Dietary Curcumin Promotes Resilience to Chronic Social Defeat Stress in a Highly Susceptible Mouse Strain

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THE CITY UNIVERSITY OF NEW YORK
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Abstract

Advisor: Dr. Nesha Burghardt

Chronic exposure to stress is a risk factor for the development of major depression and post traumatic stress disorder in humans and induces depressive- and anxiety-like phenotypes in rodents. However, there are few pharmacological interventions available that effectively treat maladaptive responses to chronic stress in the clinical setting. One therapeutic agent that has recently shown promise in treating psychiatric disorders is curcumin, a yellow-pigmented polyphenol compound found in the turmeric plant. Curcumin has been shown to prevent the development of stressed-induced depressive-like behavior in rodents and reduce symptoms of depression in clinically diagnosed patients. In this dissertation, I investigated whether dietary curcumin prevents social avoidance and anxiety-like behavior following chronic psychosocial stress in chapter 2. In studies described in Chapter 3, I placed 129/SvEv male mice on a diet of 1.5% curcumin or a control chow 5 days prior to 10 days of chronic social defeat stress and then tested them in the social interaction test, open field, and elevated plus maze. I found that curcumin effectively blocks stress-induced increases in social avoidance and anxiety-like behavior. In Chapter 4, I investigated the underlying mechanisms and found that there is a correlation between the effects of dietary curcumin on social avoidance and suppression of stress-induced increases in peripheral markers of inflammation. Interestingly, I identified two distinct responses to treatment based on social avoidance behavior (responders and non-responders). Additional experiments described in Chapter 4 reveal that social behavior prior to
social defeat stress predicts treatment response. Together, these findings suggest that curcumin may be exerting its therapeutic effect by modulating levels of inflammation in the periphery and that social behavior at baseline may be a useful tool for predicting treatment outcome in preclinical research.
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<th>Description</th>
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<tbody>
<tr>
<td>ANS</td>
<td>Autonomic Nervous System</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin-Releasing Hormone</td>
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<tr>
<td>ATCH</td>
<td>Adrenal Corticotrophin-Releasing Hormone</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
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<td>MR</td>
<td>Mineralocorticoid Receptor</td>
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<td>GC</td>
<td>Glucocorticoids</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
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<td>IL-1β</td>
<td>Interleukin-1Beta</td>
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<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
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<tr>
<td>CSDS</td>
<td>Chronic Social Defeat Stress</td>
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<tr>
<td>RSD</td>
<td>Repeated Social Defeat</td>
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<tr>
<td>BM</td>
<td>Bone Marrow</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
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<tr>
<td>MDD</td>
<td>Major Depressive Disorder</td>
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<tr>
<td>PTSD</td>
<td>Post-Traumatic Stress Disorder</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drugs</td>
</tr>
<tr>
<td>AMYG</td>
<td>Amygdala</td>
</tr>
<tr>
<td>BNST</td>
<td>Bed Nucleus of Stria Terminalis</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular Nucleus</td>
</tr>
<tr>
<td>NAC</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>PFC</td>
<td>Pre-Frontal Cortex</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB-inducing Kinase</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB Kinase</td>
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<tr>
<td>IκB</td>
<td>Inhibitor of Kappa B</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of Activated B</td>
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Chapter 1: General Introduction
Defining Stress

The term “stress” is defined as the body/brain’s response to any threat or perceived threat that disturbs an organism’s ability to maintain homeostasis (Cannon 1935). Following the perception of a stressful event, the autonomic nervous system (ANS) is rapidly activated. This results in the release of catecholamines, such as norepinephrine, from the adrenal medulla. This acts directly on the cardiovascular system triggering peripheral responses, such as an increase in heat rate, respiration, and blood pressure (Ulrich-Lai & Herman 2009). In addition, stress engages the HPA axis by stimulating the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus, which causes the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH in turn stimulates the synthesis and release of glucocorticoid hormones from the adrenal cortex, which bind to mineralcorticoid (MR) and glucocorticoid receptors (GR) located throughout the body and brain (De Kloet et al 1998). Together, these two arms of the stress response promote the ability of the organism to adapt and maintain homeostasis in the face of external challenges, a process known as allostasis (McEwen 1998). Although activation of the stress response is initially adaptive, long-term exposure to stress poses a significant risk for the development of numerous health conditions, such as heart disease, diabetes, Alzheimer’s disease, obesity, asthma, and a range of psychiatric disorders that include major depressive disorder (MDD) and post-traumatic stress disorder (PTSD) (McEwen 2004).

