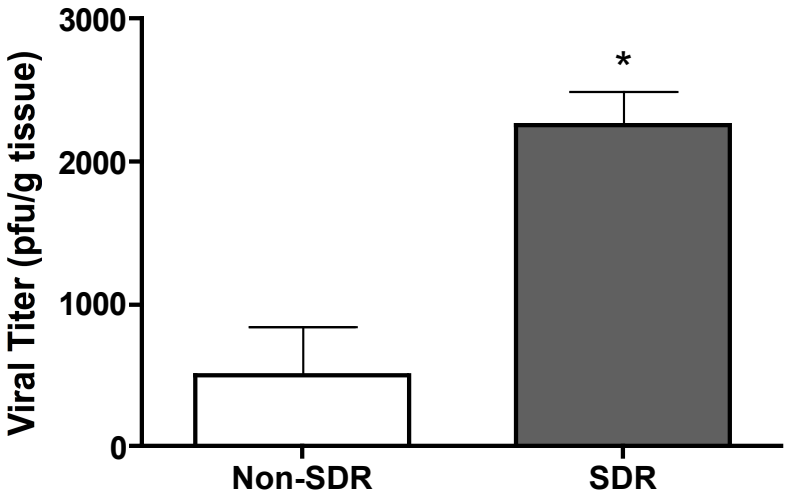


Fig. 10. SDR enhances viral titers at day 8 post-infection. SDR prior to infection with TMEV results in a significant increase in viral titers at day eight post-infection in SJL/J mice. An asterisk indicates significant differences between groups.

Procedure

A two (infected verses uninfected) by two (SDR verses Non-SDR) experimental design was employed (N=24). Mice were either exposed to chronic SDR or left undisturbed in their home cage. Two hours following the final session of SDR, mice were infected with TMEV or sham infected with sterile saline. Basic sickness behavior (e.g., body weight, sucrose preference, clinical scores, and mechanical sensitivity) was assessed following infection. At eight days post-infection subjects were sacrificed and CNS cortical tissue was microdissected for analysis by RT-PCR. Given that infiltrating T cells are generally found in the meningies of the brain, we focused on examining cortical tissue, which should include the meningies.

Results

Behavior. In order to determine if SDR exacerbates sickness behavior in response to TMEV infection in SJL/J mice, body weight, sucrose preference, clinical score, and mechanical sensitivity were assessed. Behavioral data from Experiments 4.2 and 4.3 were combined to enhance statistical power. The acute phase of TMEV infection typically results in an asymptomatic infection in SJL/J mice, though small reductions in body weight have been previously reported (Steelman et al., 2009). Similarly the present study, demonstrated that exposure to social disruption exacerbated weight loss following TMEV infection. An ANOVA showed significant main effects of stress, $F(1, 44) = 7.025$, $p \leq 0.05$, and infection, $F(1, 44) = 22.310$, $p \leq$

0.001, as well as a significant stress by infection interaction, $F(1, 44) = 7.838$, $p \leq 0.01$, on change in body weight at day one post-infection (Fig. 11A). *Post hoc* analyses indicate that subjects exposed to stress prior to infection lost significantly more weight than all other groups.

Furthermore, exposure to SDR prior to infection resulted in a reduction in sucrose preference, an indicator of anhedonia, at day one post-infection (Fig. 11B). An ANOVA confirmed a significant main effect of infection, $F(1, 12) = 5.822$, $p < 0.05$). *Post hoc* analysis indicates that the effect of infection may be partially explained significant differences between the SDR/infected subjects and the uninfected subjects (both stressed and non-stressed). No significant effects were observed for clinical score or mechanical sensitivity.

T Cell mRNA Expression. Furthermore, TMEV infection resulted in increased cortical T cell mRNA expression at day eight post-infection and SDR prior to infection significantly attenuated this increase. An ANOVA for CD4+ mRNA expression, showed a significant effect of infection, $F(1,21) = 4.726$, $p < .05$, such that infection increased the level of CD4+ mRNA expression. *Post hoc* analysis showed that the infected non-stressed condition drives this effect, such that infected non-stressed subjects had higher levels of CD4+ mRNA than all other groups. The main effect of stress and the stress ($p = .187$) by infection interaction ($p = .160$) failed to reach significance. An ANOVA for CD8+ mRNA expression showed no significant effects, however, there was a trend ($p = .106$) toward an infection

