BEHAVIORAL AND IMMUNOLOGICAL EFFECTS OF REPEATED SOCIAL DEFEAT

DISSERTATION

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By

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Mammals respond to environmental threats through complex neuroendocrine processes, collectively referred to as the stress response. Once activated, the stress response affects the regulation of many other physiological systems, including the immune system. For people living in modern, developed nations, social stress is the most common stimulus for the induction of the stress response. Subjects reporting high levels of social stress are more likely to experience increased anxiety and depression and to engage in unhealthy coping strategies and high-risk behaviors. Social disruption (SDR) is an animal model of social stress in which mice are repeatedly attacked and defeated in their home cage by an aggressive conspecific, or member of the same species. Social disruption has been reported to cause increased production of proinflammatory cytokines and glucocorticoid (GC) insensitivity in splenic macrophages. To this point however, the behavioral consequences of SDR have not been thoroughly characterized. Because social defeat has been reported to cause anxiety- and depressive-like behaviors in humans and rodents, the first study was designed to assess whether SDR causes anxiety- and depressive-like behaviors. The light/dark preference test and the open field test were used as tools to measure behaviors characteristic of anxiety. Both C57BL/6 and CD-1 male mice subjected to SDR displayed increased anxiety-like behavior. The increase in
anxiety-like behaviors persisted for at least one week after the cessation of the stressor. In contrast, depressive-like behaviors were not elicited by SDR, as assessed by the forced swim test and the tail suspension test.

The second study focused on the role of aging in social defeat. Previous research indicates that repeated social defeat of young adult mice causes increased lymphocyte trafficking to the spleen, elevated proinflammatory cytokine production, and glucocorticoid insensitivity in splenocytes. This study investigated whether repeated social defeat resulted in similar immunoregulatory changes in aged mice, as seen previously in young adults. Regardless of age, defeated mice displayed significantly higher anxiety-like behavior, as measured in the open field test, and more splenic CD11b+ Gr-1+ monocytes and neutrophils than controls. Supernatants harvested from cultured splenocytes from old mice contained comparatively higher IL-6 and TNF-α than supernatants from young animals. In addition, those same cells derived from aged defeated mice were hypersensitive to lipopolysaccharide and insensitive to glucocorticoids in vitro. These data indicate that repeated social defeat results in a proinflammatory state that is exacerbated in aged mice. The implications of these data are noteworthy, given that inflammation worsens symptoms of many age-related diseases, and is a possible root cause of Alzheimer’s disease.

The third study presented herein determined whether pharmacologically blocking SDR-induced anxiety-like behaviors also blocked the development of GC insensitivity. Neither anxiolytic nor anxiogenic drug significantly affected the development of GC insensitivity, although the chosen dose of diazepam was sufficient to have sedative effects on the mice. These data suggest that the development of anxiety-like behavior
does not directly cause the observed immunological effects of social disruption. Taken together, these data indicate that social disruption stress caused an increase in anxiety-like behaviors, but not depressive-like behaviors. This increase in anxiety-like behavior was also observed in aged mice, which were predisposed toward increased inflammation, and this effect was further exacerbated by social defeat. Pharmacologic blockade of anxiety-like behavior neither attenuated nor exacerbated the previously-observed defeat-induced changes to immune function, suggesting that the SDR-induced anxiety-like state does not causally affect immunity.
Dedicated to my family and the many friends who supported me throughout my education.
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HBSS  hank’s balanced salt solution
HPA  hypothalamic-pituitary-adrenal
IFNγ  gamma interferon
IκB  inhibitory factor kappa B
IL  interleukin
L  liter(s)
LPS  lipopolysaccharide
m  milli (10⁻³)
µ  micro (10⁻⁶)
M  moles per liter
min  minute(s)
n  nano (10⁻⁹)
NF-κB  nuclear factor kappa B
O.D.  optical density
OF  open field test
p  pico (10⁻¹²)
PE  phycoerythrin
PerCP  peridinin chlorophyll protein
PNI  psychoneuroimmunology
mRNA  messenger ribonucleic acid
RST  restraint stress
rt  room temperature
SDR  social disruption
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CHAPTER 1

INTRODUCTION

The Stress Response and Immunity

Animals live in constantly fluctuating environments. External conditions, such as temperature, food availability, and time of day, can change dramatically over time. Similarly, internal conditions such as body temperature, energy storage, and reproductive status can also be highly variable. In order to survive and thrive in such changing environments, animals use many regulatory mechanisms that collectively work to maintain homeostasis. One such regulatory mechanism is the stress response, which is activated in vertebrates when homeostasis is threatened (Selye, 1936). The perceived threat to homeostasis is referred to as a stressor.

In the short term, the stress response can be advantageous, although after being activated repeatedly, or activated for a lengthy time, the response begins to have deleterious effects on various systems, including immune function (reviewed by Bailey et al., 2003; McEwen, 1998; Padgett & Glaser, 2003). Stressors can take many forms, including physiological (e.g. food deprivation), physical (e.g. cold, pain), or psychological (e.g. restraint, social defeat, public speaking). Although these stressors differ qualitatively, perception of each can result in activation of the stress response.
Although it is generally accepted as common knowledge that long-term stress is an aversive state that is probably associated with negative health outcomes, studies of stress and immunity have recently been operationalized and studied under controlled conditions. Some of the earliest evidence for behavioral influence on immunity came from studies of illness-induced taste aversion. Cyclophosphamide, a nausea-inducing immunosuppressive drug, was paired with saccharine to condition a taste aversion to the saccharine in rats. When the conditioned rats were later injected with sheep red blood cells (SRBC), rats that were provided with saccharine showed an attenuated response to the injected SRBC, as compared with rats that did not consume saccharine (Ader, 1974). When the experiment was repeated using a non-immunosuppressive nausea-inducing drug, the taste aversion to saccharine was conditioned, but the immune response was unaffected (Ader & Cohen, 1975).

The early work in the influence of behavioral factors on immunity has grown in recent years into a multidisciplinary field of research called psychoneuroimmunology (PNI). Many researchers in the field of PNI study the bidirectional communication between the central nervous system (CNS) and the immune system, with particular interest paid to the effects of stress on immunity. Studies in humans suggest that stress has deleterious effects on immunity. In one study, dental students were given an oral wound during coursework exams, or during non-exam time. Not surprisingly, the students reported increased stress during exams, however, their wounds took roughly three days longer to heal than wounds during non-exam time (Marucha et al., 1998). Examination stress has also been reported to exacerbate asthma symptoms in college students (Liu, et al., 2002), and general self-reported stress has been connected with
increased asthma symptoms (Oh et al, 2004). Similarly, aged caregivers of Alzheimer’s
disease patients showed decreased wound healing rates (Kiecolt-Glaser et al., 1995) as
well as a blunted antibody response to influenza virus vaccine (Kiecolt-Glaser et al.,
1996). Stress can also influence health indirectly by increasing unhealthy coping
mechanisms. Low socioeconomic status, for example, is correlated with increased
production of the stress hormone cortisol and increased likelihood of smoking, regardless
of race (Cohen et al., 2006).

Animal models of stress have further expanded research in PNI, with particular
interest to areas that are more limited in human subjects, such as the mechanisms through
which stress and immunity interact. Animal models of human stressors typically place
the animal into an unpredictable situation over which it has very little control. Physical
restraint is a commonly used psychological stressor in rodents. Padgett and colleagues
(1998b) demonstrated that eight days of 12-hour restraint was sufficient to increase
plasma concentration of the stress hormone corticosterone and delay wound healing in
mice. However, although shorter duration restraint stress also increased plasma
corticosterone in hamsters, it increased wound healing rates (Kinsey et al., 2003), and
delayed type hypersensitivity (Bilbo et al., 2002). Inescapable electric shock is another
stressor that suppresses lymphocyte proliferation in response to the mitogen
phytohemagglutinin in a proportion to the severity of the stressor. That is, rats that were
placed in the tail shock apparatus but did not receive shock had higher lymphocyte
proliferation than rats that received a mild shock, which had higher proliferation than rats
that received a strong shock (Keller et al., 1981). Taken together, these data suggest that the frequency and duration of the stressor play a role in the direction and severity of the ensuing immune response.

Neuroimmune Connections

The stress response to the threat is a well-characterized psychological and physiological phenomenon that results in activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (Black, 2002; Sapolsky, 1992). After the perception of a stressor, the ventromedial hypothalamus releases the neuropeptide corticotrophin releasing hormone (CRH), which travels via the hypothalamic portal system to the pituitary gland. The anterior pituitary releases adrenocorticotropic hormone (ACTH), which is carried through the bloodstream to the adrenal cortex, prompting the release of glucocorticoids (GC) into circulation (Chrousos, 1998; DeVries, 2002, Padgett & Glaser, 2003). The primary GC released by the adrenals varies between species, and in rodents including mice, the primary GC hormone is corticosterone. There are many target organs and cell types for glucocorticoids, and GC receptors are expressed throughout the body (Muller et al., 2002). In the absence of glucocorticoid, the GC receptor is sequestered in the cytoplasm, bound by heat shock proteins (Pratt & Dittmar, 1998). Upon ligand binding, these proteins dissociate from the receptor and it translocates to the cell nucleus, where it affects gene transcription (Barnes, 2006).

One potent effect of GC is to reduce the production of proinflammatory cytokines. The production of proinflammatory cytokines including IL-1, IL-6, and TNF-α, for example, is regulated by the inflammatory mediator nuclear factor κB (NF-κB). As
NF-κB activation increases, so does the production of these proinflammatory cytokines. Activation of NF-κB is inhibited by inhibitory factor κB (IκB), which is upregulated by glucocorticoids (Castro-Caldas et al., 2003). Alternatively, GC may interfere with NF-κB by directly binding to NF-κB heterodimer subunits, and preventing translocation to the nucleus (Adcock & Caramori, 2001).

The effects of GC on proinflammatory production are evident in animal models of stress. Repeated restraint stress, for example, had a suppressive effect on the production of IL-1α in influenza-infected mice (Konstanos & Sheridan, 2001). Similarly, repeated restraint reduced wound healing and expression of IL-1β and keratinocyte growth factor (KGF-1) in the wounds (Mercado et al., 2002). However, not all stressors result in decreased cytokine production. A social stressor, social disruption, results in increased serum corticosterone, in addition to increased expression of proinflammatory cytokines (Avitsur et al., 2001).

Social Disruption

Social stress is the most commonly experienced stressors for modern day people in developed parts of the world (Kiecolt-Glaser et al., 2002; Liu, et al., 2002). Much of the human literature on social stress focuses on chronic stressors, such as low socioeconomic status (Cohen et al., 2006; Neupert et al., 2006), long-term care giving of a sick relative (Graham et al., 2006), workplace harassment and bullying (Dao et al., 2006; Hansen et al., 2006), or short-term, laboratory-induced stressors (Dickerson & Kemeny, 2004). However, human studies are limited in their scope and ability to
measure mechanisms through which stress affects immunity. Thus, animal models of
social stress have been recently developed as analogues to human stressors.
Furthermore, because social stress is so common in humans, animal stress models that
contain a social component are more ecologically relevant to modern healthcare than
models lacking a social component.

Social disruption (SDR) is a stressor that was recently developed in our lab as a
murine model of social stress (Stark, et al., 2001). During social disruption, resident
mice are group-housed and allowed adequate time to establish a social hierarchy. This
hierarchy is then disrupted by the introduction of an aggressive intruder mouse, which
repeatedly attacks and defeats the resident mice. After 2 hours of SDR, plasma
corticosterone is elevated 2-fold in the resident mice, but returns to baseline within 14
hours (Avitsur et al., 2001). Social disruption also results in splenomegaly, thymic
atrophy, increased adrenal mass and increased release of pro-inflammatory cytokines
(Avitsur, et al., 2002b). These immunological changes have strong effects on immune
function in the defeated resident mice. Mice exposed to SDR are also more susceptible to
lipopolysaccharide- (LPS) induced endotoxic shock and death (Quan et a., 2001). This
increased susceptibility to endotoxic shock is, in part, caused by an increase in the
production of the pro-inflammatory cytokines IL-1, IL-6 and TNF-α. In vitro, LPS-
activated splenocytes from mice subjected to SDR produce higher levels of these
cytokines, compared with splenocytes from non-defeated controls (Stark et al., 2001;
Avitsur et al., 2003, Quan et al., 2001).
A second mechanism that mediates the increased response to LPS is a decrease in the anti-inflammatory effects of corticosterone on mononuclear cells. Spleen cells harvested from SDR mice and cultured with LPS show a marked insensitivity to the steroid corticosterone (Stark et al., 2001). Unlike cells from non-defeated mice, these cells remain viable when cultured with high levels of glucocorticoid (Stark, et al., 2001). Depletion of specific cell phenotypes via magnetic microbead exclusion indicates that this glucocorticoid (GC) insensitivity is largely present in CD11b+ cells (Stark et al., 2001). The CD11b+ molecule is primarily expressed on monocytes, macrophages, and neutrophils. These cells are critical to the primary, or innate, immune response and appear to account for a large part of the GC insensitivity that results from SDR. The innate immune response acts as the first line of defense against microbes that enter the body. Thus, social disruption stress directly affects innate immunity.

In addition to increasing the overall inflammatory response, social defeat also has etiologically pertinent effects on infection. Mice subjected to SDR and subsequently infected with A/PR8 influenza showed reduced mortality, compared to non-stressed controls, suggesting that SDR has mildly protective effects against the virus (Hunzeker, 2004). In contrast, restraint stress had suppressive effects on immune cell recruitment in influenza-infected mice (Hunzeker et al., 2004). Theiler’s virus infection causes central nervous infection and is a murine model of multiple sclerosis. Unlike influenza infection, mice subjected to SDR prior to intracranial Theiler’s virus infection showed greater hind limb immobility and decreased viral clearance in the spinal cord (Johnson, et al., 2004). However, it should be noted that mice subjected to SDR after viral infection showed some reduced hind limb immobility and lower serum IL-6 (measured 9 days post
infection), as compared with non-defeated controls (Johnson, et al., 2006), suggesting that SDR differentially affects Theiler’s virus infection outcomes in a time-dependent manner.

Social defeat in a related model, social reorganization, caused the reactivation of latent herpes simplex virus infection, although this effect was not seen with other stressors, including restraint (Padgett et al., 1998c). Social disruption stress also affected herpes virus infection, by increasing macrophage infiltration into infected trigeminal ganglia, which in turn increased TNF-α and IFNα mRNA transcription (Dong-Newsom et al., 2007). In contrast, restraint stress suppressed IFNα production, resulting in decreased host control over herpes virus replication at the site of infection (Ortiz et al., 2003). Furthermore, although restraint has been shown repeatedly to affect wound healing rates, SDR had no effect on wound healing in mice (Sheridan et al., 2004). Taken together, these data suggest that social defeat stressors have unique effects on immunity.

Although social disruption results in many of the immunological changes outlined above, not all defeated animals exhibit these changes to the same extent. Previous work suggested that wounding was an important aspect of SDR-induced changes in immune function (Avitsur et al., 2001; Bailey et al., 2004). However, wound severity has, so far, not been associated with the severity of GC insensitivity, for example. Beyond wound severity, other, subtler changes between defeated animals, such as changes in behavior, may account for these individual differences in the development and expression of these immune changes.
Social Disruption, Anxiety, and Depression

Stress results in behavioral changes, in addition to changes in immunity. Chronic mild stress is routinely used to induce depressive-like behavior in mice (Willner, 1997). In this model, mice are subjected to a series of unpredictable stressors, including cage tilt, changed bedding, predator sounds, and reversed light cycle. Chronic mild stress also causes increased aggression (Mineur et al., 2003) and anxiety-like behavior (Mineur et al., 2006). Social defeat is not only stressful, but can also cause lasting behavioral changes.