Psychiatric Disorders and Stress

Psychiatric illnesses are complex multifactorial disorders that are influenced by multiple gene x environment interactions and manifest in numerous ways. For instance, depressed mood, anhedonia, poor sleep, fatigue, altered appetite and difficulty concentrating characterize MDD
PTSD is characterized by intense fear and avoidance, hyper-arousal, and the formation of a traumatic memory (DSM V). Over the past two decades, it has been well established that exposure to adverse/stressful life events and the way in which individuals cope in response to them play a crucial role in the development of these two disorders. For instance, the odds of experiencing a major depressive episode within the month of a major stressful life event are increased by 455% in men and 585% in woman (Kendler & Gardner 2010, Kendler et al 1999). Interestingly, different types of stress lead to distinct depressive symptoms. Psychosocial stressors, such as a romantic break up or the death of a loved one, lead to anhedonia and sadness, while other stressors do not alter mood-related symptoms (Keller et al 2007). Psychosocial stress also increases the odds of developing PTSD. For instance, individuals who experience high amounts of stress and have little social support during the time of the trauma are more likely to develop PTSD than others exposed to a similar trauma (Breslau et al 1999, Brewin et al 2000).

Previous studies in animal models suggest that chronic psychosocial stress affects the central and peripheral nervous systems through the endocrine and immune systems, which can ultimately lead to the behavioral symptoms mentioned above (Ménard et al 2017). In this chapter, I will focus on the studies that were completed in animal models and discuss their clinical implications.

Animal Models of Chronic Stress

In order to fully understand the impact of chronic stress on the nervous, endocrine, and immune systems, it is imperative to have a representative animal model (Akil et al 2017). Several chronic stress models have been used to study psychiatric disorders. The two major types of stressors used are physical and psychosocial stressors. The physical stressors include chronic
mild stress (also known as chronic variable stress) and chronic restraint stress. The psychosocial stressors include chronic social defeat stress (CSDS), social isolation and maternal separation. All of these models have been shown to induce behavioral abnormalities, with the psychosocial stressors producing longer-lasting behavioral phenotypes (Nestler & Hyman 2010). These alterations in behavior are assessed by a series of tests with different outcome measures. These measures include social behavior (social interaction test), anhedonia (sucrose preference, intracranial self-stimulation), exploratory behavior (elevated plus maze, open field) and the formation of an emotional memory (Pavlovian fear conditioning).

Although all models of chronic stress produce behavioral alterations that resemble some of the symptoms of MDD and PTSD, chronic social defeat stress (CSDS) leads to a particularly robust and long-lasting phenotype (Golden et al 2011). The advantage of using CSDS is that it is a psychosocial stressor with ecological validity. In this model, an intruder mouse is placed into the home cage of a retired CD-1 male breeder that had been pre-screened for aggressive behavior. The two mice interact for 5-10 minutes, during which time the CD-1 mouse attacks the intruder mouse. The two mice are then housed together in the same cage overnight, but are separated by a perforated Plexiglas divider that prevents further physical contact, but allows for continuous psychological stress from sensory (e.g. visual, olfactory, auditory) cues. During each day of defeat, the intruder mouse is housed with a different CD-1 mouse to prevent acclimation to the stressor. Following the last day of CSDS, mice are tested in the social interaction test, which involves exposing them to a novel CD-1 mouse and quantifying the amount of time they spend with that mouse. Mice that avoid the CD-1 are defined as susceptible to stress, and mice that interact with the CD-1 are deemed resilient. In addition to social avoidance behavior, CSDS induces anhedonia, a hallmark symptom of depression. This model therefore provides an
opportunity to study how stress affects multiple physiological systems that are dysregulated in psychiatric disorders.