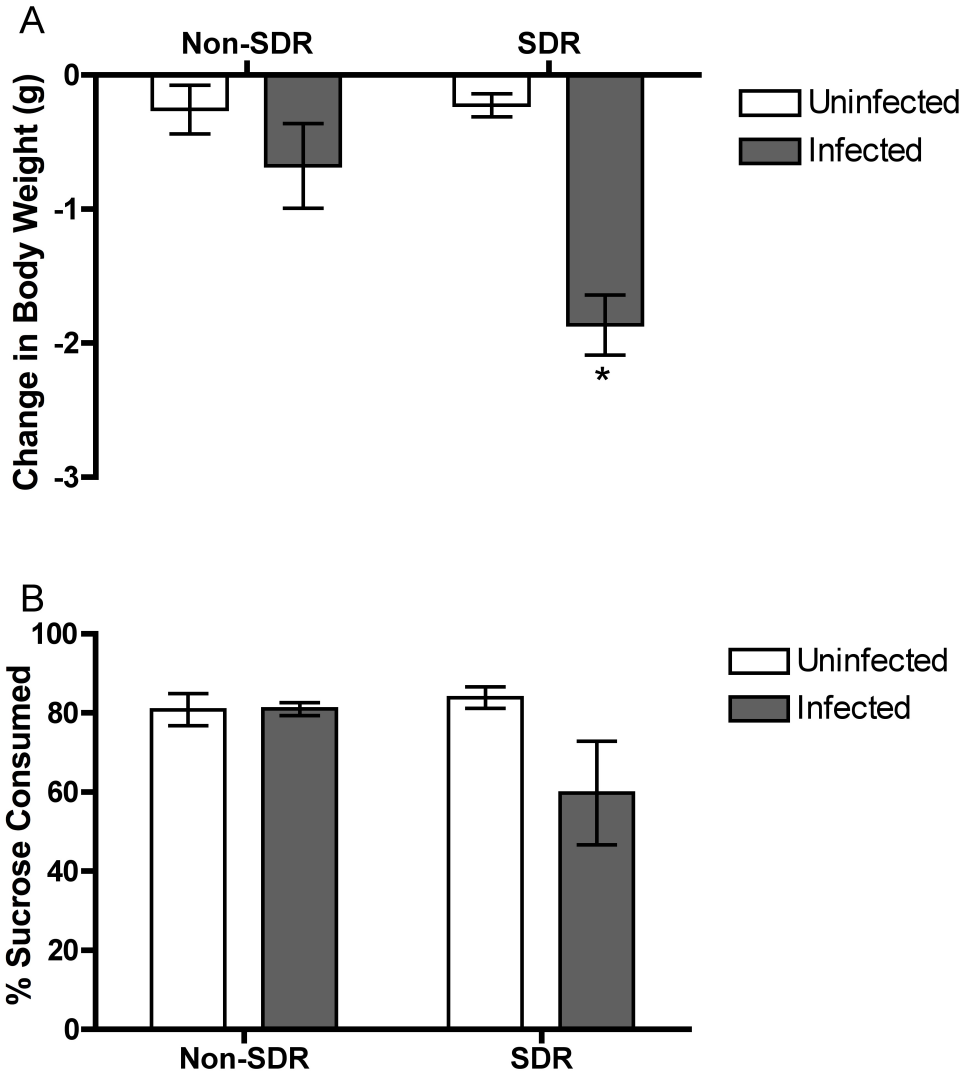


Fig. 11.. SDR exacerbates TMEV infection related sickness behavior in SJL/J mice. SDR results in a significant condition by infection interaction on body weight loss on day one post-infection, suggesting that the SDR prior to infection exacerbates weight loss (A). Furthermore, only the SDR-infected group observed a loss in sucrose preference (B).

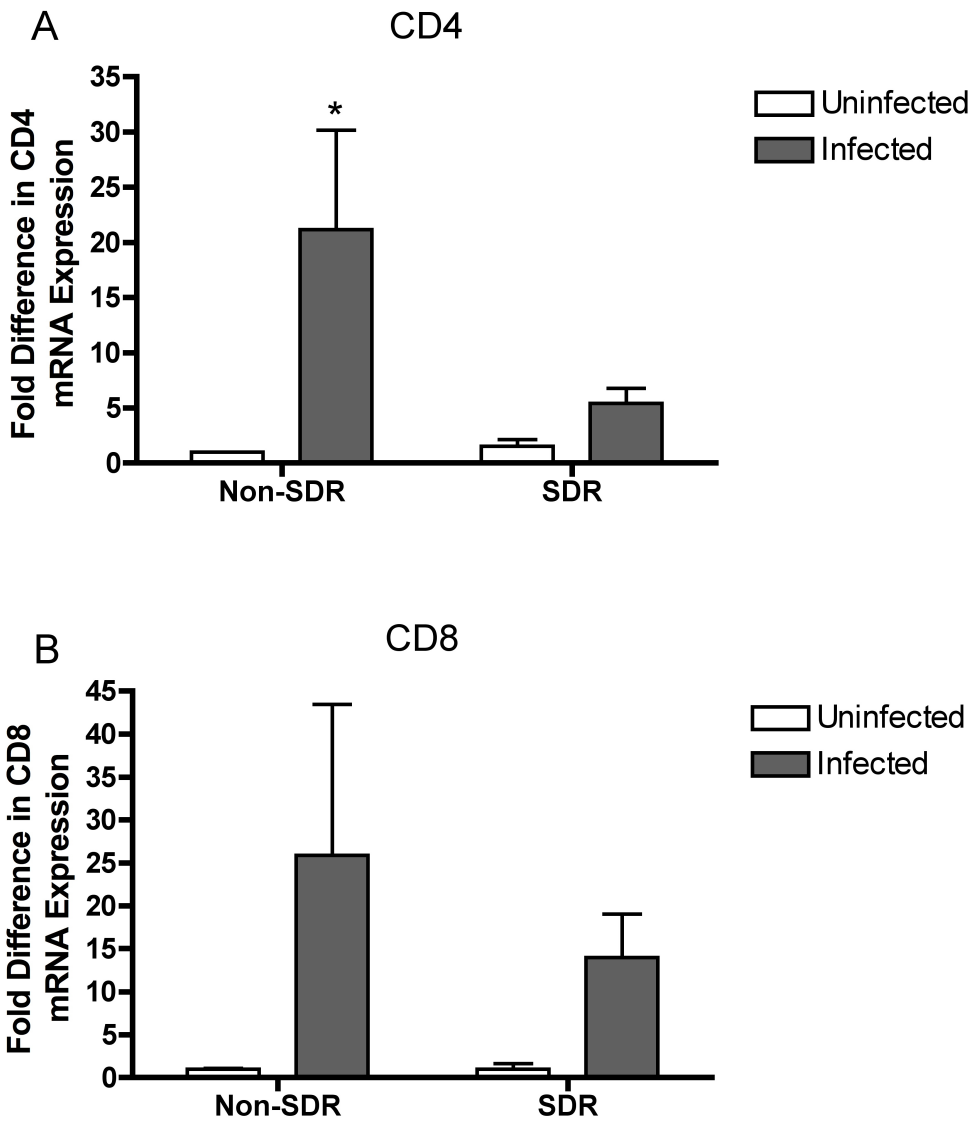


Fig. 12. SDR attenuates infection related increase in T cell mRNA expression. A MANOVA revealed a significant interaction of infection by stress. As anticipated, the data indicated that infection results in an increase in central T cell levels and that SDR prior to infection inhibits this up-regulation.

related increase in CD8+ mRNA expression. However, when analyzed together, a MANOVA revealed a significant stress by infection interaction, $F(2, 21) = 3.644$, $p \leq 0.05$, on the cortical levels of CD4+ and CD8+ mRNA expression (Fig. 12). This interaction indicates that SDR attenuates virus-induced enhancement in T cell number.