Similar behavioral effects are observed in socially defeated animals, including humans. The human literature differs from non-human animal literature in that it typically reports social defeat as bullying, although the behavioral outcomes are comparable (Björkqvist 2001). For example, children who are victims of bulling report increased anxiety and depression, as compared with non-bullied children (Hawker and Boulton, 2000). In addition to increased anxiety and depression, bullied children were more likely to develop psychosomatic conditions, including stomach pain, interrupted sleep, and reduced appetite. Furthermore, after developing these conditions, the incidence of bullying further increased (Fekkes et al., 2006). There are similar effects of bullying in the workplace. Both men and women who reported being a victim of or witness to work-related bullying also reported increased anxiety and depression (Björkqvist 2001). Bullied workers also had higher salivary cortisol concentrations than non-bullied peers (Hansen et al., 2006).

In the SDR model, resident mice are repeatedly subjected to an aversive stimulus: inescapable attack by an aggressive intruder mouse. The resident mice are often
wounded by the aggressor, which is presumably painful. Thus, the intruder mouse is potentially a fear and anxiety-inducing stimulus. The difference between fear and anxiety is somewhat controversial. However, most researchers agree that fear represents a more acute response to a specific nocuous stimulus (i.e. the intruder), whereas anxiety typically manifests as a more generalized apprehension in response to context-dependent stimuli (Berntson et al., 1998; Le Doux, 2000; McNaughton & Corr, 2004). During the sessions of inescapable attack, the aggressor obviously is a fear-inducing stimulus to the resident mice. The mice flee the aggressor and vocalize when attacked. However, the aggressor may also simultaneously act as an anxiety-inducing stimulus. If so, the anxiety response may manifest itself as a generalized anxiety-like state in the resident mice.

Indeed, repeated defeat has been shown to induce anxiety-like behavior in rodents. Anxiety is typically measured in rodents by modeling environmental approach/avoid conflicts between exploration of a novel environment and preference of dark, sheltered areas (reviewed by Ohl, 2005). In one study, rats that were repeatedly defeated in the visual burrow system showed enhanced immobility after being handled and a longer latency to right, after being overturned (Blanchard et al., 2001). Socially defeated rats showed higher defensive burying, an anxiety-like behavior, than non-defeated rats (Sgoifo et al., 2005). Similarly, in mice, chronic social defeat caused a decrease in social behavior and decreased time spent in the dark portion of the light/dark preference test (Keeney and Hogg, 1999). Mice repeatedly defeated by a conspecific showed increased anxiety-like behavior in the elevated plus maze and the partition test, as compared with mice that were not defeated (Avgustinovich et al., 1997).
In addition to anxiety, social defeat can also cause increased depressive-like behavior. Depression is typically measured by tests purported to elicit behavioral despair (lack of escape-seeking behavior) or anhedonia (reduced saccharine consumption) in mice and rats (reviewed by Cryan & Mombereau, 2004). In DBA/2 mice, acute social defeat resulted in depressive-like behaviors in the Porsolt forced swim test, with defeated mice spending more time immobile in the water, as compared with controls, which spent more time actively struggling and trying to escape the test apparatus (Hebert et al., 1998). Increased immobility was also observed in socially defeated male NIH Swiss mice (Hilakivi et al., 1989). However, social defeat has also been shown to decrease immobility in the forced swim test (Keeney & Hogg, 1999).

Defeat-induced behavioral changes may vary as a function of time between stressor and testing. For this reason, behavior was recorded both immediately after SDR and after an interval of 18 hours. In addition to anxiety, social defeat has been shown to decrease exploratory behavior (e.g. Avgustinovich et al., 1997), which can therefore bias the tests toward over reporting anxiety-like behavior (Bourin & Hascoët, 2003). The ethological paradigms in the studies outlined in Chapter 2 were chosen to test anxiety- and depressive-like behaviors while also measuring exploration and locomotion. These experiments also investigated whether the previously-observed effects of SDR on immunity and behavior could be extended to an outbred mouse strain.

**Age-Dependent Effects of Social Disruption**

As animals age, many physiological systems lose function and efficiency. This loss of function is commonly referred to as senescence, and age-dependent changes in
immune function are referred to as immunosenescence (Bauer, 2005; Castle, 2000). In humans, this compromised immunity is often evident as increased susceptibility to viral and bacterial infections and decreased response to vaccines (Miller, 1996). The specific mechanisms through which immunosenescence occurs are a topic of much current research. Of particular interest is the function and regulation of innate, or natural, immunity. The innate immune response is responsible to initially detecting and containing invading microbes, as well as recruiting other immune cells to the site of infection. The innate immune system is made up of many cell types, including natural killer (NK) cells, neutrophils, and macrophages, and these cell types communicate primarily via secreted proteins called cytokines.

Proinflammatory cytokines act to induce the activation on inflammatory cells and, in concert with chemokines, increase the activation of adhesion molecules, which aid in the recruitment and localization of other leukocytes to the site of infection (Abbas et al., 2000). Antiinflammatory cytokines, such as IL-10, act to inhibit the release of proinflammatory cytokines and thereby act as a regulatory mechanism of the inflammatory response to antigens.

One of the effects of aging is an increase in proinflammatory cytokines. In humans, increased serum IL-6 and TNF-α have been reported in healthy elderly patients and have been linked in epidemiological studies with increased mortality (Bruunsgaard et al. 2000; Harris et al. 1999; Volpato et al. 2001). Increased proinflammatory cytokines have also been reported in aged rodents. In the spleen as well as lymph nodes, aged mice have been reported to release higher levels of IL-6 than young adult mice (Daynes et al. 1993). Godbout and colleagues (2004, 2005) found that CNS immune cells of aged mice
were “primed” towards inflammation. When injected peripherally with LPS, the aged mice expressed higher amounts mRNA for IL-1β and IL-6 than young adult mice.

Similar to the effects of aging, previous work has repeatedly shown that, in mice, repeated social defeat induces an increase in the secretion of IL-1, IL-6, and TNF-α by activated splenocytes (Avitsur et al., 2002a; Quan et al., 2001; Stark et al., 2002). These effects are not limited to in vitro work. Social disruption resulted in increased mortality and endotoxic shock in LPS-injected mice, at normally sub-lethal doses (Quan et al., 2001). However, the effects of aging and stress are rarely tested together in animal models. In mice, aging has been shown to increase mortality after influenza infection, although restraint stress by itself had little effect on mortality. However, aging and restraint stress had a synergistic effect on immunity, resulting in extremely high levels of mortality after infection (Padgett et al., 1998a).

The effects of aging and repeated social defeat have not been tested together. The experiments in Chapter 3 investigate the effects of social defeat in aged and young adult mice. The hypotheses are that a) aging results in increased proinflammatory cytokine responses, and these effects are exacerbated by repeated social defeat, and b) the defeat-induced GC insensitivity previously observed in young adult mice will be increased in aged mice.

**Anxiety and Benzodiazepines**

As proposed by McNaughton and Gray (2000), fear and anxiety are distinct behaviors. Fear involves the animal moving away from a threat, and typical behaviors include fleeing and freezing. Anxiety, on the other hand, involves approach-avoidance
conflicts with the animal moving toward a potential threat and increased threat assessment. The authors further state that anxiety and fear often occur in parallel, and that anxiety is generated by activation of fear circuits. A central component of human anxiety disorders is the inability to properly regulated and interpret social and environmental stimuli, resulting in inappropriate activation of fear responses (Phelps & LeDoux, 2005).

Although the subjective experience and expression of fear and anxiety differs greatly, the underlying neurocircuitry of each response appears to overlap greatly. Because the circuits underlying fear have been successfully operationalized, they are currently among the best understood of the emotional circuits (LeDeux, 2000). The amygdala, an evolutionarily conserved bilateral structure located in the temporal lobes, is a critical component of anxiety, fear, and threat assessment. In humans, lesion or damage to amygdala leads to blunted emotional expression, decreased perception of facial expressions, and loss of normal response to fear-inducing stimuli (Adolphs et al., 1994; Amaral et al, 2003). This is supported in data from human brain imaging studies, which suggest that fearful auditory and visual stimuli increase neural activity in the amygdala (Davidson et al., 2000; Isenberg et al., 1999; but see also Adolphs, 2006).

In addition to the amygdala itself, several other cortical and subcortical brain structures including the hippocampus, prefrontal cortex, and periaqueductal gray, have also been implicated in fear and anxiety, collectively referred to as the amygdala complex (McNaughton & Corr, 2004). The prefrontal cortex appears to play a role in anxiety and behavioral inhibition of fear (Davidson et al., 2000). The basal forebrain also appears to play a modulatory role in anxiety, possibly as a relay between the cognitive aspects of
anxiety in the cortex and the autonomic aspects of anxiety in subcortical regions (Berntson et al., 1998). In rats, lesion of cholinergic neurons in the nucleus basalis magnocellularis resulted in anxiolytic effects, reducing anxiety by reducing operant suppression (Knox & Berntson, 2006). Moreover, patients with anxiety disorders such as obsessive-compulsive disorder often have increased activity in the amygdala and basal ganglia, coincident with feelings of anxiety (Breiter et al., 1996).

Anxiety disorders are commonly treated with drugs that affect the function of the amygdala. Benzodiazepines (BZ) are among the most commonly used pharmacological treatments for anxiety in humans and have also been found to decrease anxiety-like behaviors in mice (Bourin & Hascoet, 2003; Guillot & Chapouthier, 1996). Benzodiazepines are bound by the GABA<sub>A</sub> receptor complex on neurons within the amygdala and act as agonists, decreasing anxiety (Guillot & Chapouthier, 1996). GABA (Gamma aminobuteric acid) is characterized as an inhibitory neurotransmitter. The physiological action of GABA on neurons is to open Cl<sup>-</sup> channels in the cell membrane, increasing the intracellular influx of negatively charged Cl<sup>-</sup> ions, thus inhibiting action potentials. Conversely, benzodiazepine inverse agonists reduce sensitivity of the GABA receptors, commonly resulting in increased feelings of anxiety. Benzodiazepine drugs are bound by different receptors throughout the CNS, differentially affecting anxiety and sedation. However, it appears that binding of α1 and α2 benzodiazepine agonists by receptors in the fear and anxiety complex, including the amygdala, results in anxiolytic effects, whereas binding of α3 receptors in the cerebellum has sedative effects (Löw et al., 2000).
Diazepam (Valium) is a commonly used benzodiazepine agonist that has anxiolytic effects on behavior in mice and humans (Crawley & Goodwin, 1981; Witek et al., 2005). Diazepam binds to four different subtypes of GABA<sub>A</sub> receptors, α1, α2, α3, and α5, which are expressed differentially throughout the central nervous system (Chebib and Johnston, 1999; Löw et al., 2000). In mice, the GABA<sub>A<sub>α2</sub> receptor subtype is expressed throughout the amygdala complex, including the cortex, striatum, and periaqueductal gray, and deletion of the GABA receptor induces anxiety-like behavior. Point mutations within the GABA receptor gene ablate the anxiolytic effects seen in the light/dark preference and elevated plus maze tests, with no effect on locomotion (Löw et al., 2000). Furthermore, genetic knock-out mice lacking GABA receptors spent less time in the lighted portion of the light/dark preference test and reduced immobility in the forced swim test, both interpreted as anxiogenic, or anxiety-inducing, effects by the authors (Mombereau et al., 2005). Therefore, it appears that, in mice, binding of the α2 GABA<sub>A</sub> receptor by diazepam within the fear and anxiety circuits results in a reduction in anxiety-like behavior.

The major goal of the studies outlined in Chapter 4 was to determine whether the behavioral changes associated with social stress and the development of GC insensitivity, as outlined above, could be reversed, induced, or exacerbated by pharmacological manipulation. Mice were treated with anxiolytic and anxiogenic drugs, subjected to six cycles of SDR, and then individually tested for previously observed SDR-induced behavioral and immunological changes. The hypotheses were that (a) repeated administration of anxiolytic drugs decreases SDR-induced anxiety-like behavior and GC
insensitivity in defeated mice; and (b) anxiogenic drugs induce anxiety-like behavior and mimic the effect of SDR on GC insensitivity in non-defeated mice.
CHAPTER 2

SOCIAL DISRUPTION STRESS CAUSES INCREASED ANXIETY-LIKE BEHAVIOR AND ALTERS SPLENOCYTE FUNCTION IN C57BL/6 AND CD-1 MICE

Introduction

Social defeat stress causes changes in immune function and behavior. In an animal model of social stress termed social disruption (SDR), resident mice are subjected to repeated attack and defeat in their home cage by an aggressive intruder mouse (Stark et al., 2001). Mice that display submissive behaviors during SDR are still attacked by the aggressor (Avitsur et al., 2001). After the two-hour session of SDR, defeated mice show a two-fold increase in plasma corticosterone (Avitsur et al., 2001) demonstrating the high magnitude of the ensuing stress response.

Social defeat has been shown to have immunosuppressive effects. For example, socially defeated mice had reduced mitogen-induced splenocyte proliferation, as compared with handled controls (Beitia et al., 2005). Similarly, splenocytes from socially defeated mice immunized with sheep red blood cells formed significantly fewer plaque-forming and rosette-forming cells than controls (Devoino et al., 2004). Defeat in the SDR model, however, causes an increase in the production of the proinflammatory
cytokines IL-1α, IL-6, and TNF-α (Avitsur et al., 2002b; Avitsur et al., 2003) and a marked insensitivity to the glucocorticoid hormone corticosterone (Stark et al., 2001). Similar to the observed differences in the immune effects of SDR, the biobehavioral response to SDR may also differ from that of other social stressors.

Social defeat has also been shown to cause lasting behavioral changes in rodents, including the development of anxiety-like behaviors. For example, rats that observed a partner rat as it received inescapable foot shock displayed increased defensive burying, a behavior associated with anxiety (Guttiérrez-García et al., 2006). Similarly in mice, repeated social defeat increased anxiety-like behavior in the light/dark preference test, with defeated mice spending more time in the light area of the apparatus, but had no effects on depressive-like behavior in the Porsolt forced swim test (Keeney and Hogg, 1999). However, mice that received just one eight-minute session of social defeat showed increased immobility in the Porsolt forced swim test (Hebert et al., 1998).

The effects of repeated social defeat in the social disruption model on anxiety- and depressive-like behaviors have not been explored. Like the immunological effects of SDR, the behavioral response to social disruption may differ from other social stressors. Thus, this study tested the effects of SDR on anxiety-like and depressive-like behaviors. Changes in cytokine production, splenic cell populations, and glucocorticoid insensitivity were also measured. These effects were measured in both inbred and outbred mouse strains.
Methods

Animals. Male C57BL/6 and CD-1 mice were purchased from Charles River and housed in groups of 3-5 in polycarbonate cages and maintained under 12:12 light cycle in a temperature (21 ± 1 °C) and humidity (50 ± 5%) controlled, Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility at the Ohio State University for the duration of this experiment. Standard lab diet and tap water were available ad libitum. All mice were aged 8-10 weeks and were experimentally naïve at the beginning of the experiments. Mice were handled minimally, for the purpose of general husbandry. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) at the Ohio State University.

Social Disruption (SDR). The social disruption paradigm was described previously (Avitsur et al., 2001, Stark et al., 2001). Briefly, social disruption consisted of introducing an aggressive intruder mouse into the home cage of the resident mice. The resident mice were the subjects of these experiments. Aggressors were introduced to each cage for six daily cycles lasting two hours each, starting at approximately 1700 EST. The aggressor mouse usually attacked the resident mice within 5 minutes of being introduced into the cage. Aggressors were C57BL/1 or CD-1 males, selected based on having a history of agonistic behavior. Aggressors were single housed for a period of several weeks to further induce aggressive behavior. Sessions were monitored to ensure that the intruder repeatedly attacked and consistently defeated the resident mice. If the aggressor did not attack the residents, or if the residents defeated the aggressor, then the aggressor was removed and replaced by a new aggressor mouse. During SDR, the
resident mice generally displayed submissive behaviors, including upright submissive posture, fleeing, and crouching. A different aggressor was used during each cycle to prevent habituation. Control mice were left undisturbed in their home cages during SDR sessions.

\textit{Wound Severity}. Cutaneous wounds were assessed at sacrifice, as described previously (Avitsur et al., 2001), with some changes. Wounding was quantified by a trained observer on a 5-point scale, ranging from 0 to 4, with 0 representing no visible wounds, and 4 representing large wounds (Hilakivi et al., 1989).