**Stress, Inflammation, and the Immune System**

*Features of the Immune System*

The immune system can be broken down into two main subcomponents: the innate and the adaptive immune system. The innate immune system comes equipped with cells (monocytes, macrophages, dendritic cells, innate lymphocytes, and granulocytes) that rapidly respond to injuries or foreign pathogens. This is accomplished through the use of pattern recognition receptors that respond to pathogen associated molecular patterns and/or danger associated molecular patterns (Portou et al 2015). Activation of these receptors and others lead to the stimulation of the transcription factor nuclear factor kappa beta (NFκB). NFκB is comprised of five proteins: p50, p65 (RelA), p52, RelB, and c-Rel (Oecminghaus & Ghosh, 2009). Under basal conditions, the NFκB is in a complex with the inhibitor of kappa B protein kinase (IκB). The activation of this pathway occurs in response to numerous inflammatory stimuli. Activation of NF-κB induced kinase (NIK) subsequently activates IκB kinase (IKK) that degrades IκB and phosphorylates NF-κB. (Bekhbat et al 2017, Yang et al 2003). This frees NFκB from the cytoplasm allowing for nuclear translocation and enhanced gene transcription of numerous genes coding for cytokines, chemokines, adhesion molecules, and receptors involved in inflammatory signaling cascades (Bhatt & Ghosh 2014, Matsusaka et al 1993).

When the innate immune system is unable to eliminate a foreign pathogen, the adaptive immune system mounts an immune response by generating antigen-specific lymphocytes (T and B cells) and memory cells that can prevent infection when re-exposed to the same pathogen (Mueller & Mackay 2016). Re-exposure to a previously encountered pathogen activates memory
T cells. T cells then respond to the local pathogen by initiating cytokine release and the recruitment of natural killer and dendritic cells which are able to phagocytize foreign pathogens (Mueller & Mackay 2016). Interestingly it has been proposed that the adaptive immune system stores an immunological “memory “of a stressful experience, enabling protection against future exposure to the same stressful experience (Lewitus & Schwartz 2009).

Chronic Stress and the Innate Immune System

In recent years, there has been a surge in the number of studies demonstrating that repeated social defeat (RSD) activates the innate immune system. RSD is a variant of the CSDS model, during which an aggressive CD-1 is placed into the home cage of a group of C57BL/6J mice for two hours a day for 6 days. During these two hours, the CD-1 attacks the resident mice and disrupts the social hierarchy within the cage. RSD has been shown to enhance the proliferation of monocytes and granulocytes out of the bone marrow and into circulation where they are delivered to secondary immune organs and the brain (Kinsey et al 2007, Wohleb et al 2013). This effect has been shown to dependent on adrenergic signaling, which increases the expression of genes that are associated with monocyte cell growth and proliferation (Powell et al 2013). In addition to enhancing the proliferation of innate immune cells, chronic stress has also been shown to up-regulate genes that are associated with their function. This is evidenced by the finding that there is an increase in the expression of cytokines, such as IL-6, IL-1 β, and TNF α in the blood of chronically stressed mice (Brachman et al 2015, Hodes et al 2014, Wohleb et al 2011). Furthermore, when mice are exposed to an immunological challenge in the form of lipopolysaccharide (LPS), a bacterial endotoxin that stimulates cells of the innate immune system, mice with a history of stress release higher levels of pro-inflammatory cytokines than mice without a history of stress (Powell et al 2009, Wohleb et al 2012).
Interestingly, it has been demonstrated that alterations in the number and function of innate immune cells are only seen in mice that develop a stress-susceptible phenotype (Hodes et al 2014). For instance, mice that went on to exhibit social avoidance had higher levels of circulating IL-6 prior to and after CSDS than resilient mice. The role of IL-6 in the development of a stress-susceptible phenotype was further explored by transplanting stem cells from stress susceptible mice to stress-naïve mice that had their native immune system eliminated via irradiation. It was found that these mice developed a stress-susceptible phenotype following a sub-threshold social defeat protocol that does not produce this phenotype on its own (Hodes et al 2014). A subsequent study utilizing the learned-helplessness paradigm in rats provided further support for the role of IL-6 in the expression of a stress-susceptible phenotype. In this paradigm, rats are exposed to inescapable foot shocks for two days. On the third day, the rats are once again exposed to foot shocks, however the rats have a chance to escape the chamber that is delivering the foot shocks. Rats that fail to escape the chamber are categorized as susceptible. As in the social defeat study discussed above, it was found that only the rats that failed to show escape behavior had an increase in circulating IL-6 (Yang et al 2015). Collectively, these studies highlight a role for basal and stress-induced circulating cytokines in the development and expression of a stress-susceptible phenotype.