Experiment 4.3: Social Disruption Impairs Virus-Specific Adaptive Immunity in the CNS but Not the Spleen

T cell responses, particularly the CD8+ T cell response, have been shown to play a vital role in the clearance of TMEV from the CNS. Given that prior research has demonstrated that restraint stress attenuates virus specific T cell responses (Steelman et al., 2009), this study sought to determine if SDR also affects T cell response. It has been demonstrated that SDR disrupts viral clearance and reduces T cell number, however, it is possible that a general reduction does not necessarily result in a reduction in virus specific T cells. Therefore, this study evaluated the effect SDR on virus-specific T cells through the use of the ELISPOT assay. This assay evaluates IFN-γ secretion by virus specific T cells by stimulating lymphocytes from the spleen and the CNS with Theiler's virus specific CD4+ and CD8+ T cell peptides. Each peptide will cause virus specific T cells to produce IFN-γ that can be quantified.

Procedure

A two (infected verses uninfected) by two (SDR verses Non-SDR) experimental design was employed (N=24). Mice were either exposed to chronic SDR or left undisturbed in their home cage. Two hours following the final session of SDR, mice were infected with TMEV or sham infected with sterile saline. Body weight, sucrose preference, clinical scores, and mechanical sensitivity were assessed following infection. Eight days post-infection subjects were sacrificed and the brain, spinal cord, and spleen were collected. Lymphocytes from the spleen and CNS (brain and spinal cord) were isolated and an ELISPOT assay was performed.

Results

TMEV infection increased the number of cells responding to both CD4+ and CD8+ immunodominant peptide while SDR profoundly decreased T cell responses in the CNS. Specifically, infection significantly increased virus-specific CD4+ cells in the CNS, but this increase was significantly attenuated in subjects previously exposed to social disruption. An ANOVA verified significant main effects of stress, $F(1, 20) = 18.668$, $p < 0.001$, and infection, $F(1, 20) = 17.552$, $p < 0.001$, as well as a significant stress by infection interaction, $F(1, 20) = 18.417$, $p < 0.001$, for virus specific CD4+ T cells in the CNS (Fig. 13A). *Post hoc* analyses indicate that the non-stressed infected subjects have significantly higher levels of virus-specific CD4+ cells in the CNS.

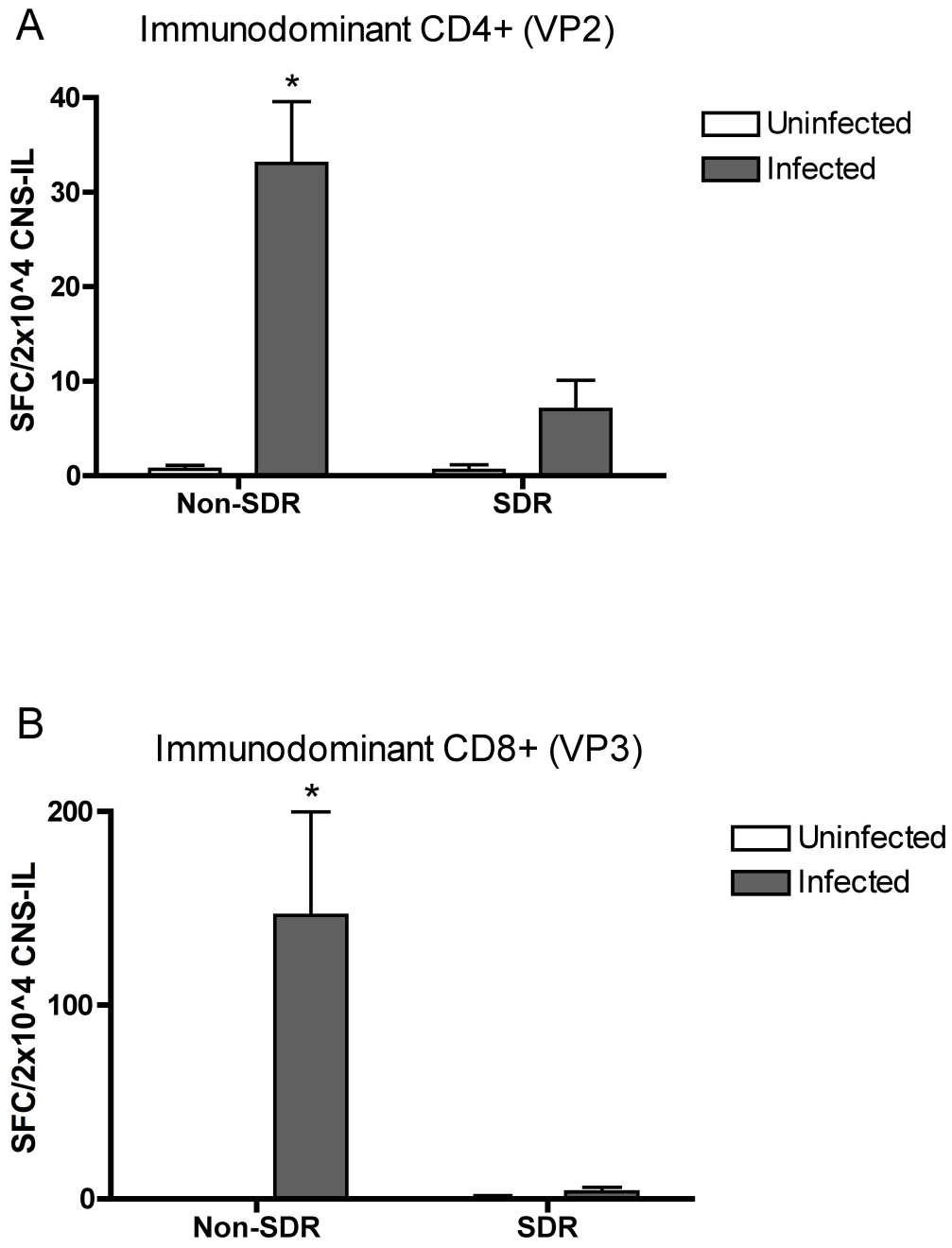


Fig. 13. SDR suppresses CNS T cell effector function. Within the cortex of the brain infection results in a significant increase in both CD4+ and CD8+ T cell effector function. This increase in inflammation was significantly attenuated in the SDR group.