\textit{Glucocorticoid Insensitivity Assay}. Glucocorticoid (GC) insensitivity can be quantified by measuring the viability of cells cultured with various physiological and pharmacological concentrations of glucocorticoid in the absence or presence of LPS (lipopolysaccharide). High doses of corticosterone typically reduce the viability of LPS-stimulated splenocytes by inducing apoptosis. However, splenocytes harvested from SDR mice remain viable even in the presence of high concentrations of corticosterone (Stark et al., 2001). In the present study, mice were sacrificed by CO\textsubscript{2} asphyxiation on the morning following the last day of behavioral testing (for all groups: 15 days after the first cycle of SDR). Wound severity and individual body weights were assessed at this time. Spleens were harvested, weighed, and homogenized for one minute by a stomacher (Stomacher 80 Biomaster, Seaward, London, England) as per manufacturer’s instructions (Bailey et al. 2004; Stark et al. 2001). Red blood cells were lysed with a hypotonic
solution (0.16M NH₄Cl, 10mM KHCO₃, and 0.13 mM EDTA). The cell suspension was washed in HBSS/10% FBS and passed through a 70 µm nylon filter. Triplicate samples of splenocytes were cultured 100 µl/well at 2.5 x 10⁵ cells per well in flat-bottom 96-well tissue culture plates in complete RPMI (containing 10% heat-inactivated fetal bovine serum, 0.075% sodium bicarbonate, 10mM Hepes buffer, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 1.5 nM L-glutamine, and 0.0035% 2-mercaptoethanol). Cultures were stimulated with 0.40 µg/ml LPS and corticosterone (dose range 0.005 - 0.05 µM) for 48 hr at 37°C and 5% CO₂. Cell viability was measured with a tetrazolium substrate solution (Cell Titer 96 non-radioactive proliferation kit, Promega), and read at 490 nm by an ELISA plate reader. Viability of each sample was expressed as the mean optical density (OD) of each LPS-stimulated sample, minus OD of non-stimulated samples treated with the same corticosterone concentration. For comparison purposes, data were converted into a corticosterone resistance index, which represents the viable cells in each corticosterone treatment group, as a percentage of non-corticosterone treated cultures from the same treatment group (Avitsur et al., 2001, 2002b).

Cytokine ELISAs: Splenocytes were processed as above, suspended 2.5 x 10⁶ cells/ml in RPMI, plated 200 µl/well in cell culture treated 96-well plates, and incubated for 18 hr at 37°C in 5% CO₂. Pro-Inflammatory cytokines (IL-6 and TNF-α) from splenocytes culture supernatants were quantified by a standard sandwich ELISA, as described previously (Avitsur et al., 2005; Stark et al., 2001). For IL-6 determination, rat anti-mouse IL-6 antibody was used (BD Pharmingen, San Diego, CA). The ELISA for
TNF-α used rat anti-mouse TNF-α antibody (BD Pharmingen, San Diego, CA) and was also performed as per manufacturer’s instructions, with the modification that assay diluent was phosphate-buffered saline plus 2% bovine serum albumin.

**Flow Cytometry:** Cell suspensions of 2.5 x 10⁵ cells in RPMI were incubated for 45 minutes at 4°C with FITC-conjugated Gr-1/Ly-6G (clone RB6-8C5), PE-conjugated anti-mouse Pan-NK (clone DX5), PerCP-conjugated anti-mouse CD45R/B220 (clone RA3-6B2) and APC-conjugated anti-mouse CD11b/Mac-1 (clone M1/70). All monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA). Cells were stained using a standard lyse/wash procedure using PBS (Dulbecco’s PBS without Ca and Mg, 2% FBS, and 0.1% NaN₃). A total of 10,000 events from each sample were analyzed on a dual-laser flow cytometer (FACSCalibur, BD Immunocytometry Systems) using Cell Quest Pro and Attractors software. Forward and side scatter characteristics as well as differences in antibody staining were used to identify lymphocytes, neutrophils, and monocytes/macrophages. Matched isotype controls were used to set negative staining criteria.

**Tests of anxiety-like behavior and locomotion.** Two tests were used to assess anxiety-like behaviors in defeated mice: the light/dark preference test and the open field test. The light/dark preference test, also known as the black-white test, is a commonly used test for anxiety-like behavior (Crawley, 1981; Crawley and Goodwin, 1980; Flint, 2002; Holmes et al., 2001; Ohl, 2005). The apparatus consisted of two Plexiglas boxes, connected by a
small passage at floor level. The larger box (30 x 20 cm) was lit by a bright incandescent light bulb (4100 lux) directly overhead, and the smaller box (30 x 10 cm) was made of black Plexiglas and was covered, making it much darker (< 3 lux). Mice that express anxiety-like behavior tend to spend less time in the brightly lit box and take longer to emerge from the dark box after entering. These effects are reversed by anxiolytic compounds (Bourin and Hascoët, 2003; Crawley, 1981; Crawley and Paylor, 1997). The open field apparatus consisted of a 30 x 30 x 25 cm Plexiglas box with a solid floor and was lighted by overhead room lighting (1300 lux). A grid was drawn on the floor that divided the floor into 36 squares. The open field test is designed to take advantage of a rodent’s natural tendencies to explore the environment while avoiding open spaces. Mice that express anxiety-like behavior tend to spend less time in the center of the open field and also tend to locomote near the walls of the apparatus (thigmotaxis). These effects are also reversed by anxiolytic compounds (Bhatnagar et al., 2004; Crawley, 1999; Dulawa et al., 2004; Prut and Belzung, 2003; Sullivan et al., 2003). For this experiment, the dependent variables were [a] total number of line crosses, [b] rearing, [c] total time spent in the center/perimeter of the open field, and [d] number of transitions between the center/perimeter of the open field. Each test apparatus was cleaned with water-dampened cloths between subjects. Both tests were recorded for 5 minutes.

Tests of depressive-like behavior. Two tests were used to assess depressive-like behaviors in defeated mice: the Porsolt forced swim test and the tail suspension test. In the forced swim test, individual mice were placed in a glass cylinder (43 cm high, 22 cm diameter) filled with room-temperature water to a depth of 15 cm. The rim of the cylinder
was high enough that the mouse could not climb or jump out, and the water was deep enough that the mouse could not touch the bottom of the cylinder with its tail (Crawley and Paylor, 1997; Hebert et al. 1998; Porsolt, 1977; Porsolt, 1997). This test was recorded for 5 minutes in the dark (< 3 lux), using infrared lighting. Struggling behavior was measured as time spent actively swimming around the apparatus, climbing the walls of the tank (forepaws break the surface of the water), or floating (which included minor movements to keep the head above water). The water was replaced between subjects.

Like the Forced Swim test, the tail suspension test models depression in mice by placing the animal in an inescapable, uncomfortable situation and measuring passive behavior (Dalvi and Lucki, 1999; Mayorga and Lucki, 2001; Steru et al., 1985). Individual mice were suspended by their tails with adhesive tape, from a horizontal bar 25 cm above the tabletop. This test was recorded for 6 minutes under ambient room lighting. Struggling behavior was measured as time spent actively kicking and struggling vs. hanging motionless.

**General Procedure.** In the first experiment, anxiety-like behavior was measured on the morning immediately following the sixth or final cycle of social disruption. Mice were randomly assigned to either home cage control (HCC; n=19) of social disruption (SDR; n=22) treatment groups. All behavioral tests began at approximately 1000 h. Mice were transported to the testing area and left undisturbed for 30 minutes before testing began. Behavior was measured in the light/dark preference test. During testing, the investigators left the room and behavior was videotaped for later analysis. The videos were digitized and scored by a trained observer blinded to the treatment groups, using the Observer
software (Noldus Information Technologies, the Netherlands). On the following
morning, mice were sacrificed by CO₂ asphyxiation at which time spleens were
harvested, weighed, and processed as described above for assays of GC insensitivity,
cytokine production, and phenotyping by flow cytometry.

The second experiment focused on temporal defeat-induced changes in behavior.
Separate groups of mice were tested for anxiety- or depressive-like behaviors in the open
field, forced swim test, or tail suspension test, on the mornings following day 1 and day 6
of SDR. A third group received 6 cycles of SDR and was tested after 8 days of rest (day
14 after the first cycle of SDR). A total of 51 C57Bl/6 mice were used in the open field
test (mice were randomly assigned to HCC and SDR groups as follows: Day1 (10, 10);
Day6 (9, 9); Day14 (3, 10)). A total of 48 C57Bl/6 mice were used in the forced swim
test (mice were randomly assigned to HCC and SDR groups as follows: Day1 (10, 10);
Day6 (5, 10); Day14 (3, 10)). A total of 60 C57Bl/6 mice were used in the tail
suspension test (mice were randomly assigned to HCC and SDR groups as follows: Day1
(10, 10); Day6 (10, 10); Day14 (10, 10)). Because separate groups of mice were tested
on each day, there was no habituation to either test. The open field test was scored by
two trained observers and inter-observer reliability was > 96%. Mice were sacrificed on
the morning following the last day of behavioral testing (for all groups: 15 days after the
first cycle of SDR), at which time spleens were harvested, weighed, and processed as
described above for assays of GC insensitivity, cytokine production, and phenotyping by
flow cytometry.

The third experiment measured SDR-induced changes in anxiety- and depressive-
like behaviors in the outbred CD-1 strain. As in the first experiment, mice were
randomly assigned to SDR or HCC treatment, and the SDR groups were subjected to 6 cycles of SDR over 6 days. On the morning following the 6th cycle of SDR, a total of 60 CD-1 mice were tested in the open field test (HCC(6); SDR(8)), light/dark preference test (HCC(10); SDR(10)), forced swim test (HCC(10); SDR(16)), or tail suspension test (HCC(10); SDR(10)). Mice were sacrificed 15 days after the first cycle of SDR, at which time spleens were harvested, weighed, and processed as described above for assays of GC insensitivity, cytokine production, and phenotyping by flow cytometry.

Statistics. A repeated measures 2-way (stress vs. corticosterone concentration) ANOVA was used to assess glucocorticoid insensitivity. Between groups 1-way ANOVA was used to compare spleen mass, cytokine concentrations, and cell population phenotypes. A between groups, 2-way (stress vs. ethological measure) ANOVA was used to compare behavioral measures on separate time points. Correlational comparisons were made between behavioral measures and immunological measures using Fisher’s r to z conversion to establish statistical significance. Follow-up comparisons were performed using Fisher’s PLSD tests, where appropriate. All statistical comparisons were made using Statview software (SAS Institute, Inc.). All results are presented as mean ± SE. Statistical significance was set at \( p < 0.05 \).

Results

Experiment 1: Effects of social disruption on anxiety-like behavior in C57BL/6.

Social disruption stress caused an increase in anxiety-like behaviors in the light/dark preference test. Individual mice were placed in the corner of the brightly lit
box, and allowed to freely explore the apparatus. Each mouse moved about and explored the apparatus. Defeated mice spent significantly more time in the smaller, dark box of the apparatus than did non-defeated controls ($F(1,39) = 4.13; p < 0.05$; Fig. 1A). Latency to enter the dark box did not differ between treatment groups; both defeated and non-defeated mice entered the dark box after approximately 1 minute ($p > 0.96$). However, after entering the dark box, the defeated mice took significantly longer to reemerge than did controls ($F(1,39) = 6.16; p < 0.05$; Fig. 1B).

**Experiment 2: Temporal effects of social disruption on immune function and behavior in C57BL/6.**

*Immunological measures.* Consistent with previous data from our lab and others, SDR caused an increase in spleen mass. Mean spleen mass was $133.0 \pm 11.3$ mg in SDR mice and $84.8 \pm 6.1$ mg in HCC mice ($F(1,45) = 10.15; p < 0.01$); this effect persisted when corrected for individual body mass ($F(1,45)=10.68; p < 0.01$; Fig. 2A). Also consistent with previous findings, the cellular makeup of the spleen, as measured by flow cytometry, indicated that the enlarged spleens of SDR mice had more macrophages/monocytes than control mice, with SDR mice having a mean number of $4.79 \times 10^6$ cells per spleen, compared with $1.58 \times 10^6$ ($F(1,44) = 6.07; p < 0.05$). Glucocorticoid insensitivity developed in SDR mice and not in controls ($F(1,43) = 5.18; p < 0.05$; Fig. 2B). More specifically, follow-up comparisons revealed significantly higher cell viability in the SDR splenocytes stimulated with 0.5µM corticosterone and 5.0µM corticosterone. In addition, supernatants from cultured splenocytes stimulated
with LPS revealed an increased production of the proinflammatory cytokine IL-6
\((F(1,24) = 11.75; p < 0.01; \text{Fig. 2C})\) and TNF-\(\alpha\) in SDR mice compared to controls
\((F(1,39) = 7.62; p < 0.01; \text{Fig. 2D})\). As reported previously, cytokine production and cell
proliferation were greatly reduced in non LPS-stimulated samples (Avitsur et al., 2003).
Mean wound severity within SDR treatment groups was 1.73 ± 0.22, and did not differ
significantly between behavioral tests.

**Open field test.** Social disruption (SDR) caused an increase in anxiety-like behaviors in
the open field test. As in the light/dark preference test, all mice moved about and
explored the open field. However, on all days tested, mice subjected to SDR spent
significantly less time in the center of the open field than controls \((F(1,45)= 14.89; p <
0.001; \text{Fig. 3A})\). Although mice from both treatment groups entered and explored the
center of the open field, SDR mice entered the center fewer times than controls \((F(1,45)
= 7.50; p < 0.01; \text{Fig. 3B})\). This difference was not due to changes in locomotion or
activity as line crosses did not differ between treatment groups on any of the days tested
\((p > 0.62; \text{Fig. 3C})\).

**Porsolt forced swim test.** Social disruption did not have a strong depressive-like effect in
the forced swim test. After being placed in the water tank, defeated mice displayed a
shortened latency to stop swimming and become immobile after 1 and 6 cycles of SDR
compared with controls \((F(1,18) = 4.80; p <0.05 \text{ and } F(1,13) = 7.29; p < 0.05; \text{Fig. 3D})\).
However, the total time spent immobile did not differ between treatment groups \((p >

0.12; Fig. 3E). Similarly, overall time spent swimming and climbing the walls of the tanks did not differ between groups ($p > 0.33$ and $0.23$, respectively).

**Tail suspension test.** Like the forced swim test, social disruption did not affect depressive-like behavior in the tail suspension test. Social disruption had no effect on time spent immobile on all days tested ($p > 0.42$; Fig. 3F). There were no observed cases of tail climbing during testing.

*Experiment 3: Effects of SDR on immune function and anxiety-like behavior in outbred mice.*

*Immunological measures.* Social disruption also caused an increase in spleen mass in outbred CD-1 mice. Mean spleen mass was $195.4 \pm 33.7$ mg in SDR mice and $104.1 \pm 7.9$ mg in HCC mice ($F(1,13) = 6.03; p < 0.05$), and this effect persisted when corrected for individual body mass, ($F(1,13) = 6.23; p < 0.05$; Fig. 4A). Glucocorticoid insensitivity developed in defeated outbred CD-1 mice and not in controls ($F(1,13) = 9.46; p < 0.01$; Fig. 4B). Follow-up comparisons revealed significantly greater cell viability in the SDR mice splenocytes stimulated with 0.005, 0.1, 0.5, and 5.0µM corticosterone. Also consistent with previous findings from inbred mouse strains, supernatants from cultured splenocytes stimulated with LPS revealed an increased production of the proinflammatory cytokines IL-6 ($F(1,13) = 12.56; p < 0.01$; Fig. 4C) and TNF-α ($F(1,13) = 18.24; p < 0.001$; Fig. 4D) in SDR mice compared to controls.
Cytokine production and cell proliferation were greatly reduced in non LPS-stimulated samples. Mean wound severity within SDR treatment groups was 2.22 ± 0.15, and did not differ significantly between behavioral tests.

Open field and light/dark preference tests. Outbred CD-1 mice subjected to six sessions of SDR showed anxiety-like behavior in the open field test, but not in the light/dark box. In the open field test, defeated outbred CD-1 mice spent significantly less time in the center of the open field than controls (F(1,12) = 13.90; p < 0.01; Fig. 5A). Defeated mice entered the center of the open field about as many times as controls (p > 0.22; Fig 5B). As in C57BL/6 mice, social disruption had no effect on locomotion (p > 0.85; Fig. 5C). In the light/dark box test, there was no difference between the SDR and control groups, which both spent a mean 164 ± 27 s and166 ± 28 s, respectively, in the dark box (p > 0.83). Similarly, latency to reemerge from the dark box after entering did not differ (p > 0.80). Mean time to reemerge was 28.3 ± 6 s in SDR and 26.2 ± 6 s in HCC mice.

Forced swim and tail suspension tests. As with the inbred mouse, no observable differences were found in depressive-like behaviors in defeated outbred CD-1 mice. In the forced swim test, there was no difference in latency to become immobile (p > 0.75; Fig. 5D) or total time spent immobile (p > 0.93; Fig. 5E). Overall time spent swimming and climbing the walls of the tanks did not differ between groups (p > 0.93). Similarly, the tail suspension test revealed no differences in immobility (p > 0.88; Fig 5F). As with the C57BL/6 strain, no tail climbing behaviors were observed in outbred CD-1 mice.
Discussion

The data confirm and extend previous reports on the effects of repeated social defeat on the immune system (Avitsur et al., 2001; Bailey et al., 2004; Padgett et al., 1998) showing that the noted effects are not limited to inbred mouse strains. Social disruption (SDR) stress resulted in a significant enlargement of the spleen, due to an increase in trafficking of CD11b+ myeloid cells from the bone marrow to the spleen (Engler et al., 2005). In addition, splenocytes from mice exposed to SDR produced higher levels of IL-6 and TNF-α upon in vitro stimulation with LPS and remained viable in culture, even in the presence of high pharmacological doses of corticosterone, a phenomenon referred to as glucocorticoid resistance (Stark et al., 2001).

Beyond simply extending the observations concerning SDR from the inbred to the outbred CD-1 mouse strain, the goal of this study was to assess whether the stress of SDR affected behavior. The data reveal that on the one hand, SDR had little to no effect on depressive-like behavior as assessed by forced-swim or tail-suspension testing. However, on the other hand, by using measures commonly used to assess anxiety-like behaviors in rodents (Bhatnagar et al., 2004; Bourin and Hascoët, 2003; Crawley, 1981, 1999; Crawley and Paylor, 1997; Ohl 2005), the data described herein reveal that SDR of either inbred C57BL/6 or outbred CD-1 male mice resulted in an increase in anxiety-like behavior. The development of this anxiety-like behavior was evident in C57BL/6 mice after a single cycle of SDR and lasted for at least one week following the cessation of the stressor. In C57BL/6 mice, but not CD-1 mice, SDR caused a decrease in the latency to become immobile in the forced swim test. However, overall immobility was not significantly affected by SDR. Likewise, no significant differences were found as a
result of SDR in the tail suspension test in either mouse strain. Taken together, these data suggest that SDR does not cause an increase in depressive-like behavior. However, there are some limitations of these two tests, which both evaluate behavioral despair. The effects of SDR on depressive-like behaviors in anhedonia models of depression, such as sucrose consumption, will be explored in future studies.

The extant literature indicates that forms of social defeat can be associated with decreased locomotion (Avgustinovich et al., 1997; Kudryatseva et al., 2004). If true for SDR, such reduced activity would undoubtedly bias the interpretation of our tests for anxiety. However, data from the open field test indicated that SDR had no effect on locomotion, regardless of strain thus suggesting that SDR increases anxiety-like behaviors without affecting overall activity levels. This further indicates that the observed reduction in time spent in the center of the open field was not just an artifact of decreased mobility, but was directed by different exploratory patterns of defeated vs. non-defeated mice. The observed differences in mobility indicate that there are qualitative differences between SDR and other social stressors.

The SDR-induced increase in time spent in the dark box of the light/dark preference test seen in C57BL/6 mice did not replicate in outbred CD-1 mice. This is possibly due to strain-specific differences in the baseline expression of anxiety-like behaviors. Compared to other strains, C57BL/6 mice are considered to be a relatively “low anxiety” strain in the L/D box (Bouwknecht and Paylor, 2002; Rodgers et al., 2002). In fact, data from the present study indicate that during the 5 minute test, control C57BL/6 mice spent 125 seconds (41.6%) in the dark box whereas outbred CD-1 controls spent 165 seconds (55%) in the dark. Thus, because of the higher baseline ‘anxiety’ of
the CD-1 mouse, which spends comparatively more time in the dark, the effects of SDR may be too subtle to detect in this strain of mouse. This reduced effect can not be explained by strain-specific wound severity, as both C57Bl/6 and CD-1 mice were wounded to a similar extent in all experiments.

Although a direct causal relationship between anxiety-like behavior and GC insensitivity has not yet been established in this model, high expression of anxiety-like behaviors was seen only in defeated mice, and not in non-defeated controls. Mice subjected to SDR that expressed high levels of GC insensitivity and splenomegalay also tended to express higher levels of anxiety-like behavior, whereas control mice did not develop GC insensitivity and expressed low levels of anxiety-like behavior. However, at an individual level within the SDR-treated group, the data did not reveal statistically significant correlations between the development of anxiety and any immunological measures, including wound severity. Submissive behavior was not measured in the present study, although previous research has demonstrated that mice that showed high amounts of submissive behavior during SDR more often developed GC insensitivity (Avitsur et al., 2001). Similarly, within the SDR treatment groups, no statistically significant correlations were found between wound severity and any immunological measures. Further studies are needed to be able to draw any such causal links among submissive behaviors, anxiety-like behaviors, and the immunological consequences of SDR.

The stress response is a well-characterized psychophysiological reflex that affects immunological regulation in most animal species, including humans. These stress-induced effects are not inconsequential; high stress reactivity has been linked to increased
susceptibility to infection and immune dysfunction (Black, 2002; Padgett and Glaser, 2003; Glaser and Kiecolt-Glaser, 1998; Kiecolt-Glaser et al., 2002; Liu et al, 2002; Rohleder et al., 2001; Cohen and Hamrick, 2003). Some of these effects can be modeled in the laboratory, and as we and others have shown, social defeat activates the core stress responses and can have deleterious immunological effects on defeated mice (Devoino et al., 2003; Merlot et al., 2003; Padgett et al., 1998c; Sheridan et al., 2000), including increased proinflammatory cytokine production, splenomegaly, trafficking of lymphocytes to the spleen, and insensitivity to the antiproliferative effects of glucocorticoids in LPS-stimulated splenocytes (Avitsur et al., 2003; Bailey et al., 2006; Engler et al., 2005; Stark et al., 2001). Although slight differences occurred between C57BL/6 mice and CD-1 mice, the present data indicate that the development of anxiety-like behavior can be added to the list of consequences mediated by social disruption. Whether or not the immune and behavioral changes associated with social disruption are covariates or are dependent upon one another remains to be evaluated fully.
Introduction

A search of the extant literature reveals that advanced age is associated with the deterioration of many biological systems, a phenomenon typically referred to as senescence. In the brain, senescence is characterized by neuronal loss and cognitive deficits; these deficits often manifest as senile dementias. Similarly, the immune system is subject to age-dependent functional deficits referred to as immunosenescence; older individuals respond poorly to immune challenges. As a result, opportunistic infections that are easily defended in the young adult can be life threatening to the aged (Bauer 2005; Castle 2000).

Among the contributing factors to the progression of immunosenescence is decreased cytokine regulation. In healthy young adults absent of apparent disease, serum concentrations of proinflammatory cytokines are generally undetectable. However, with advancing age, these cytokines can reach high concentrations and can have untoward effects on the overall health of an individual. For example, high serum concentrations of the proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha
(TNF-α) are predictive of atherosclerosis in the aged adult population (Castle 2000; Malaguarnera et al. 2001). These cytokines are associated with increased mortality due to a myriad of causes including coronary artery disease (Bruunsgaard et al. 2000; Harris et al. 1999; Volpato et al. 2001). Similarly, there is an age-dependent loss of proinflammatory cytokine regulation in rodents. Elevated TNF-α and IL-6 mRNA gene expression has been detected in the coronary arteries of aged Fisher 344 rats (Csiszar et al. 2003), and cells from the lymph nodes and spleens of aged mice produce comparatively more IL-6 than cells from young adults (Daynes et al. 1993). These age-related cytokine changes are not limited to the periphery. Recently, Godbout and colleagues showed that aged mice have higher levels of the proinflammatory cytokines in their brains when the animals were challenged peripherally with LPS (Godbout et al. 2005; Godbout and Johnson 2004). In fact, the brains of aged mice expressed twice as much mRNA for IL-1β and IL-6 as compared to the brains of young adult mice (Godbout et al. 2005). This noted elevation in brain IL-1β and IL-6 contributed to neuroinflammation. In sum, aged individuals have higher basal pro-inflammatory cytokine expression and are hyper-responsive to mitogenic stimulation. Taken together, these observations indicate that there is a progressive loss of cytokine regulation with advancing age that is characteristic of immunosenescence.

In addition to aging, psychological stress also disrupts immune regulation, and can result in immunological decrements. There is a wealth of knowledge on the effects of aging in immunity, and there are many studies on the effects of stress in aged individuals (reviewed by Graham et al., 2006). However, stress and aging are rarely compared side by side within the same experiment. Recent findings from our laboratory
indicate that a social stressor (social disruption, SDR) results in dysregulation of proinflammatory cytokine responses in young adult mice (Avitsur et al., 2003; Bailey et al., 2004; Stark et al., 2001). In contrast to many experimental stressors, which are generally immunosuppressive, SDR causes an increase in proinflammatory cytokine production, hyper-responsiveness to mitogenic stimulation, and glucocorticoid insensitivity. Similar to the effects of immunosenescence, this hyperinflammatory response is characterized by an increase in IL-6 concentrations in serum and increased production of IL-6 and TNF-\(\alpha\) in response to LPS-stimulation (Stark et al. 2001, 2002). These effects can have a potentially negative influence on the health of the young adult animals. For example, mice subjected to SDR and injected with LPS were more likely to die from endotoxic shock than were control animals (Quan et al. 2001). The increased production of pro-inflammatory cytokines was shown to be the mechanism for this increased susceptibility to endotoxic shock. Thus, the stress of repeated social defeat (i.e., SDR) induces immune changes in young mice that parallel those of immunosenescence in the old.

Because of the similarity of these findings, we hypothesized that the detrimental effects of aging and stress on immune function would interact, causing negative health outcomes. In fact, studies have already shown that aged mice are more susceptible to influenza-induced mortality than younger mice, and mortality increased when aged mice were subjected to repeated restraint stress (Padgett et al. 1998a). However, the increased mortality of the aged animal subjected to restraint stress was attributed to a severely depressed immune response to viral challenge. More specifically, restraint stress suppressed the expression of cytokines necessary for the activation of anti-viral immune
responses. Therefore, because of the apparent increase in pro-inflammatory cytokines due to the stress of social defeat, the objective of the present study was somewhat different. In sum, experiments were designed to test whether social defeat of aged animals resulted in hyperinflammatory immune changes similar to those seen in young adult animals. In addition, the data were analyzed to determine whether aging exacerbated the effects of social defeat.

Methods

**Animals.** Male CD-1 mice (n=40) were ordered from Charles River at 6-8 weeks of age. Young adult mice were 2 months old, and aged adult mice were 14±1 months of age at the beginning of the experiments. The 14-month-old mice were housed in our facility for one year prior to the experiment. Mice were initially group-housed, and then randomly assigned by age group into Young Adult Defeat (n=6), Young Adult Control (n=10), Aged Defeat (n =15), or Aged Control (n=9) treatment group, singly housed, and left undisturbed for at least 2 weeks before testing began. The mice were singly housed for this experiment, in order to reduce the incidence of spontaneous fighting within the cages. Previous observations from our lab indicated that the aged mice were more likely than the young adult mice to engage in such fighting within the home cage. Because fighting is itself an independent variable in the present study, the mice were singly housed to avoid this potential confound. All mice were kept in an American Association for Accreditation of Laboratory Animals Care (AAALAC) accredited facility at the Ohio
State University and provided standard lab diet and tap water *ad libitum*. The Institutional Laboratory Animal Care and Use Committee (ILACUC) at the Ohio State University approved all experimental protocols.

*Social Defeat.* The social disruption paradigm was described previously (Avitsur et al. 2001). An aggressive intruder mouse was introduced to the home cage of each resident mouse for 30 minutes for six daily sessions, starting at the beginning of the nighttime active cycle (1600 EST). The defeated resident mice were the subjects of this study. The aggressor mice were singly housed for several weeks prior to the experiment to induce territoriality and agonistic behavior toward unfamiliar male mice. The aggressor typically started to attack the resident mice within 5 minutes of being introduced into the cage. Behavior was observed to ensure that the resident mice were attacked and defeated by the aggressor. Defeated mice did not initiate attacks but exhibited defensive behaviors, such as fleeing, freezing, and upright submissive postures in response to the aggressor (Avitsur et al. 2001, 2002a, Engler et al., 2004; Stark et al. 2001). In cases in which the aggressor did not attack the resident mouse, or the resident attacked and defeated the aggressor, the aggressor was replaced. After each 30-min defeat session, the aggressor was removed and returned to its home cage. Each mouse was defeated in its home cage each night, although a different aggressor was used during each defeat cycle to prevent habituation (Avitsur et al. 2001; Quan et al., 2003; Stark et al. 2001). Control mice were left undisturbed in their home cages during defeat sessions.
Glucocorticoid Sensitivity Assay. The glucocorticoid sensitivity assay was performed as described previously (Avitsur et al. 2001; Stark et al. 2001). Briefly, at sacrifice, spleens were harvested and weighed. A single cell suspension was prepared by homogenizing the spleens in a stomacher (Biomaster #80, Seaward, London, England), and red blood cells were lysed with a hypotonic salt solution. Triplicate samples of splenocytes were cultured at 2.5 x 10^5 cells per well in flat-bottom 96-well tissue culture plates in complete RPMI (containing 10% heat-inactivated fetal bovine serum, 0.075% sodium bicarbonate, 10mM Hepes buffer, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 1.5 nM L-glutamine, and 0.0035% 2-mercaptoethanol). Cultures were stimulated with 0.40 µg/ml LPS and increasing concentrations of corticosterone (dose range 0.005 - 0.05 µM) for 48 h at 37°C and 5% CO2. Cell viability was measured with a tetrazolium substrate solution (Cell Titer 96 non-radioactive proliferation kit, Promega), and read at 490 nm on an ELISA plate reader (Stark et al. 2001). Viability for each sample was expressed as the mean optical density (OD) of each LPS-stimulated sample, minus OD of non-stimulated wells treated with the same corticosterone concentration. For comparison purposes, data were converted into a corticosterone resistance index, which represents the viable cells in each corticosterone treatment group, as a percentage of non corticosterone treated cultures from the same age and defeat group (Avitsur et al., 2001, 2002a).

Cytokine Assays. Pro-Inflammatory cytokines (IL-6 and TNF-α) from splenocytes culture supernatants were quantified by a standard sandwich ELISA as described previously (Avitsur et al. 2005; Stark et al. 2001). Briefly, for IL-6 determination, 96-well ELISA plates were coated with 50 µl/well of 2 µg/ml rat anti-mouse IL-6 antibody
(BD Pharmingen, San Diego, CA) overnight at 4°C and then washed 2X with PBS-Tween wash buffer. Non-specific binding was blocked with 200 µl/well of PBS/10% FBS for 2 hours at room temperature. After two washings, 50µl of samples or standards were added, and plates were incubated overnight at 4°C. After 4 washes, 100 µl/well of biotinylated anti-mouse capture antibody (BD Pharmingen) was added and incubated for 45 min at room temperature. Plates were washed 6X with wash buffer, and 100 µl/well of 1:1000 avidin-peroxidase (BD Pharmingen) was added and incubated for 30 min at room temperature. Plates were washed 8X with wash buffer, and 100 µl/well of 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt substrate (Sigma, St. Louis, MO) plus 10% H2O2 was added and developed in the dark for 30 minutes. Plates were then read at 405 nm on an ELISA plate reader. The IL-6 concentrations for each sample were calculated using a polynomial curve generated from known standards. The ELISA for TNF-α was similar with the exception of using 3,3’,5,5’ tetramethylbenidine (TMB) as the substrate and 1M H3PO4 as a stop solution; TNF-α plates were read at 450 nm.