Furthermore, infection resulted in a significant increase in virus-specific CD8+ cells in the CNS, an effect that was significantly attenuated by prior exposure to social disruption. ANOVA verified significant main effects of stress, $F(1, 20) = 10.197$, $p < 0.01$, and infection, $F(1, 20) = 12.338$, $p < 0.01$, as well as a significant stress by infection interaction, $F(1, 20) = 10.344$, $p < 0.01$ (Fig. 13B). Follow up *post hoc* analyses demonstrate that the non-stressed infected subjects have significantly higher levels of virus-specific CD8+ cells in the CNS than all other groups.

These effects were not explained by SDR-induced alterations in peripheral T cell responses. Infection significantly increased the number of virus-specific CD4+T cells in the spleen, $F(1, 19) = 8.833$, $p < 0.01$, but this increase was not significantly altered by SDR exposure prior to infection, all other Fs < 0.862 (Fig. 14A). A main effect of infection on virus-specific CD8+ T cell populations in the spleen approached significance, $F(1, 18) = 3.905$, $p = 0.064$), but no other significant main effects or interactions were present, all Fs < 0.574, ps > .05 (Fig. 14B).

Discussion

These experiments provide compelling evidence that pre-exposure to social stress can disrupt the adaptive immune response to infection. They are the first to demonstrate the SJL/J mice, like BALB/cJ mice, infected with TMEV exhibit increased sickness and poorer viral clearance when pre-exposed to SDR. These data suggests that this results from increased viral load. It was also demonstrated that

infection results in an increase in CD4+ and CD8+ T cells within the CNS. This increase is necessary for clearing the virus. Pre-exposure to SDR resulted in a blunting of infection related increase in CNS T cell mRNA expression. Furthermore, TMEV infection resulted in increases in IFN-γ producing virus specific CD4+ and CD8+ T cells in the CNS, and SDR prior to infection significantly blunts this response. SDR did not significantly alter the levels of T cells in the spleen at day eight post-infection. This would suggest that the adverse effects of SDR on the disease course of TMEV infection are primarily centrally mediated, however, it is possible that if a different time point (e.g., day two) was evaluated a peripheral effect of SDR may be observed.

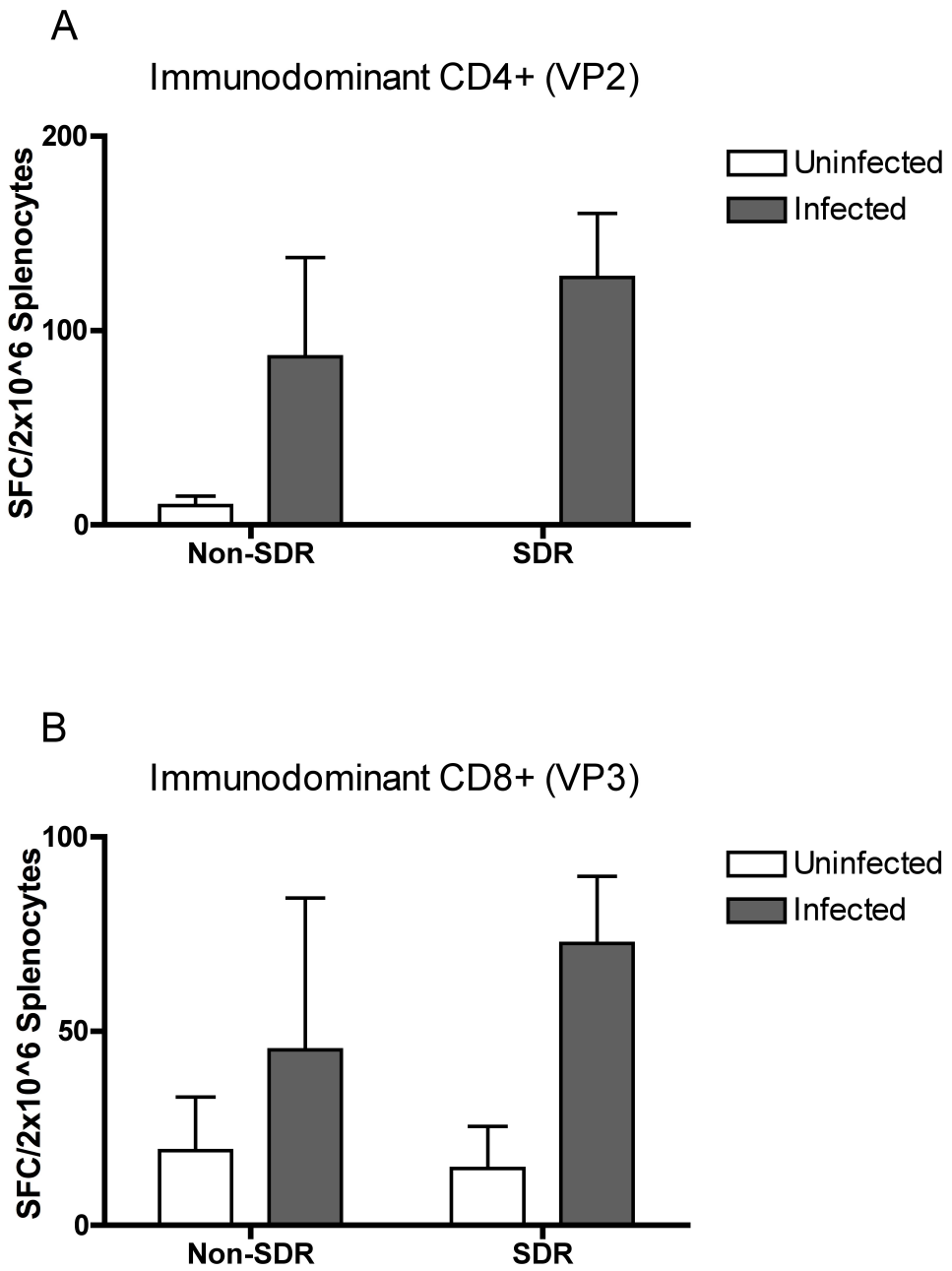


Fig. 14. SDR does not significantly impact peripheral T cell effector function. There was a significant infection related increase in CD4+ T cell effector function and trend toward an increase in CD8+ T cell effector function, however, SDR did not significantly alter these responses.