**Flow Cytometry.** Cell suspensions containing 2.5 x 10⁵ cells were incubated at 4°C for 45 minutes with FITC-conjugated Gr-1/Ly-6G (clone RB6-8C5), PE-conjugated anti-mouse Pan-NK (clone DX5), PerCP-conjugated anti-mouse CD45R/B220 (clone RA3-6B2), and APC-conjugated anti-mouse CD11b/Mac-1 (clone M1/70). A second panel was run in parallel, using FITC-conjugated anti-mouse CD3e (clone 145-2C11), PE-conjugated anti-mouse CD8a (clone 53-6.7), and PerCP-conjugated anti-mouse CD4 (clone RM4-5). All monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA). A standard lyse-wash procedure using PBS (Dulbecco’s PBS without Ca
and Mg, 2% FBS, and 0.1% NaN₃) was used to stain the cells. A total of 10,000 events from each sample were analyzed on a dual-laser flow cytometer (FACSCalibur, BD Immunocytometry Systems) using Cell Quest Pro and Attractors software. Lymphocytes, neutrophils, and monocytes/macrophages were identified by forward and side scatter characteristics and differences in antibody staining. Matched isotype controls were used to set negative staining criteria.

*Serum Corticosterone Determination.* Serum corticosterone was measured with a radioimmunoassay (RIA) kit (ImmuChem Double Antibody corticosterone ¹²⁵I RIA kit; MP Biomedicals, Orangeburg, NY), per the manufacturer’s protocol (Stark et al., 2001; Jasnow et al., 2000; Padgett et al., 1998b). Blood was collected from the retroorbital plexus within 3 minutes of opening the cage immediately following the first cycle of defeat (i.e., at 1830 EST). A baseline sample was collected from each animal at the same time of day but one week before the start of the experiment. Blood samples were immediately put on ice, allowed to clot for one hour, the clots were removed, and the samples were centrifuged at 4000 x g at 4° C for 20 minutes. Serum was stored at -70° C until analysis. Blood samples were collected from each animal in this study, and a random selection of samples from each treatment group was assayed in duplicate. The minimum sensitivity of the assay was 23.5 ng/ml.
Behavior testing. Behavior was assessed on the morning following the sixth defeat session. Anxiety-like behavior was measured in the open field test, and depressive-like behavior was measured in the forced swim test. Both tests were carried out as detailed in Chapter 2.

Statistics. Spleen size, cytokines, and cell population data were compared by 2 X 2 between measures (age vs. defeat treatment) ANOVA. Repeated measures 2 X 2 (age vs. defeat treatment) ANOVA tests were used to assess statistical significance for the GC insensitivity, behavioral measures, and serum corticosterone. Behavioral data were compared using one-way between-groups ANOVA. Fisher’s PLSD tests were used for post hoc comparisons of pair-wise differences. All results are presented as mean ± SE. Statistical significance was set at \( p < 0.05 \).

Results

The first set of experiments was designed to confirm that the aged animals in this study showed classical signs of immunosenescence before they were subjected to the stress of social defeat. Whereas the typical mouse aging study uses animals greater than 20 months of age, the social stress model used in these studies precluded the use of such old mice for multiple reasons. Most important was our concern for their ability to survive the stress of repeated aggressive interactions with an intruder. Thus, the mice chosen for the experiment were 14 months old. Even so, the data revealed that the 14-month-old CD-1 mouse used in the following studies showed multiple signs of immunosenescence, including thymic involution and increased proinflammatory cytokine production. Mean
relative thymus mass, expressed as thymus mass/body mass, from aged mice was significantly lower than in the younger mice (F(1,25) = 112.65; \( p < 0.0001 \); Fig 6A). The proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) secreted by spleen cells stimulated with LPS were measured via ELISA and compared between groups. As would be predicted in an aged mouse, production of both IL-6 (F(1,17) = 4.40; \( p < 0.05 \); Fig 7) and TNF-α (F(1,17) = 8.64; \( p < 0.01 \)) was increased in aged mice, compared with young adult mice.

In addition to confirming that the 14-month-old animal showed signs of immunosenescence, it was important to verify that our model of social defeat was a stressor for the aged animals. One of the core stress responses typically noted after exposure to a stressor is activation of the hypothalamic-pituitary-adrenal axis resulting in increased circulating glucocorticoids. In rodents, the predominant glucocorticoid is corticosterone. As compared to age-matched control animals, both young and old mice, subjected to social defeat, had significantly elevated levels of plasma corticosterone immediately after social defeat (F(1,26) = 42.81; \( p < 0.0001 \); Fig 8). There was no apparent age effect with regard to the corticosterone response to social defeat (\( p > 0.10 \)).

Previous studies showed that SDR of young mice causes splenomegaly (Avitsur, et al., 2001; Quan, et al., 2001; Stark, et al., 2001). Therefore, we tested whether aging exacerbated this effect. Although non-stressed old mice had a lower spleen/body mass than non-stressed young adult animals (F(1,17) = 7.29; \( p < 0.05 \); Fig 6B), social defeat of either age group caused an increase in relative spleen mass (splenomegaly) (F(1,36) = 16.00; \( p < 0.01 \)). Previously, we also reported that splenomegaly was associated with increased accumulation of neutrophils, macrophages and B cells in the spleen (Avitsur et
al., 2002b). Flow cytometric analysis of the cells in the spleens from both young and aged mice revealed that total cell numbers were higher in both defeated groups regardless of age (F(1,36) = 24.39; p < 0.0001; Fig 9A). The stress-induced increase in cell number was predominantly due to an accumulation of granulocytes (F(1,36) = 9.30; p < 0.01; Fig 9B) and monocytes (F(1,36) = 10.49; p < 0.01; Fig 9C). Social defeat had no effect on T cell populations in young adult mice. However, in aged mice, there was a significant increase in CD8+ cells (F(1,22) = 6.54; p < 0.05; Table 1) and a marginally significant increase in CD4+ T cells (F(1,22) = 3.92; p < 0.06). Although not statistically significant, there was a trend suggesting an interaction between aging and defeat in granulocyte (F(1,36) = 3.58; p < 0.07) and monocyte (F(1,36) = 2.77; p < 0.10) populations in the spleen.

Previous studies also showed that SDR increased proinflammatory cytokine production from cultured splenocytes. Both IL-6 and TNF-α were higher in supernatants derived from splenocytes of defeated young mice as compared to those from home cage (non-defeated) young controls (Avitsur et al., 2003; Bailey et al., 2004; Stark et al., 2002). Again, the data from this current study agreed with these previous findings in young mice -- defeat increased production of both IL-6 (F(1,16) = 8.73; p < 0.01; Fig 7) and TNF-α (F(1,16) = 8.84; p < 0.01). Similarly, social defeat of aged mice significantly increased TNF-α production (F(1,22) = 9.34; p < 0.01) and increased IL-6, a trend toward statistical significance (F(1,22) = 3.46; p < 0.08). There was no statistically significant interaction between aging and stress.

Of particular interest to us has been the observation that SDR results in the development of functional glucocorticoid insensitivity in splenocytes from young mice.
Specifically, LPS-stimulated CD11b+ monocytes from SDR animals remain viable, even when co-cultured with increasing concentrations of corticosterone. Therefore, the effects of aging on the development of stress-induced GC insensitivity were assessed in the present study. The data showed that aging, by itself, had no effect on GC sensitivity. Cells obtained from either old or young non-stressed control animals were equally sensitive to corticosterone culture (F(1,17) = 0.72; p > 0.40; Fig 10). However, there was an interaction between defeat and age treatments (F(1,36) = 7.22; p < 0.01). Follow-up comparisons revealed that this interaction was driven primarily by an increased in cell viability in the presence of high concentrations of corticosterone (aged defeated vs. all other groups: p < 0.01 at 5.0 µM, p < 0.001 at 0.5 µM concentration). There was a main effect of aging (F(1,36) = 4.66; p < 0.05), due primarily to the high GC insensitivity in the aged, defeated group (F(1,22) = 35.56; p < 0.0001). Cell viability (i.e., glucocorticoid insensitivity) was high in both defeated groups, compared to non-defeated controls (F(1,38) = 42.96; p < 0.0001). There was also a main effect of aging, with higher GC insensitivity in aged mice compared with young mice. Follow up comparisons confirmed higher GC insensitivity in aged defeated mice than in all other treatment groups [young defeated mice (p < 0.01) and young and old control groups (p < 0.0001)].

As in young adult mice, social defeat resulted in increased anxiety-like behavior, although the effects were not as pronounced. Time spent in the center of the open field test was decreased in defeated mice (F(3, 36) = 3.36; p < 0.05; Fig 11A), and follow-up comparisons revealed a significant decrease in aged, defeated mice (p < 0.05). These mice also entered the center of the open field fewer times than non-defeated mice (F(3, 36) = 2.86; p < 0.05; Fig 11B). However, locomotion was also significantly reduced by
social defeat (F(3, 36) = 3.37; p < 0.05; Fig 11D). Depressive-like behavior, reported here as immobility in the forced swim test, was unaffected by social defeat (F(3, 36) = 1.80; Fig 11E). Similarly, latency to become immobile was not affected by social defeat (F(3, 36) = 0.34; Fig 11F).

Discussion

As reported herein and in previous studies of SDR (Quan et al., 2001; Sheridan et al., 2004; Stark et al., 2001), social defeat has unique physiologic and immunologic effects that are not characteristic of other stressors. Whereas most experimental stressors result in activation of the hypothalamic-pituitary-adrenal axis (i.e., elevated corticosterone) and an associated suppression of inflammation, social disruption (SDR) contrastingly results in higher levels of TNF-α and IL-6 being produced from LPS-stimulated splenocytes (Avitsur et al., 2005). Furthermore, SDR results in the development of functional glucocorticoid resistance in splenic CD11b+ macrophages (Avitsur et al. 2003; Bailey et al 2004). In other words, SDR results in higher pro-inflammatory cytokine secretion that is refractory to the innate feedback control mechanisms that are typically engaged to limit inflammatory cytokine production (Webster et al., 2004). Therefore, because of the link between excessive inflammation and age-related diseases, we tested whether the effects of social defeat would be exacerbated in aged individuals.

In the present study, both aging and SDR significantly decreased thymus mass. Although these separate effects have been reported previously (Castle 2000, Engler et al., 2005), this is the first study to examine both effects in young and aged, defeated, and
non-defeated mice. Social defeat had no effect on thymus mass in the aged mice. This is most likely because these aged mice were undergoing thymic involution, any defeat-induced reduction of thymus mass was masked by the relatively large age-dependent reduction in spleen mass.

Although mice over 20 months of age are typically used in aging research, mice aged 14 months were chosen for this series of experiments for several reasons. First, there was a concern that older mice (e.g. 20 months) would not be able to defend themselves or would not engage the aggressor mouse during the social defeat paradigm used in this study. Second, there was a strong desire to study the direct effects of aging, not age-related disease. Thus, mice aged 14 months were old enough to show some early signs of immunosenescence (e.g. thymic involution; Castle 2000), yet young enough to show low levels of apparent age-related disease. Evidence from our lab suggests an increase in mortality in mice as early as 10 months old when infected with a sub-lethal dose of influenza (Padgett et al. 1998a). The third reason for using 14 month-old mice for the aged group was that we did not wish to bias our sample by using only those mice that survived to a very old age. In any animal population, only a few individuals survive to very late life, and these individuals consist of a small, very healthy subset of the population (Coe 2004; Coe and Ershler 2001). Indeed, age-related alteration of NK activity and IL-2 and TNF-α release observed in “old” mice disappear in “very old” mice (Puerto et al., 2005). Thus, by using adult animals that are aged, but not geriatric, our results are indicative of age-dependent trends that are applicable to all members of the population and not just a subpopulation of extraordinarily healthy animals.
In sum, both young adult and aged adult mice were subjected to social defeat – they were repeatedly defeated by an aggressive conspecific for six consecutive days. Confirming previous reports (Avitsur et al., 2003; Stark et al., 2002), splenocytes from defeated mice produced higher levels of the proinflammatory cytokines IL-6 and TNF-α. In addition, social defeat resulted in a marked insensitivity to the anti-proliferative effects of corticosterone when splenocytes were co-stimulated with LPS. Moreover, the data indicate that aging exacerbates the stress effects. Most evident was a substantially marked decrease in sensitivity to corticosterone in the aged animal. Even though the only cell that has, thus far, been shown to become insensitive to glucocorticoids after social defeat is the CD11b+ macrophage (Stark et al., 2001), it should translate that if the macrophage is insensitive to the anti-inflammatory effects of GC, proinflammatory cytokine production should increase. In fact, that is just what happens as we age. It was reported by Quan and colleagues (2003) that the expression of the glucocorticoid receptor (GR) in CD11b+ cells was not affected by social defeat. However, the translocation of the GR to the nucleus was impaired in defeated mice. Thus, exogenous corticosterone was unable to reduce activation of the NF-κB complex, which in turn drove the production of proinflammatory cytokines. Although age-specific effects of social defeat on the GR were not examined in the present study, clear parallels exist in the development of GC insensitivity and increased IL-6 and TNF-α production suggest that the same mechanisms are active in aged animals as previously identified in young adult mice.

The present study used unfractionated spleens for the GC sensitivity and cytokine production assays. Stark and colleagues (2001) demonstrated that exclusion of CD11b+
cells reversed GC insensitivity and restored IL-6 production in defeated mice to that of control mice. Depletion of B cells by CD19+ magnetic microbead exclusion had no effect on either measure. It was also reported previously that social defeat causes CD11b+ cells to traffic from the bone marrow and into the spleen (Engler et al., 2004). Concomitant with this trafficking of CD11b+ cells was an increase in GC sensitivity in the bone marrow and a decrease in GC sensitivity in the spleen (Engler et al., 2005). Previous research in other labs has found age-dependent increases (Krabbe et al., 2004; Liang et al., 1998; O’Mahony et al., 1998, Roubenoff et al, 1998), decreases (Boehmer et al., 2004; Chelvarajan et al., 2006; Delpedro et al., 1998), or has no effect on production of these proinflammatory cytokines (Beharka et al., 2001). There are some methodological differences in these studies, although it appears that isolated macrophages and monocytes from aged individuals tend to down-regulate TNF-α and IL-6 production. On the other hand, unfractionated splenocytes from aged mice appear to increase production of TNF-α and IL-6 (Liang et al., 1998). In the present study, whole spleen cell populations were studied for cytokine release. The observed increases in IL-6 and TNF production by splenocytes from socially defeat mice were likely due to activated macrophages, which presumably received proinflammatory signals from other splenocytes including T cells in the cell culture. There is evidence that macrophages respond differently to mitogen in vivo as compared to in vitro (reviewed by Gomez et al., 2005), and in fact, depletion of CD11b+ macrophages from the spleens of socially defeated mice restores cytokine production to non-defeated levels (Stark et al., 2001). Peritoneal and splenic macrophages from aged mice have been shown to express lower toll-like receptors (TLR) than young mice (Renshaw et al., 2002; but see also Boehmer et
al., 2004; Chelvarajan et al., 2005). Of particular interest the age-dependent reduction in TLR-4, which binds LPS. However, social defeat has been shown previously to increase TLR-4 expression (Bailey, unpublished observation). Further studies are warranted to determine the defeat-specific effects of aging on TLR expression among slenocytes.

Along with this change in cytokine regulation, advancing age brings increased risks from a variety of diseases. These diseases range from those of infectious origin such as influenza virus, to those of an inflammatory nature such as coronary artery disease. Although we can vaccinate against those of the infectious nature and presumably lessen morbidity and mortality in the elderly population, those diseases of an inflammatory nature are more insidious and difficult to ameliorate. In fact, chronically elevated systemic inflammation can have a substantial impact on health for older individuals. For example, with regard to heart disease, which is the leading cause of mortality in the population over the age of 65 (Hoyert et al., 2003), circulating levels of IL-6 and TNF-α predict the development of atherosclerosis (Bruunsgaard et al. 2000; Harris et al. 1999; Volpato et al. 2001). Such an effect of altered regulation of inflammation is not limited to the peripheral immune system. There is now a solid link between excessive and uncontrolled inflammation in the brain and the development of clinical symptoms of Alzheimer’s (Griffin 2006). Thus, if inflammation contributes to the development of many of the diseases of aging such as coronary artery disease, arthritis, Alzheimer’s disease, etc., then it is reasonable to assume that conditions that exacerbate inflammation might worsen those diseases.

In other words, the inability to limit activation of inflammatory mediators would exacerbate diseases associated with aging. The stress of social defeat weakens one of
those balancing systems; it decreases an animal’s sensitivity to the natural anti-inflammatory effects of corticosterone. The loss of sensitivity to corticosterone is further magnified in the aged animal, where inflammatory conditions are more likely to contribute to disease. In conclusion, the findings delineated herein suggest that the stress of social defeat, which is exacerbated by aging, would contribute to the development of age-associated disease.
CHAPTER 4

ACUTE BENZODIAZEPINE TREATMENT IN SOCIAL DISRUPTION STRESS

Introduction

Previous work from our lab and others had demonstrated that repeated social defeat causes an increase in anxiety-like behavior. As shown in Chapter 2, defeat in the SDR model causes increases in proinflammatory cytokines, glucocorticoid (GC) insensitivity, and anxiety-like behavior in the open field test and the light/dark test in multiple mouse strains. However, questions remain as to whether anxiety mediates or covaries with the development of these SDR-induced changes in immune regulation.

In mice and humans, expression of anxiety and anxiety-like behaviors can be alleviated with pharmacological treatments (McNaughton & Gray, 2000). Benzodiazepine drugs are one of the most commonly prescribed treatments for anxiety (Witek et al., 2005). Benzodiazepine (BZ) drugs have been used repeatedly to reduce anxiety-like behaviors in mice, and because of this, they are often used as a standard by which new ethological paradigms involving anxiety-like behaviors are tested. In the open field test, benzodiazepine agonists including diazepam have been shown to increase time spent in the center of the field (Rex et al., 1996). Transversely, BZ antagonists tend to decrease time spent in the center of the open field (Prut & Belzung, 2003; Stout &
Diazepam is bound by GABA receptors, which are found throughout the central nervous system. Unlike most neurotransmitters, which have pluripotent effects on neural function, GABA is the main inhibitory neurotransmitter of the mammalian central nervous system (Mombereau et al., 2004). Benzodiazepine agonists, including diazepam (DZ), mimic GABA and have inhibitory effects on neural activity. Conversely, antagonists such as the partial inverse agonist β-carboline FG7142 (FG) reduce the inhibitory effects of GABA neurons, and therefore have excitatory effects (Hart, et al., 1998, Mikkelsen et al., 2005). Although receptors for benzodiazepines are expressed throughout the CNS, different subtypes are expressed in various regions, and binding of these specific subtypes has been reported to have different effects on behavior. Although there is recent evidence that suggests that the GABA\textsubscript{B(1)} also contributes to the expression of anxiety-like behavior in mice (Mombereau et al., 2004), the GABA\textsubscript{A} subunit is expressed throughout the limbic system and is necessary for the expression of anxiety-like behaviors (Mikkelsen et al., 2005).

The following experiments were designed to elucidate the role of anxiety in the development of glucocorticoid insensitivity. The first hypothesis was that pharmacological blockade of anxiety-like behavior would result in reduced GC insensitivity. The second hypothesis was that pharmacologically increasing anxiety-like behavior could, in itself, induce SDR-like immunological effects, including GC insensitivity.
Methods

General Procedure. In this experiment, C57BL/6 males were subjected to six consecutive 2-hour cycles of social disruption stress (SDR). Prior the SDR, the mice were administered a weight-dependent dose of drug or vehicle. On the morning following the final session of SDR, the mice were tested for anxiety-like and exploratory behaviors. The next day, the mice were sacrificed, and spleens were harvested and processed for GC insensitivity and flow cytometry.

Animals. A total of 47 male C57BL/6 mice were purchased from Charles River and housed in groups of 2-3 in polycarbonate cages and maintained under 12:12 light cycle in a temperature (21 ± 1 °C) and humidity (50 ± 5%) controlled, AAALAC-accredited facility at the Ohio State University for the duration of this experiment. The mice were randomly assigned into the following treatment groups: SDR/DZ (n = 6), SDR/FG (3), SDR/VEH (8), SDR/NI (4), HCC/DZ (9), HCC/FG(3), HCC/VEH(9), or HCC/NI(5). Standard lab diet and tap water were available ad libitum. All mice were aged 8-10 weeks and were experimentally naïve at the beginning of the experiments. Mice were handled minimally, for the purpose of general husbandry. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) at the Ohio State University.

Social Disruption (SDR). Upon arrival, mice were group-housed 2-3 animals per cage and randomly assigned to either the social disruption (SDR) or home cage control (HCC) condition. Social disruption consisted of introducing an aggressive intruder mouse into
the residents’ home cage. The resident mice were the subjects of these experiments. Sessions were monitored to ensure that the intruder repeatedly attacked and defeated the resident mice. Aggressors were introduced to each cage for six daily cycles of defeat, each lasting one hour, starting at approximately 1700 EST. A different aggressor was used during each cycle to prevent habituation. Control mice were left undisturbed in their home cages during SDR sessions.

*Drugs.* Mice were subjected to six consecutive sessions of SDR. At least 30 min prior to the onset of SDR, each mouse was weighed injected i.p. with the benzodiazepine agonist diazepam (DZ; 1.0 mg/kg), the inverse agonist FG7142 (FG; 16 mg/kg), or vehicle (PBS + 5% Tween 80) at a volume of approximately 0.20 ml. Two additional non-injected (NI) groups of mice were neither weighed nor injected, but otherwise received identical treatment as the other stress treatment groups.

*Glucocorticoid Insensitivity Assay.* Glucocorticoid (GC) insensitivity can be quantified by measuring the viability of cells cultured with various physiological and pharmacological concentrations of glucocorticoid in the absence or presence of LPS (lipopolysaccharide). High doses of corticosterone typically reduce the viability of LPS-stimulated splenocytes by inducing apoptosis. However, splenocytes harvested from SDR mice remain viable even in the presence of high concentrations of corticosterone (Stark et al., 2001). In the present study, mice were sacrificed by CO$_2$ asphyxiation on the morning following the last day of behavioral testing (for all groups: 14 days after the first cycle of SDR). Wound severity and individual body weights were assessed at this
Spleens were harvested, weighed, and homogenized for one minute by a stomacher (Stomacher 80 Biomaster, Seaward, London, England) as per manufacturer’s instructions (Bailey et al. 2004; Stark et al. 2001). Red blood cells were lysed with a hypotonic solution (0.16M NH₄Cl, 10mM KHCO₃, and 0.13 mM EDTA). The cell suspension was washed in HBSS/10% FBS and passed through a 70 µm nylon filter. Triplicate samples of splenocytes were cultured 100 µl/well at 2.5 x 10⁵ cells per well in flat-bottom 96-well tissue culture plates in complete RPMI (containing 10% heat-inactivated fetal bovine serum, 0.075% sodium bicarbonate, 10mM Hepes buffer, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 1.5 nM L-glutamine, and 0.0035% 2-mercaptoethanol). Cultures were stimulated with 0.40 µg/ml LPS and corticosterone (dose range 0.005 - 0.05 µM) for 48 hr at 37°C and 5% CO₂. Cell viability was measured with a tetrazolium substrate solution (Cell Titer 96 non-radioactive proliferation kit, Promega), and read at 490 nm by an ELISA plate reader. Viability of each sample was expressed as the mean optical density (OD) of each LPS-stimulated sample, minus OD of non-stimulated samples treated with the same corticosterone concentration. For comparison purposes, data were converted into a corticosterone resistance index, which represents the viable cells in each corticosterone treatment group, as a percentage of non-corticosterone treated cultures from the same treatment group (Avitsur et al., 2001, 2002a).

**Flow Cytometry:** Cell suspensions of 2.5 x 10⁵ cells in RPMI were incubated for 45 minutes at 4°C with FITC-conjugated Gr-1/Ly-6G (clone RB6-8C5), PE-conjugated anti-mouse Pan-NK (clone DX5), PerCP-conjugated anti-mouse CD45R/B220 (clone RA3-6B2) and APC-conjugated anti-mouse CD11b/Mac-1 (clone M1/70). A second panel
was run in parallel, using FITC-conjugated anti-mouse CD3e (clone 145-2C11), PE-
conjugated anti-mouse CD8a (clone 53-6.7), and PerCP-conjugated anti-mouse CD4
(clone RM4-5). All monoclonal antibodies were purchased from BD Pharmingen (San
Diego, CA). Cells were stained using a standard lyse/wash procedure using PBS
(Dulbecco’s PBS without Ca and Mg, 2% FBS, and 0.1% NaN₃). A total of 10,000
events from each sample were analyzed on a dual-laser flow cytometer (FACSCalibur,
BD Immunocytometry Systems) using Cell Quest Pro and Attractors software. Forward
and side scatter characteristics as well as differences in antibody staining were used to
identify lymphocytes, neutrophils, and monocytes/macrophages. Matched isotype
controls were used to set negative staining criteria.

Tests of anxiety-like behavior and locomotion. The open field test is designed to take
advantage of a rodent’s natural tendencies to explore the environment while avoiding
open spaces. Mice that express anxiety-like behavior tend to spend less time in the center
of the open field and also tend to locomote near the walls of the apparatus (thigmotaxis).
These effects are also reversed by anxiolytic compounds (Bhatnagar et al., 2004;
Crawley, 1999; Dulawa et al., 2004; Prut and Belzung, 2003; Sullivan et al., 2003). An
automated open field (VersaMax, AccuScan Instruments, Columbus, OH) test was used
to assess anxiety-like behavior, locomotion, and exploratory behaviors. The apparatus
consisted of a 30 x 30 x 25 cm Plexiglas box with a solid floor and was lighted by
overhead room lighting (1300 lux). Two sets of optical sensors were used to record the
horizontal and vertical movement of the mouse while in the open field. Behavior was
measured on the morning immediately following the sixth cycle of social defeat.
Behavioral tests began at 1000 h (± 2 h) and lasted 5 min per mouse. Mice were transported on a cart to the testing area and left undisturbed for 30 minutes before testing began. Data were later exported and analyzed using the VersaDat program for total distance traveled and rearing, and the VersaMap program to define and measure the center and perimeter of the open field. For this experiment, the dependant variables were [a] total distance traveled, [b] number of rears, [c] total time spent in the center/perimeter of the open field, and [d] number of transitions between the center/perimeter of the open field. Each test apparatus was cleaned with water-dampened cloths between subjects.

Statistics. A repeated measures ANOVA was used to assess glucocorticoid insensitivity. A between groups, 2-way ANOVA was used to compare behavioral measures on separate time points. Post hoc analyses were performed using Fisher’s PLSD tests, where appropriate. All statistical comparisons were made using Statview software (SAS Institute, Inc.). All results are presented as mean ± SE. Statistical significance was set at \( p < 0.05 \).

Results

Daily injections of vehicle had no effect on any of the measures taken. Thus, vehicle-injected and non-injected control groups were combined in the following analyses.

Immunological effects. As previously shown, social disruption resulted in splenomegaly \( (F(1,39) = 34.94; p < 0.0001; \text{Fig 12A}) \), and this effect persisted when controlling for body mass \( (F(1,39) = 37.14; p < 0.0001; \text{Fig 12B}) \). Follow-up comparisons revealed that
this effect was not affected DZ treatment, although splenomegaly did not develop in FG treated mice. Analysis of splenic cellularity by flow cytometry indicated that, as shown previously, SDR caused an increase in granulocytes \((F(1, 41) = 19.19; p < 0.0001)\) and macrophages \((F(1, 41) = 14.97; p < 0.001)\). Diazepam had no effect on the previously-observed SDR-induced increases in granulocyte and macrophage populations (Fig. 13, detailed in Table 2). Similarly, glucocorticoid insensitivity developed mice subjected to SDR but did not develop in any of the non-SDR control groups, regardless of anxiety-inducing or anxiety-reducing drug treatment \((F(1,39) = 4.07; p < 0.05;\) Fig 14). Diazepam did not affect the development of GC insensitivity, although FG-treated mice did not develop GC insensitivity.

**Anxiety-like behavior.** As shown in chapter 2, six cycles of SDR caused a statistically significant increase in anxiety-like behavior, thus validating the automated open field test. Time in the center of the open field was decreased in SDR treatment groups \((F(1,39) = 20.81; p < 0.001;\) Fig 15A), as compared with control mice. Total entries to the center of the open field were also decreased in SDR mice \((F(1,39) = 14.19; p < 0.001;\) Fig 15B). Rearing was significantly decreased in SDR mice \((F(1,39) = 5.67; p < 0.05;\) Fig 15C). There was a main affect of SDR on locomotion, here reported as total distance traveled \((F(1,39) = 7.51; p < 0.05;\) Fig 15D). Follow-up comparisons revealed that this effect was driven by significantly reduced distance traveled by defeated control mice.
Discussion.

The mechanisms that cause the immunological and behavioral effects previously seen after SDR were neither attenuated nor exacerbated by benzodiazepine treatment. The benzodiazepine agonist, diazepam, had no significant effect on spleen mass, GC insensitivity, or anxiety-like behavior in the open field test, even at doses that were previously found to have sedative effects on locomotion (Prut & Belzung, 2003). Similarly, the benzodiazepine partial inverse agonist FG7142 did not exacerbate the effects of SDR and had no statistically significant effect on any of the measured outcomes, likely due to limited sample size. These data suggest that a) the drugs had no effect on anxiety during SDR, b) diazepam had only a transient anxiolytic effects, because anxiety-like behavior appeared in the open field test after the cessation of SDR, or c) anxiety is a covariate of the observed immunological effects of SDR.

Preliminary data suggested that the dose of diazepam (1 mg/kg) was more than sufficient to have anxiolytic effects, and may have had slight sedative effects on the mice. Like many anxiolytics, high doses of diazepam cause sedation in mice. However, low doses increase exploratory behavior without affecting locomotor activity (Blumstein & Crawley, 1983). The chosen dose has also been reported by others to have sedative effects on locomotion in the open field (Prut & Belzung, 2003). However, preliminary data indicated that both higher (4 mg/kg) and lower dose (0.25 mg/kg) diazepam had even less effect on blocking the development of SDR-induced splenomegaly than the dose used herein. In addition, there is recent evidence that diazepam may have limited anxiolytic efficacy when used in stressors that have a strong social component. Male C57BL/6 mice that were repeatedly defeated over 20 days showed an increase in anxiety-
like behavior in the elevated plus maze (EPM) and decreased social contact in the “partition” test, in which the mouse is placed in a cage with a novel mouse, separated by a partition. If the mouse spends more time avoiding the partition (and the novel mouse on the other side of the partition), it is presumably avoiding social contact (Avgustinovich et al., 1997; Kudryavtseva, 2000, 2004). Diazepam (1 mg/kg) restored EPM behavior to non-defeated values, but had no significant effects on social contact in the partition test (Amikishieva & Semendyaeva, 2005). This suggests that, even if diazepam treatment would have reversed the anxiogenic effects of SDR in the open field test, these effects may have persisted throughout the SDR sessions themselves. Further, it suggests that any sedative effects that were observed at this dose of diazepam in the open field may have not been present during SDR defeat sessions. To address this concern, the effects of anxiolytic drugs on mice subjected to SDR should be measured in tests that also contain a social component.