CHAPTER V

SUMMARY AND DISCUSSION

Evidence indicates that a prior history of stress is linked to onset and exacerbation of autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis, (Ackerman et al., 2002; Grant et al., 1989; Herrmann et al., 2000; Mohr et al., 2000; Mohr et al., 2004; Rimón and Laakso, 1985; Uno et al., 1989; Urrows et al., 1994; Warren et al., 1982). Our laboratory has sought to understand the mechanisms by which social stress exerts these effects using TMEV infection, an animal model of MS (Johnson et al., 2006; Johnson et al., 2004b; Meagher et al., 2007). Prior research demonstrated that social disruption stress leads to higher viral titers and increased inflammatory lesions, which result in an exacerbation of behavioral and immunological measures of the acute and chronic phases of TMEV infection (Johnson et al., 2006; Johnson et al., 2004b; Meagher et al., 2007). Furthermore, IL-6 neutralization during the stress exposure period can reverse the effects of SDR on acute phase TMEV infection (Meagher et al., 2007).

To extend these findings, my dissertation examined the impact of SDR exposure on elements of the innate and adaptive immune response to Theiler's virus infection. Results suggest that the adverse effects of social stress are mediated by stress-induced enhancement of CNS cytokines and suppression of CNS T cell function. First, the impact of SDR on sensitization of proinflammatory cytokines during the stress-exposure period was examined. It was demonstrated that chronic

SDR results in an up-regulation of IL-6 mRNA expression. It was also demonstrated that SDR sensitizes infection-related increases in central and peripheral proinflammatory cytokines. The protective effects of IL-6 neutralizing antibody appear to be partially mediated by reversing the central, but not the peripheral, increase in proinflammatory cytokine expression. Next, the impact of SDR on adaptive immune function was also examined by evaluating T cell function. It was demonstrated that SDR disrupts viral clearance and enhances sickness behavior in response to infection. Furthermore, it was demonstrated that SDR prior to infection attenuates infection related increases in cortical T cell mRNA expression and central CD4+ and CD8+ T cell effector function. Overall, these data suggest that SDR prior to TMEV infection stimulates the inflammatory component of the innate immune system, while suppressing the virus-specific adaptive immune system respone.

Social Disruption Induces CNS Inflammation

In Chapter III the effect of SDR on inflammation was examined. Prior studies have shown that social stress results in peripheral increases in pro-inflammatory cytokines (Engler et al., 2008; Gaab et al., 2005; Merlot et al., 2003; Merlot et al., 2004a; Stark et al., 2002). For example, Stark et al. showed that social disruption stress results in an increase in IL-6 level within plasma and liver. Additionally, Engler et al. (2008) demonstrated that SDR results in an increase in splenic and hepatic IL-1β. The current study extends this research by showing a significant up-

regulation of IL-6 mRNA expression in the hippocampus following chronic exposure to SDR. Furthermore, it was demonstrated that there is a robust increase of hippocampal IL-1β following a single session of SDR. However, this effect appears to habituate, such that by the sixth session of SDR no significant up-regulation of IL-1β was observed.

This effect may be driven by the development of glucocorticoid resistance (Avitsur et al., 2001; Miller et al., 2002; Quan et al., 2001; Sheridan et al., 2000; Stark et al., 2002). It has been demonstrated that chronic exposure to SDR results in a blunting of the anti-inflammatory effects of glucocorticoids on splenocytes (Avitsur et al., 2001; Johnson et al., 2004b; Meagher et al., 2007; Miller and Chen, 2006; Quan et al., 2001). Data from Quan et al. (2001) suggest that the blunting of this effect may occur due to a down-regulation of GC receptors. It has been suggested that the effect of GCR is not limited to the spleen, as a reduction of GC receptor mRNA was also observed in the brain following SDR (Quan et al., 2001).

Prior research has shown that GCR depends upon IL-1β (Engler et al., 2008). Mice lacking the IL-1 type 1 receptor showed an elevation in serum corticosterone, but did not shown an accumulation of CD11b+ cells in the spleen or the development of GCR. Despite research indicating that SDR increases IL-6 release, it is not essential in the development of SDR-induced GCR within the spleen (Stark et al., 2002). Prior research from our laboratory is in line with this, showing the development of GCR even when IL-6 neutralizing antibody is administered (Meagher et al., 2007).

The development of GCR does not occur following all stressors, but appears to develop uniquely in response to social stress (Avitsur et al., 2001; Quan et al., 2001). Chronic exposure to restraint stress does not alter the responsivity of cells to the anti-inflammatory effects of glucocorticoids (Quan et al., 2001). This may explain why social disruption stress has proinflammatory effects (Gaab et al., 2005; Merlot et al., 2003; Merlot et al., 2004a; Stark et al., 2002), in contrast to the anti-inflammatory effects of restraint stress (Mi et al., 2004; Mi et al., 2006a).