In addition to GABA, serotonin has been implicated in anxiety disorders. Some commonly-used anti-depressants that increase synaptic serotonin also have anxiolytic effects. The specific mechanisms through which serotonin affects anxiety-like behavior are not well understood at this time. However, McNaughton and Gray (2000) suggest that serotonin-dependant modulation of theta wave activity in the hippocampus is gated by a serotonergic pathway and has indirect effects on arousal. In humans and animals, serotonin (5-HT) agonists and reuptake inhibitors have anxiolytic effects on behavior, whereas antagonists generally have anxiogenic effects (Bhatnagar et al., 2004;
Peng, et al., 2004). The serotonergic system was not addressed in the present study, although future studies may address the relative importance of this system in SDR-induced anxiety-like behaviors.

Administration of both a benzodiazepine agonist and an inverse agonist had no effect on SDR-induced changes in immunity or anxiety-like behavior in the open field test. This suggests the SDR-induced increases in anxiety-like behavior, as presented in the previous chapters, are not directly causing “downstream” immune changes seen previously in mice subjected to SDR. However, because the drug treatments did not affect overall time in the center of the open field after the cessation of the stressor, the overall efficacy of these drugs within this stress paradigm is suspect. Further experiments are required to conclusively delineate the mechanisms through which social defeat alters immune function and behavior in the social disruption paradigm.
CHAPTER 5
DISCUSSION

Introduction

The experiments presented herein investigated the effects of social defeat on anxiety- and depressive-like behavior and immunity in mice, and whether these effects were altered with aging. Social defeat in the social disruption model revealed significant increases in anxiety-like behaviors in the open field test and the light/dark preference test. However, no increased depressive-like behaviors were observed in the forced swim test or the tail suspension test. These data also replicated and expanded previous findings on the effects of social disruption stress on immunity. In both inbred C57BL/6 and outbred CD-1 strains, social disruption caused an increase in spleen mass, increased IL-6 and TNF-α production by LPs-stimulated splenocytes, and insensitivity to the pro-apoptotic effects of corticosterone. These effects were larger in aged mice, which demonstrated a similar increase in serum corticosterone after defeat sessions, compared with young adult mice. Benzodiazepine treatment had no effect on the development of GC insensitivity or anxiety-like behavior, as measured in the open field test.
Behavioral Effects of Social Defeat

In the first set of experiments, it was hypothesized that social defeat in the social disruption paradigm would cause increases in anxiety- and depressive-like behaviors. Social disruption caused an increase in anxiety-like behaviors, but not depressive-like behaviors. Although not an objective in the current study, social disruption presumably also induced fear in the resident mice. During SDR the resident mice display defensive behaviors, and the development of anxiety-like behavior presupposes the concurrent development of a fear response (McNaughton & Gray, 2000). The development of anxiety-like behavior in the SDR model is consistent with results from other social defeat models, including the sensory contact model of defeat (Avgustinovich et al., 1998). Perhaps more importantly, however, the development of anxiety-like behavior in SDR mice mirrors the development of anxiety in socially defeated humans. One of the strengths of the SDR model is that, compared with many other laboratory stressors, it is ecologically relevant within the model species (i.e. increased face validity). Although mice do not tend to live in large social groups, agonistic interactions are presumably quite common, especially in circumstances where resources are limited (Nelson & Chiavegatto, 2001). Extending the experimental model to outbred mice further increased the validity and reliability of the model.

The lack of any observed depressive-like effects in the present studies suggests that mice defeated after six sessions of SDR do not become passive. One reason for this is that the tests used herein are purported to measure “behavioral despair” or the failure to attempt to escape from aversive stimuli (Crawley, 1999; Cryan & Mombereau, 2004; Porsolt et al., 1977). Thus, although these studies provided no evidence to support the
hypothesis that SDR affects depressive-like behavior, it is possible other models of
depression, including the anhedonia model, may later provide evidence for that SDR does
affect depression in mice. Another reason why no depressive-like behavior emerged is
that six days of SDR may not be a long enough time to induce behavioral despair.
Repeated social defeat has been shown to increase immobility in the forced swim test in a
dose-dependent-like fashion (Hebert et al., 1998). Although some logistical problems
(e.g. excessive wounding) would have to be overcome, a longer exposure to the aggressor
mouse, or SDR lasting longer than six days, may induce a depressive-like state in
defeated mice.

The present studies were unable to account for individual differences in the
development of anxiety-like behavior and SDR-induced immune changes. Mice that
developed GC insensitivity, for example, also expressed anxiety-like behaviors, though
the two were not correlated significantly on an animal-by-animal basis. The same was
also true of wound severity. However, the role of wound severity may have been
previously overstated. Johnson and colleagues write that “GC resistance has previously
been highly correlated with the degree of wounding (Avitsur et al., 2002).” Although
wounding was not measured in the article cited, when Avitsur and colleagues (2001) did
measure wounding in the SDR model, the authors did not measure wound severity, per
se, but concluded that wounded animals developed GC insensitivity, while non-wounded
animals did not. Data from our lab and others suggest that wounding is necessary, but
not sufficient for the development of GC insensitivity (Bailey et al., 2004; Merlot et al.,
2003). Evidence from socially defeated rats also suggests that wounding, but not wound
severity, leads to an increased chronic stress state (Zelena et al., 1999). Like wound
severity, anxiety-like behavior does not correlate with GC insensitivity on an animal-to-animal scale, but the presence of anxiety-like behavior is higher in mice that develop GC insensitivity, as compared with those that do not.

Alternatively, it is possible that the tests of anxiety-like behavior employed herein were not sensitive enough to predict SDR-induced changes in immune function. With the groundwork laid with these data on anxiety-like behavior, a battery of tests can be designed and run on large samples of mice in experiments using SDR. The recent acquisition and implementation of automated ethological testing equipment would help greatly to this end. Although mice habituate to some of the common tests of anxiety-like behavior (e.g. the open field test (Holmes et al., 2001; Paylor et al. 2006)), a rapid behavioral battery could be designed to explore, for example, the effects of SDR on defensive burying, behavior in the elevated plus maze and hole board test, novel object exploration, and tests of depressive-like behavior, such as anhedonia models.

Aging and Social Defeat

The second set of experiments addressed the effects of aging on SDR-induced immune and behavioral changes previously observed in young mice. Aged-dependent decreases in immune regulation, commonly referred to as immunosenescence, are worsened by stress in animals, including humans (reviewed by Hawkley & Cacioppo, 2004). This study was designed to address interactions between aging and social defeat. In searching the somewhat disparate literature on aging and stress, it was apparent that, although anecdotal evidence abounds, empirical evidence for the interactions between stress and aging is relatively rare, and that the two fields do not tend to overlap (see
Graham et al., 2006 for review of the human literature). This presented an excellent opportunity, and the data revealed some interesting effects of aging and defeat on inflammation.

For example, social defeat stress and aging interacted synergistically, causing increased defeat-induced release of proinflammatory cytokines and GC insensitivity, compared with non-defeated, young adult mice. Non-defeated aged controls also exhibited increased cytokine release by activated splenocytes. The role of aging and proinflammatory cytokine release is somewhat controversial. Previous studies have found that aging causes either increases (Roubenoff et al, 1998; Walston et al., 2005) or decreases in proinflammatory cytokines (Delpedro et al., 1998). Similarly, isolated splenic and peritoneal macrophages from aged animals have been shown to express lower IL-6 gene expression than cells from younger animals (Chelvarajan et al., 2006). However, when whole splenocytes were stimulated with mitogen, IL-6 concentrations were increased in aged animals (Boehmer et al., 2004; Daynes et al., 1993; Liang et al., 1998). These data underscore some of the critical differences seen between in vitro and in vivo assay environments (Gomez et al., 2005). Although the present studies assay cytokine release and GC insensitivity in vitro, our methods more closely model the in vivo splenic environment than do assays using fractioned splenocytes. This consistency is important as we make inferences about the overall stress response.

As with young adult mice, in aged mice social defeat resulted in increased anxiety-like behavior, with no effect on depressive-like behavior. This is consistent with previous studies of stress in aged humans, although the increased anxiety and depression in aged individuals tends to have more to do with illness and reduced social networks
than social defeat (Hawkley & Cacioppo, 2004). Still, these data replicated the effects seen in young adult animals in the previous chapter and indicate that the behavioral effects of social defeat are constant throughout adulthood.

Benzodiazepine Treatment of SDR

The third study investigated whether pharmacological blockade or activation of central anxiety signals affected SDR-induced immune changes. We hypothesized that, if the development of an anxiety-like state was necessary for the development of GC insensitivity, then blocking or inducing anxiety pharmacologically would affect GC insensitivity. However, even at relatively high doses, anxiolytic and anxiogenic drugs had no effect on immunity or the post-SDR expression of anxiety-like behaviors.

Pilot data indicated that the chosen dose of diazepam was sufficient to have lightly sedative effects on locomotion, and therefore more than sufficient to reduce anxiety. Assuming that the chosen dose of diazepam was sufficient to block the activation of the anxiety circuits, this study indicates that an SDR-induced anxiety-like state does not cause GC insensitivity. However, there are some problems drawing such conclusions based on the current data, especially given that anxiety-like behaviors did emerge after SDR, as measured by the open field test. A primary assumption of this model is that SDR-induced increases in anxiety-like behavior are a result of defeat during SDR sessions. However, although SDR sessions are presumably fear-inducing, other neural signals could contribute to the development of anxiety-like behavior, such as pain and discomfort that the resident mice experience as a result of wounding during SDR. Chronic pain has also been associated with anxiety and depression in humans (Gallagher
et al., 1995), and mice subjected to sciatic nerve transection showed increased
nociception and anxiety-like behavior in the light/dark preference test (Narita et al.,
2006). In the present studies, although anxiety-like behavior was treated during SDR
sessions, the behavioral changes could have emerged as a result of central activation
throughout the six day SDR treatment.

It is also possible that, although the dose of diazepam was chosen based on pilot
data and literature suggesting that it was an appropriate dose to suppress anxiety-like
behavior, the drug had no effect on anxiety in the context of intense social defeat. In
socially defeated rats, DZ had anxiolytic effects in the elevated plus maze, but had no
effect on the partition test, a test of social anxiety (Amikishieva et al., 2005). In other
words, although the diazepam treatment caused the rats to spend more time exploring the
open arms of the novel environment, it did not restore the amount of time these same
animals would spend in proximity to an unfamiliar rat. If DZ has no affect on the social
aspects of anxiety-like behavior, then anxiety was not blocked during SDR.

Repeated activation of the HPA axis by restraint stress may increase anxiety-like
behavior, although it does not result in GC insensitivity. Swiss mice subjected to three
cycles of 2 hour restraint displayed increased anxiety-like behavior in the elevated plus
maze and light/lark preference test, and increased stress reactivity no novel environment
(Chotiwat & Harris, 2006). However, CD-1 mice subjected to six cycles of 2 hour
restraint did not show significantly increased anxiety-like behavior in the open field test
and did not develop GC insensitivity, although the RST mice did lose weight (see

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Appendix A). These data suggest that repeated activation of the HPA axis can result in anxiety-like behavior, but that additional peripheral signals are needed for the development of GC insensitivity.

Another possibility is that peripheral signals, such as proinflammatory cytokines or other inflammatory mediators, such as NF-κB, activate the anxiety circuitry, resulting on the expression of anxiety-like behavior. If this is the case, IL-6 is not a likely candidate as an inducer of anxiety-like behavior. Genetic IL-6 knockout mice have been tested extensively and do not differ from wild type mice in measures of anxiety-like (open field, elevated plus maze), depressive-like behaviors (forced swim test, tail suspension), or exploratory behaviors (Swiergiel & Dunn, 2006). Instead, increased IL-6 has been linked with sickness behaviors, such as decreased locomotion, social exploration, and weight loss (Bluthe et al., 2000; Godbout et al., 2005).

Mice lacking the p50 (NF-κB1) component of NF-κB showed decreased anxiety-like behaviors in the elevated plus maze and the open field test, as well as increased exploration of a novel object, as compared with wild type mice (Kassed et al., 2004). These NF-κB mice are more prone to infection than wild type mice (Artis, et al., 2003) and also display reduced macrophage NF-κB activation (Kato et al., 2002). In contrast, macrophages from mice subjected to SDR have increased NF-κB nuclear translocation, as compared with control mice (Quan et al., 2003).

Central activation, resulting in anxiety-like behavior, also resulted in activation of CD11b+ macrophages in the periphery, resulting in GC insensitivity and increased pro-inflammatory cytokine production. Alternatively, periphery activation of the inflammatory response led to activation of central anxiety circuitry, resulting in anxiety-
like behavior. These two mechanisms are not mutually exclusive. Although the third study presented herein attempted to address this issue, a causal link between the observed behavioral and proinflammatory immunological changes has yet to be established. Future studies are needed to further explore the neuroimmune response to social defeat.

Conclusions

The studies presented herein were designed to test the hypothesis that social disruption causes behavioral changes in mice, in addition to altering immune regulation. Social defeat caused an increase in anxiety-like behavior, increased proinflammatory cytokine production, and glucocorticoid insensitivity. These effects were observed in young and aged, inbred and outbred strains or mice. The SDR-induced anxiety-like behavior is a novel finding in this model, and sets it apart from other immune-altering models of stress, which have no effect on anxiety-like behavior or result in depressive-like behavior. However, although there is no current data to support the development of depressive-like behavior as a result of SDR, further investigation is needed to completely rule it out.

The present data support the hypothesis that higher-order processes in the central nervous system contribute to the development of glucocorticoid insensitivity, and that wounding is, in itself, not sufficient to induce or predict glucocorticoid insensitivity. However, wounding does appear to play some role in the development of GC insensitivity, as mice not wounded during SDR (Avitsur et al., 2001; Bailey et al., 2004), or mice subjected to repeated two hour restraint (Appendix A) do not express GC insensitivity (although some potentially interesting trends emerged). Further
investigation will reveal insights into the effects that the stress response and signal proteins, including corticosterone, play on the development of GC insensitivity, as expressed in the SDR model.

The field of psychoneuroimmunology focuses on the study of the interactions between the central nervous system and immunity. Stress has been shown repeatedly to affect health outcomes in people, leading to shortened lifespan, slowed wound healing, increased heart disease, and increased disease incidence. For this reason, much current human research investigates the role of anti-stress intervention therapies on restoring health in stressed individuals. Research in animals, on the other hand, probes the mechanisms by which stress and immunity affect one another. Some of the stressors that are commonly used in mice, such as chronic restraint, undoubtedly activate the stress response in the animals, although these models lack a direct behavioral parallel to a human stressor. Thus, the social disruption model was developed to incorporate social components into the stressor. The increased anxiety-like behavior reported herein bolsters the use of social disruption as a model of social stress and defeat in humans, which has also been shown to increase both anxiety and depression. In turn, these data further the contemporary understanding of the intricate cross-talk between multiple regulatory physiological systems, which were previously thought to behave independently from one another.
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APPENDIX A

EFFECTS OF RESTRAINT ON ANXIETY-LIKE BEHAVIOR AND GC INSENSITIVITY IN CD-1 MICE

Introduction

The experiments presented herein demonstrated that social defeat in the social disruption (SDR) paradigm caused increased anxiety-like behavior in the open field test and the light/dark preference test. Social disruption also resulted in many of the previously-observed immune regulatory changes, including increased proinflammatory cytokine production and glucocorticoid insensitivity in cultured splenocytes (Avitsur et al., 2001; Stark et al., 2001).

One of the proposed mechanisms for both behavioral and immune changes is the repeated activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS). However, repeated activation of the HPA axis by restraint stress (RST) did not have the same effects on wound healing as SDR (Sheridan et al., 2004). In this study, restraint was repeatedly applied for 12 hours on eight consecutive days, resulting in decreased wound healing rate. In contrast, SDR is typically applied for two hours on six consecutive days, and did not appear to affect wound healing in healthy male mice. So, although the HPA axis was activated in both paradigms, the pattern of activation was quite different, resulting in very different affects.
The following experiment was performed to examine whether RST had any effects on anxiety-like behavior or SDR-induced GC insensitivity. We hypothesized that repeated activation of the HPA axis and SNS via repeated RST would result in increased anxiety-like behavior and GC insensitivity.

Methods

Animals. Male CD-1 mice (n=38) were ordered from Charles River at 6-8 weeks of age. Mice were housed 3-5 per cage, and randomly assigned into either restraint (RST; n=24) or food and water deprived (FWD; n=14) treatment group and left undisturbed for at least 1 week before testing began. All mice were kept in an American Association for Accreditation of Laboratory Animals Care (AAALAC) accredited facility at the Ohio State University and provided standard lab diet and tap water ad libitum. The Institutional Laboratory Animal Care and Use Committee (ILACUC) at the Ohio State University approved all experimental protocols.

Restraint stress. Mice were restrained in polypropylene tubes, as described previously (Padgett et al., 1998b; Sheridan et al., 2004), with modifications made to the timing and direction of restraint treatment. In order to simulate the effects of the SDR model, mice were subjected to six cycles of daily restraint, each lasting 2 h starting at approximately 1700 h EST. Because the restrained mice were deprived of access to food and water during restraint, control cages were also food and water deprived during daily restraint treatments. All mice were weighed daily, prior to restraint, to control for handling.
Glucocorticoid Sensitivity Assay. The glucocorticoid sensitivity assay was performed as described in chapter 2.

Open field test. The open field test was performed as described in chapter 2.

General Procedure. The timeline of this study was designed to parallel previous studies in mice subjected to SDR. Thus, mice were subjected to six days of RST, and behavior in the open field test was measured on the morning following the last cycle of restraint stress. All mice were sacrificed on the morning flowing behavior testing. At sacrifice, spleens were harvested, weighed, and prepared for the GC insensitivity assay, as described in chapter 2.

Statistics. Spleen size was compared by one-way between measures (stress treatment) ANOVA. A repeated measures ANOVA was used to assess statistical significance for the GC insensitivity assay. Fisher’s PLSD tests were used for post hoc comparisons of pair-wise differences. All results are presented as mean ± SE. Statistical significance was set at $p < 0.05$.

Results

Five of the mice in the FWD group were excluded from all analyses, because, due to unknown causes, these animals lost significant body mass on days 2 and 3 of the experiment.
Six days of two hour restraint stress caused significant weight loss during testing (Fig 16A), and this effect is more evident when controlling for body weight on day 1 (F(1,192) = 24.01; p < 0.0001; Fig 16B). However, restraint stress did not significantly alter spleen mass. Spleen mass for RST and FWD mice was 115.50 ± 11.89 and 108.11 ± 5.59 respectively (Fig. 16C). No effect emerged when controlling for individual body mass. Similarly, RST had no affect on GC insensitivity. Stimulated splenocyte proliferation did not differ between RST and FWD treatments, even at high pharmacological doses of corticosterone (Fig. 16D).

Restraint stress did not significantly affect anxiety-like behavior or locomotion in the open field test. Time in the center of the open field appeared to be reduced in the RST group, though not to a statistically significant degree (p > 0.35; Fig 17A). Similarly, total entries to the center of the open field were not significantly reduced by RST (p > 0.25; Fig 17B). Locomotion, here reported as line crosses did not differ between treatment groups (Fig. 17C).

**Discussion**

The open field test is commonly used to measure locomotion, exploratory behaviors, and anxiety-like behaviors in many different species. Based previous data (Chapter 2), there were no strain differences in open field behavior. In the CD-1 mouse strain, SDR-induced increases in anxiety-like behavior were most pronounced in the open field test. However, a separate lab found RST-induced increases in anxiety-like behavior in the light/dark preference test and elevated plus maze in the NIH Swiss mouse strain (Chotiwat & Harris, 2006). Although an outbred mouse strain was used, these effects
were evident after just three days of restraint. Future studies using inbred strains and different ethological tests may replicate previous findings of RST-induced anxiety-like behavior. However, the current data indicate that repeated activation of the stress response is not sufficient to induce splenomegaly or GC insensitivity.

In the present study, six days of two hour restraint did not significantly affect immunity in any of the measures used. Although HPA and SNS activation were not directly measured, previous studies suggest that RST as short as one hour is sufficient to activate these systems (e.g. Tuli et al., 1995). Based on these data, it can be inferred that repeated, short-term increases in corticosterone are not sufficient to induce GC insensitivity, as seen in the SDR model.
APPENDIX B

NOTES ON MANUAL VS. AUTOMATED BEHAVIORAL TESTING

In the first two experiments, behavioral data were coded manually, by trained observers. The experiment presented in Chapter 4, however, used an automated system for recording and coding behavior in the open field. The automated open field test had many advantages over manual behavioral coding, including extremely fast data output and a reduction in any possible observer bias. The automated system tracked locomotion differently than manual coding, and could give read-outs of total horizontal activity (beam beaks) or total distance traveled. Because the optical beams were placed more closely together (approx 2.5 cm apart) than the lines drawn on the floor of the manual open field (approx 8 cm apart), total distance traveled is probably the most accurate measurement of locomotor activity. Rearing behaviors were coded very similarly by the automated system as they were by manual coding methods. Although the automated light/dark box test apparatus was not used in the present studies, it presumably offers the above mentioned advantages over manual coding, in addition to allowing measurement of behavior inside the dark box, which is currently impossible with manual coding.

However, due to the nature by which the automated system determines the location of the mouse, this method of behavioral assessment may be less sensitive than
manual coding. For example, when manually coding the location (i.e. center vs. periphery of the open field) of a mouse, the definition is very clearly defined in the ethogram as the mouse having all four of its feet on one side of the line drawn on the floor of the apparatus. Because the automated system is not sensitive enough to detect foot location, it estimates the location of the center of the mouse mathematically. Thus, in some cases, the automated system coded the mouse as being in the center of the open field, whereas a trained observer might have coded the mouse being in the periphery.

Because the automated system coded mouse location differently, it appeared to be less sensitive to anxiety-like behaviors in the open field than manual coding. Although the two systems were not tested side-by-side on the same animals, the automated system required a higher degree of overall fighting during the SDR sessions to find anxiogenic effects that were easily detectable with manual coding in cages with less fighting. Whereas manual coding in the open field consistently found SDR-induced increases in time in the center of the open field in cages of 5 CD-1 or C57BL/6 mice, the automated system found this effect in C57BL/6 mice housed 3 per cage, but not 5 per cage.

The automated system also appeared to give more consistent effects with C57BL/6 mice than in CD-1 mice. Strain differences in SDR-induced anxiety or differences in body size ay have caused technical issues. Due to their larger size, the mathematical center of a CD-1 mouse placed it in the center of the open field, whereas manual coding placed the same mouse in the periphery. As suggested in chapter 2, the light/dark test may not have been sensitive enough to detect anxiogenic effect of SDR in CD-1 mice. Again, these differences may be strain-specific or may represent shortcomings of the specific ethological paradigms used.
Figure 1. SDR increases anxiety-like behavior in the light-dark preference test. Male C57BL/6 mice were subjected to repeated social disruption (SDR) for 6 consecutive days. On the morning following the sixth day of defeat, the mice were individually tested in the light/dark preference test for 5 minutes. (a) Defeated mice (SDR) spent significantly more time in the dark box of the test apparatus than non-defeated home cage controls (HCC). (b) Defeated mice took longer to emerge from the dark box than controls. *$p < 0.05$ vs. controls.
Figure 2. Social disruption caused changes in immune regulation in C57BL/6 mice. Mice were subjected to six consecutive days of social disruption stress (SDR), then spleens were harvested, weighed, and cultured. (a) Mice subjected to SDR had heavier spleens than controls (HCC) mice, even when corrected for body weight. (b) Splenocytes were cultured for 48 hours, stimulated with LPS and various concentrations of the glucocorticoid hormone corticosterone. Splenocytes from defeated mice were insensitive to the pro-apoptotic effects of corticosterone. (c, d) Splenocytes were cultured for 18 hours, stimulated with LPS. Supernatants from these cells were assayed for IL-6 and TNF via sandwich ELISA. *p < 0.05; **p < 0.01 vs. control.
Figure 3. Behavioral effects of social disruption in C57BL/6 mice. Mice were subjected to 6 consecutive days of social disruption (SDR; filled bars). Separate groups of mice were tested on the day after 1 day of defeat (Day 1), after the 6th day of defeat (Day 6), or after 6 days of defeat, then rested for 8 days with no SDR (Day 14). Non-defeated controls (HCC; open bars) were also tested on each of these days. (a) Mice subjected to SDR spent less time in the center of the open field and (b) entered the center of the open field less often than controls. (c) Locomotion was not affected by SDR. (d, e) In the forced swim test, defeated mice became immobile more quickly than controls on Day 1 and Day 6, although total time spent immobile did not differ between groups. (f) Immobility in the tail suspension test was not affected by SDR. * p < 0.05 vs. controls.
Figure 4. Immune effects of SDR in CD-1 mice. Mice were subjected to 6 consecutive days of social disruption (SDR). Spleens were harvested, weighed, and cultured. (a) As with inbred strains, spleens from SDR mice were larger than those from control (HCC) mice. (b) Cultured splenocytes from SDR mice were insensitive to the pro-apoptotic effects of corticosterone, as compared with controls, even at high concentrations. (c, d) Supernatants from LPS-stimulated splenocytes from defeated mice contained higher concentrations of IL-6 and TNF than those from control mice. * p < 0.05; † p < 0.001
Figure 5. Behavioral effects of SDR in CD-1 mice. Mice were subjected to 6 consecutive days of social disruption (SDR), and behavior was measured on the morning following the 6th day of defeat. (a) In the open field test, defeated mice spent less time in the center of the open field than controls (HCC) and (b) Entered the center of the open field fewer times than controls. (c) Locomotion was not affected by SDR. (d, e) In the forced swim test, CD-1 mice showed no differences in latency to become immobile or in overall time immobile, regardless of defeat treatment. (f) No differences were found in time immobile in the tail suspension test. **p < 0.01
Figure 6. Aging and stress affect thymus and spleen mass. Male CD-1 mice were subjected to repeated social defeat for 6 days. (a) Mice aged 14 months show thymic involution, a typical feature of immunosenescence. Repeated social defeat caused a reduction in relative thymus mass (thymus mass/body mass) in young adult mice (2-months-old), but not aged mice. (b) Relative spleen mass (spleen mass/body mass) is lower in aged mice than young adult mice. Repeated social defeat causes an increase in both young and aged mice. *p < 0.05 vs. controls.
Figure 7. Aging and social defeat cause increased proinflammatory cytokine production in cultured splenocytes. Spleen cells were harvested from young adult and aged adult mice, stimulated with LPS, and incubated for 18 hours. Cytokines were quantified by ELISA. (a) Aging caused an increase in the production of both IL-6 and TNFα. (b,c) Social defeat caused increased production of IL-6 and TNFα in both young and aged mice. *p < 0.05 vs. young adults; **p < 0.05 vs. age-matched controls.
Figure 8. Serum corticosterone concentrations from young adult (age 2 months) and aged adult (14 month) male mice subjected to repeated social defeat. Samples were obtained via retro orbital plexus immediately after defeat session (approximately 1830 EST) and quantified from serum via radioimmune assay. \(*p < 0.05\) vs. baseline.
Figure 9. Social defeat alters splenic cellularity in both young and aged adult mice. Splenocyte cell populations were phenotyped by flow cytometry. *p < 0.05; † p < 0.001 vs. controls.
**Table 1.** Social defeat alters splenic cellularity in both young and aged adult mice. Splenic cell populations were phenotyped by flow cytometry and expressed as mean cell count with standard error in italics. *p < 0.05 vs. controls.

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Figure 10. Social defeat causes insensitivity to the glucocorticoid (GC) hormone corticosterone. Spleen cells were harvested from young adult and aged adult mice, costimulated with LPS and corticosterone and incubated for 48 hours. GC insensitivity is defined as high cell viability in the presence of increased concentrations of corticosterone. Cell viability was higher in aged, defeated mice than any other group. Both defeated groups demonstrate GC insensitivity, as compared with control groups, with an interaction between age and defeat treatments. *p < 0.05 vs age-matched control; **p < 0.05 vs all groups.
Figure 11. Defeat increased anxiety-like behavior in aged mice. Young and aged adult mice were subjected to six days of repeated social defeat, and then assessed in the open field test. Data from young adult mice area presented here, but were not analyzed due to abnormally high anxiety-like behavior. (a) Defeated mice spent less time in the center of the open field. (b) Defeated mice entered the center of the field fewer times than controls. (c) Locomotion was reduced in defeated mice. (d, e) The Porsolt forced swim test indicated that defeat did not affect time immobile or latency to become immobile, two tests of depressive-like behavior. * $p < 0.05$ vs. age-matched control.
Figure 12. Effects of social disruption (SDR) and benzodiazepines on spleen mass. Mice were randomly assigned to stress and drug treatment groups. All mice were weighed daily and injected with either drug or vehicle, then subjected to SDR or returned to their home cage. (a) SDR caused an increase in spleen mass, regardless of drug treatment. (b) This effect persists when controlling for body mass. ** $p < 0.01$; † $p < 0.001$ vs. same drug control.
Figure 13. Effects of social disruption (SDR) and benzodiazepines on splenic cellularity. Mice were randomly assigned to stress and drug treatment groups. All mice were weighed daily and injected with either drug or vehicle, then subjected to SDR or returned to their home cage. At sacrifice, spleen cells were isolated and phenotyped by flow cytometry. (a,b) SDR caused an increase in granulocytes and macrophages in vehicle and diazepam-treated mice. (c,d) SDR and drug treatments had no effect on CD4+ or CD8+ T cells. * p < 0.05; † p < 0.001 vs. same drug control.
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Table 2. Effects of social disruption (SDR) and benzodiazepines on splenic cellularity. Mice were randomly assigned to stress and drug treatment groups. All mice were weighed daily and injected with either drug or vehicle, then subjected to SDR or returned to their home cage. At sacrifice, spleen cells were isolated and phenotyped by flow cytometry. Values are expressed as mean ± S.E. ** $p < 0.01$; † $p < 0.001$ vs. same drug control.
Figure 14. Effects of social disruption (SDR) and benzodiazepines on glucocorticoid insensitivity. Mice were randomly assigned to stress and drug treatment groups. All mice were weighed daily and injected with either drug or vehicle, then subjected to SDR or returned to their home cage. At sacrifice, spleen cells were stimulated with LPS and cultured with various concentrations of corticosterone. Cell viability was quantified at 48 hours. * p < 0.05
Figure 15. Effects of social disruption (SDR) and benzodiazepines on anxiety-like behaviors and locomotion. Mice were randomly assigned to stress and drug treatment groups. All mice were weighed daily and injected with either drug or vehicle, then subjected to SDR or returned to their home cage. Behavior was assessed using an automated open field test. (a) SDR caused a decrease in time spent in the center of the open field in vehicle and diazepam treated mice. (b) SDR decreased the number of entries to the center of the open field in vehicle and diazepam treated mice. (c,d) SDR caused a decrease in rearing behavior and locomotion in vehicle treated mice. * $p < 0.05$; † $p < 0.001$ vs. same drug control.
Figure 16. Repeated two hour restraint stress affects body mass. Male CD-1 mice were subjected to six consecutive days of two hour restraint stress (RST). Control mice were food and water deprived (FWD). All mice were weighed daily, prior to restraint or food and water deprivation, respectively. (a, b) Body mass was significantly reduced in RST mice. Because the RST group weighed more on day 1, controlling for body weight reveals this trend more clearly. (c) However, spleen mass was not significantly increased as a result of restraint. (d) Similarly, GC sensitivity was not significantly decreased in RST mice, as shown by splenocyte proliferation.

† p < 0.001 vs. FWD control.
Figure 17. Repeated restraint does not affect anxiety-like behavior. Male CD-1 mice were subjected to six consecutive days of two hour restraint stress (RST). Control mice were food and water deprived (FWD). All mice were weighed daily, prior to restraint or food and water deprivation, respectively. On the morning of the day following the sixth cycle of RST, mice were individually tested for anxiety-like behavior and locomotor differences in the open field test. (a) Restraint did not decrease time in the center of the open field to a statistically significant extent. (b) Similarly, entries to the center of the open field were not decreased to a statistically significant extent. (c) Locomotion (line crosses) was unaffected by restraint stress.