Social Disruption Sensitizes TMEV-induced Inflammatory Responses

Furthermore, this dissertation sought to determine if SDR sensitized Theiler's virus-induced proinflammatory cytokine release and if the protection conferred by IL-6 neutralizing antibody is through reversal of this stress-induced sensitization. This hypothesis is supported by a growing body of evidence suggesting prior exposure to social stress can sensitize or prime the inflammatory pathway, such that a subsequent inflammatory stimulus results in an exaggerated inflammatory response (Bailey et al., 2009; Dong-Newsom et al., 2010; Mays et al., 2010; Powell et al., 2011; Quan et al., 2001). For example, repeated social disruption stress resulted in increased HSV-1 infection-related CD11b+ macrophages and proinflammatoy cytokines, IFN-α and TNF-α (Dong-Newsom et al., 2010). Furthermore, SDR enhances allergen-induced airway inflammation (Bailey et al., 2009) and LPS-induced proinflammatory cytokines (Quan et al., 2001).

It has also been shown that other inflammatory-stimuli can sensitize the innate immune response to a challenge (Cunningham et al., 2005; Deak et al., 2005; Johnson et al., 2002; Matsumoto et al., 2006; Perry, 2007). For example, exposure to inescapable shock prior to administration of LPS results in enhanced plasma IL-1β (Johnson et al., 2002). Additionally, administration of IL-6 or exposure to stress has been shown to sensitize inflammatory response to a stressor (Deak et al., 2005b; Matsumoto et al., 2006). Furthermore, exposure to LPS can exacerbate local brain inflammation in prion disease infected mice (Cunningham et al., 2005).

Previous research suggests that SDR may also enhance inflammatory response to TMEV infection (Meagher et al., 2007). It was demonstrated that SDR results in increased IL-6 in sera and increased inflammatory lesions within the CNS that depend upon stress-induced IL-6. This dissertation extended these findings by demonstrating that chronic SDR sensitizes the inflammatory response to TMEV infection. Exposure to SDR prior to TMEV infection resulted in increased infection related central IL-6 and IL-1β mRNA expression and increased peripheral IL-1β and TNF-α mRNA expression. Furthermore, the protective effect of IL-6 neutralizing antibody administration during the stress exposure period reversed the enhancement of central inflammation, but not peripheral inflammation. This suggests that SDR-induced IL-6 serves to sensitize the inflammatory pathway. Furthermore, given that administration of IL-6 neutralizing antibody has been shown to reverse the adverse behavioral effects of IL-6 (Meagher et al., 2007), this data suggests that the sensitization of neuroinflammatory processes mediate the

adverse effects of social stress on TMEV infection, including enhanced sickness behavior, increased histological markers of CNS inflammatory lesions, and reduced CNS viral clearance (Meagher et al., 2007).

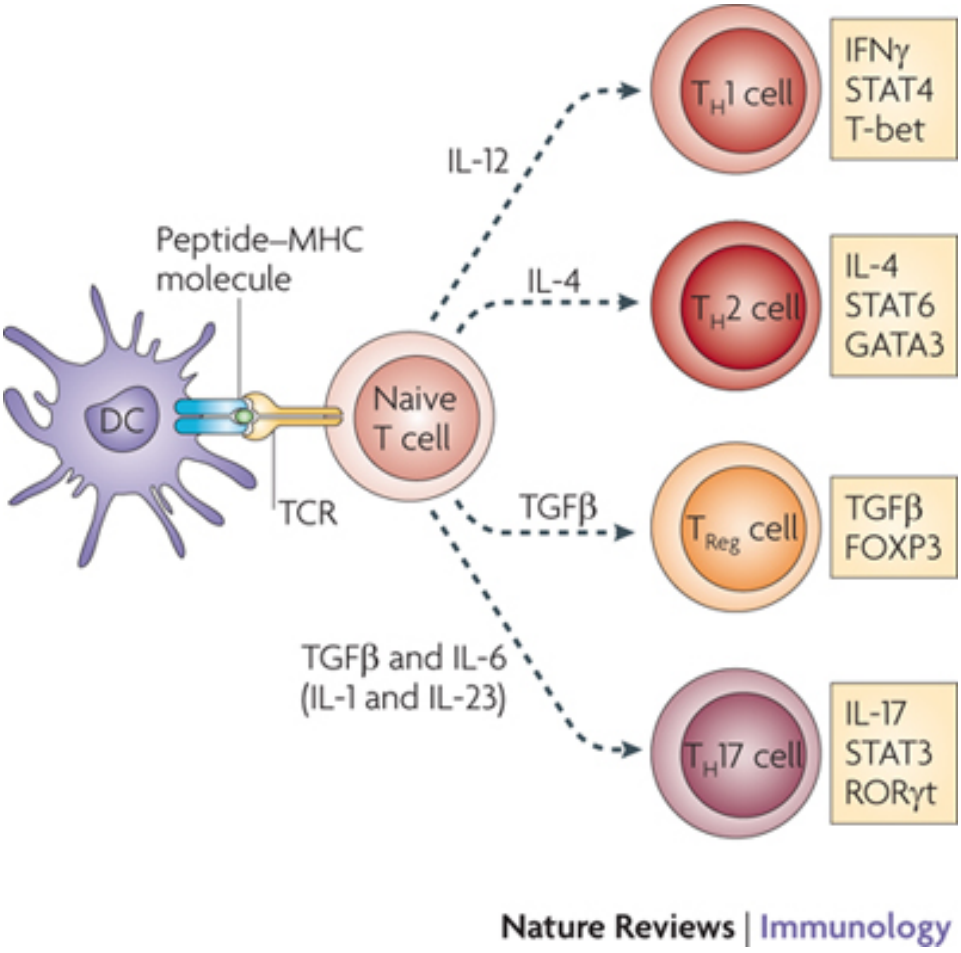
The activation of microglia may underlie this sensitization effect. Microglia are the primary immune cells of the brain and are the primary producers of CNS inflammatory cytokines (Kreutzberg, 1996; Van Dam et al., 1995). It has been demonstrated that administration of minocycline, a glial cell inhibitor, reverses shock-induced increases in central proinflammatory cytokine expression (Blandino et al., 2006). This suggests that they are important mediators of stress induced-neuroinflammation. It has also been demonstrated that stress can induce a hyperinflammatory microglial phenotype (Frank et al., 2007; Nair and Bonneau, 2006; Perry, 2007; Sugama et al., 2007), which may underlie stress-induced exacerbations of neuroinflammation in various diseases, including MS.

The present studies examined the expression of CD11b, a Mac-1 marker associated with microglia and macrophage activation, to indirectly assess the role of microglia. A trend towards an increase in CD11b mRNA expression was observed in the chronic SDR group; however, the effect failed to reach significance. Furthermore, although the interaction between SDR and IL-6 neutralizing antibody treatment for CD11b was not statistically significant, there was (1) a trend suggesting that SDR may increase TMEV-induced CD11b mRNA expression and (2) that this can be reversed with IL-6 neutralization during the stress exposure period. However, given the inconclusive nature of this finding, future research is

needed to determine if priming of microglia is the cellular mechanism underlying stress-induced sensitization of TMEV infection-induced inflammation. Given that many cell types, such as astrocytes and neurons, are also able to release proinflammatory cytokines, these cells may also be involved in stress-induced enhancement of neuroinflammation.

Social Disruption Attenuates Infection Related Adaptive Immune Response

Given the important role the adaptive immune system has in the pathogenesis of TMEV infection, my dissertation also evaluated the impact of SDR on elements of the adaptive immune system. The adaptive immune system has two branches: cell mediated immunity and humoral immunity. Cell mediated immunity includes the actions of cytotoxic T cells (CD8+) in the destruction of infected cells, while humoral immunity is characterized by B cell antibody production. Different helper T cell (CD4+) subtypes (see Fig. 15) help to direct and coordinate these responses. For example, Th1 cells are essential for the development of cytotoxic memory T cells. Both Th1 and cytotoxic T cells produce high levels of IFN-γ that act to stimulate immune function and inhibit viral replication. For effective clearance of the virus, this process should occur early in TMEV infection. Th2 helper T cells assist B cells in their production of antibodies. The Th2 cell response should come after the production of Th1 cells for effective viral clearance to occur. Furthermore,



Fig, 15. Differentiation of naïve T cells. A naïve T cell has the potential to become one of many phenotypes. This decision is based upon the cytokine profile in the extracellular environment. Furthermore, each differentiated cell type results in a different profile of cytokine release. Image borrowed from Zou and Restifo (2010).

a response of regulatory helper T cells (Tregs) should follow the Th2 response. Tregs act to suppress the immune response. Consequently, the presence of Tregs improves disease outcomes by reducing inflammation and decreasing the likelihood of auto-reactive T cell development.

Prior research has demonstrated that stress is capable of inhibiting T cell function. For example, restraint stress results in a reduction in TMEV-specific IFN-γ producing CD4+ and CD8+ T cells in the periphery and CD8+ T cells in the brain (Steelman et al., 2009). Furthermore, chronic social stress has been shown to result in a suppression of plasma IFN-γ and reduced CD8+ T cell expansion in the spleen (Sommershof et al., 2011). This effect appears to be mediated by stress-induced glucocorticoids. To determine if SDR suppresses the adaptive immune response to TMEV infection, Chapter IV examined the role of SDR in modifying TMEV-induced T cell mRNA expression and virus-specific IFN-γ T cell production.

This series of studies was conducted with SJL/J mice. Given that prior work examining the impact of SDR on disease progression was carried out in BALB/cJ mice, it was important to verify that SDR modifies disease pathogenesis in this strain. As anticipated, SDR enhanced sickness immediately following infection (e.g., increased weight loss and decreased sucrose preference) and resulted in increased viral titers at day eight post-infection. No SDR-induce differences were observed on other measures of sickness (i.e., mechanical sensitivity and clinical score); however, this is not surprising given the attenuated acute phase symptomatology

that SJL/J mice display, when compared to BALB/cJ mice, when infected with TMEV.

Prior exposure to SDR altered central T cell mRNA expression and function in response to TMEV infection. As anticipated, infection resulted in robust CD4+ and CD8+ T cell mRNA expression in the cortex on day eight post-infection, a time point where viral titers are high. Prior exposure to SDR resulted in a significant attenuation of this increase in mRNA expression. Furthermore, infection increased virus-specific CD4+ and CD8+ T cell IFN-γ production and prior exposure to SDR significantly attenuated this increase. This suppression likely underlies the poorer viral clearance and increased disease pathogenesis observed in mice exposed to SDR prior to infection.

A stress-induced suppression of T cell expression was not observed in the periphery at day eight, suggesting that SDR may affect TMEV disease course primarily by altering the CNS immune response to infection. However, given that the immune response is generally initiated in the periphery, it is possible that assessing levels at day eight post-infection was too far removed from infection such that the peripheral alterations were missed. Future research evaluating other time points is needed to determine if the effect of SDR on T cell function also extends to the periphery.

Interaction Between the Innate and Adaptive Immune Systems

It has been demonstrated that the initial innate immune response to infection shapes the later adaptive immune response (Biron, 1998; Biron, 1999). Thus, exaggerated infection-related neuroinflammation might have cascading effects, including a dysregulation of T cell and antiviral responses during early infection. Furthermore, a poorer antiviral response during the acute phase is likely to result in increased severity of the later Theiler virus induced demyelination (Lipton et al., 2005; Sieve et al., 2004; Trottier et al., 2004). Given that administration of neutralizing antibody to IL-6 can reverse the adverse effects of SDR on viral clearance, it appears that dysregulation of the innate, inflammatory response to infection results in a dysregulation of the acquired, T cell mediated response.

One mechanism by which increased neuroinflammation may affect T cell function is through altering the CD4+ subtype expressed, shifting the balance away from mounting the most effective immune response. There is evidence that a high level of IL-6 could suppress Th1 responses in favor of Th17 responses. A Th17 immune response can be induced by the presence of IL-6 and TGF-β (or IL-1 and IL-23) (Zou and Restifo, 2010). Given that there is a central up-regulation of IL-6 and IL-1β, this seems a likely possibility. The Th17 cells release IL-17 and IL-21, which serve essential functions in antimicrobial immunity at epithelial and mucosal barriers. However, it has recently been demonstrated that Th17 promote

persistent TMEV infection and exacerbate pathogenesis of chronic phase demyelinating disease (Hou et al., 2009).

This effect may be through the promotion of autoreactive T cells. Interestingly, Th17 has also been linked to the development of a variety of autoimmune diseases, including multiple sclerosis, collagen-induced arthritis, rheumatoid arthritis, and autoimmune myocarditis (Afzali et al., 2007; Bettelli et al., 2006; Bettelli et al., 2007; Chen et al., 2007; Chen and Wood, 2007; Chen et al., 2006; Kleinschek et al., 2007). Specifically, studies of patients with MS confirm the presence of increased IL-17 expression in active brain lesions (Lock et al., 2002) and within the CSF (Ishizu et al., 2005; Matusevicius et al., 1999). Furthermore, cytokines released by Th17 cells have been shown to be associated with disease exacerbation in the EAE model of multiple sclerosis (Komiyama et al., 2006; Vollmer et al., 2005). Further research is needed to determine if social stress prior to infection exacerbates disease by shifting the balance from an efficient acquired immune response and toward Th17.

Another mechanism by which SDR-induced neuroinflammation may disrupt the T cell response to disease is by altering the efficacy of antigen presenting cells (APCs). If microglia and other CNS APCs were less effective at presenting virus to the immune system, this would result in an inadequate T cell response. A trend toward increased CD11b expression would be suggestive of increased activation and presumably primed viral clearance. However, research is beginning to reveal that different stimuli can cause varied microglia activational profiles. For example,

it is possible that this increase may not represent traditionally activated microglia or macrophages that are effective antigen presenting cells, but rather CD11b+Ly6C+ cells. These are immature myeloid cells that exit the bone marrow without maturing and are the predominant innate immune cell to infiltrate into the CNS after infection with TMEV (Bowen and Olson, 2009). Unlike traditional Cd11b+ cells, CD11b+Ly6C+ cells have been shown to suppress T cell responses (Bingisser et al., 1998; Delano et al., 2007; Movahedi et al., 2008; Terabe et al., 2003). Therefore, an increase in CNS infiltration during acute phase disease may result in a disruption of viral clearance.

SDR-induced neuroinflammation may also disrupt trafficking of T cells into the CNS. Chemokines play well-established roles in T cell trafficking and can be suppressed by glucorticoids (Smith and Herschman, 1997). Furthermore, restraint stress results in a suppression of chemokines in response to TMEV infection (Mi et al., 2004). Stress-induced inflammatory cytokines may alter chemokine expression and, subsequent, T cell trafficking. Experiment 4.3 shows that SDR exposure reduces virus specific CD4+ and CD8+ T cells within the CNS but did not alter peripheral T cell response to infection. This pattern could be explained by a reduction in chemokine activity within the CNS decreasing migration of these cells and, thereby, impairing viral clearance.

Impact of Stress on Theiler's Virus Induced Demyelination

Future studies are needed to determine the mechanism by which SDR-induced immune system modifications affect the immune response during the chronic phase of TMEV infection. Prior research has shown that SDR prior to TMEV infection results in an exacerbation of chronic phase disease (Johnson et al., 2006). The literature suggests that a dysregulated adaptive immune system is responsible for viral persistence and chronic phase disease exacerbations. Immunological manifestations of chronic phase disease exacerbation include enhanced inflammation and increased autoreactive T cells. Increased inflammation, as is observed in TMEV infected mice pre-exposed to SDR, can may shift the balance from Tregs, which serve to suppress both inflammation and T cell function, toward Th17 cells, which are prone to autoreactivity. Both pathways require TGF-β, however, increases in IL-6 will cause naïve T cells to preferentially differentiate into Th17 cells (see Fig. 15). The combination of enhanced inflammation with increased autoreactive T cells may explain how stress exacerbates demyelination in TMEV infection.

Furthermore, research is needed to examine the effect of SDR presented at different time points on the immune response to TMEV infection and TVID. The MS literature suggests that stress not only increases susceptibility to developing MS, but also to disease onset and exacerbations in disease course (Ackerman et al., 2002; Brown et al., 2005; Brown et al., 2006; Grant et al., 1989; Li et al., 2004; Mohr et al., 2004; Mohr, 2007). Understanding the mechanisms underlying these effects

would benefit from studies looking at the timing of stress with animal models. Prior data has shown that social disruption stress presented concurrently with infection improves TMEV pathogenesis. It is possible that by presenting the stress concurrent with infection, the anti-inflammatory actions of stress predominated and shifted the balance toward an effective Th1/Th2 anti-viral response. Furthermore, given that T cell suppression is observed in response to SDR, it would be interesting to evaluate the effect of social stress presented during the demyelinating phase of the disease. Based on the suppression of the acquired immune response it may improve disease, however, the enhance inflammation and possible Th17 switch may instead exacerbate disease pathogenesis.

Summary and Implications

In summary this dissertation demonstrates that exposure to SDR modifies components of the innate and adaptive immune response to Theiler's murine encephalomyelitis virus infection. It suggests that SDR-induced release of IL-6 sensitizes virus-initiated proinflammatory cytokine release. This enhanced CNS proinflammatory cytokine release appears to initiate a less effective adaptive immune response and, consequently, results in an exacerbation of disease course.

This line of work may have broad implications for understanding the role of stress and cytokine expression in altering vulnerability to MS and other inflammatory neurodegenerative diseases. Elucidating the mechanisms by which social stress exacerbates the severity of a virally-initiated autoimmune disease may

lead to the development of new interventions to prevent or reverse the adverse effects of stress. This line of research suggests that anti-inflammatory behavioral and/or pharmacological interventions may improve neurodegenerative and inflammatory disease course. Furthermore, this line of research may provide insight into the personal and immunological individual profile that is more likely to benefit from particular therapeutic interventions, such that interventions can be better targeted. This series of experiments brings us closer to understanding these mechanisms by demonstrating that social disruption stress sensitizes Theiler's virus-induced neuroinflammation and suppresses virus-specific T cell function within the CNS.

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