Chronic Stress and the Adaptive Immune System

As previously mentioned, it has been proposed that the adaptive immune system stores an immunological “memory” of a stressful experience thereby protecting the organism from the deleterious effects of repeated stress exposure. For instance, it has been shown that activation of T-cells by inoculating rats with a segment of myelin basic protein prior to chronic mild stress protected against the development of depressive-like behavior. This was associated with an
increase in neurogenesis and levels of brain-derived neurotrophic factor (Lewitus et al 2009). In line with this finding, transplantation of lymph node cell suspensions from mice that were previously exposed to social defeat into Rag-2 knockout mice (mice that lack mature lymphocytes) reduced levels of peripheral pro-inflammatory cytokines and anxiety-like behavior (Brachman et al 2015). Although intriguing, these findings should be interpreted with caution. Rag-2 knockout mice that received lymphocytes from home-cage controls actually displayed an increase in anxiety-like behavior and higher levels of pro-inflammatory cytokines compared to mice that did not receive the transplant. This suggests that mice that received transplants from defeated mice may be compensating for the pro-inflammatory state induced by removal of Rag-2. Additional work is thus needed to gain a better understanding of the role of the adaptive immune system in preventing stress-induced phenotypes.

**Stress, Inflammation and the Neuroendocrine System**

As discussed above the ANS is rapidly activated and results in the release of epinephrine and adrenalin, which initiates the “fight or flight” response. Following the activation of the ANS, the HPA axis releases corticosterone, which terminates the stress response. Interestingly, cells of the immune system contain both adrenergic and GC receptors, making them sensitive to neuroendocrine signals. In this section I will discuss the co-regulation of stress-induced inflammation by signals from the ANS and HPA-axis.

*Adrenergic Signaling Drives Monocyte Proliferation*

In recent years, it has been well documented that adrenergic signaling drives the pro-inflammatory phenotype observed following repeated social defeat (RSD) (Wohleb et al 2011, Wohleb et al 2015). RSD enhances adrenergic signaling which leads to the proliferation of a sub-population of monocytes which express the cell surface marker Ly-6C (Powell et al 2013). The
presence of this cell surface marker indicates that these cells are “primed”, resulting in the release of high amounts of pro-inflammatory cytokines when confronted with an immune challenge (Bailey et al 2007). One mechanism by which adrenergic signaling drives monocyte proliferation is through the down-regulation of the chemokine CXCL12, which inhibits monocyte proliferation (Nie et al 2008). This was demonstrated in a study where mRNA and protein levels of CXCL12 were restored when the \( \beta_3 \) adrenergic receptor was blocked (Heidt et al 2014). Further supporting the role of adrenergic signaling is the observation that pre-treatment with propranolol, a \( \beta \) adrenergic receptor antagonist, prior to each session of RSD prevents the number and reactivity of monocytes within the blood and spleen (Wohleb et al 2011).

**Chronic Social Stress Leads to Glucocorticoid Resistance**

In contrast to the pro-inflammatory effects of adrenergic signaling, corticosteroids exert powerful anti-inflammatory effects through the GR. Under normal physiologic conditions, the GR is able to inhibit NF\( \kappa \)B transcription factor prior to and following nuclear translocation (Bekhbat et al 2017). Prior to nuclear translocation, the GR is able to physically interact with IKB which results in the enhancement of its binding affinity to NF\( \kappa \)B thereby inhibiting nuclear translocation (Scheinman et al 1995a, Scheinman et al 1995b). Within nucleus, the GR is able to repress the transcription of pro-inflammatory gene targets such as IL-6 and IL-8. This occurs by preventing the binding of NF\( \kappa \)B to DNA promoter regions and the interaction with basal transcriptional machinery (Mukaida et al 1994, Ray & Prefontaine 1994).

Interestingly, chronic stress can lead to glucocorticoid resistance, which diminishes the ability of glucocorticoids to suppress pro-inflammatory processes. Previous studies have shown that innate immune cells from mice exposed to RSD show decreased sensitivity to exogenous application of corticosterone (Avitsur et al 2001, Engler et al 2005). For example, when
stimulated with the pro-inflammatory endotoxin LPS, splenocytes show enhanced cell survival and an increase in the production of IL-6 and TNF-a in the presence of corticosterone compared to cells from control mice (Engler et al 2005).

A more recent study explored the molecular mechanisms by which RSD induces GC resistance in splenocytes. It was found that splenocytes had decreased expression of GR mRNA as well as the co-chaperone protein FKBP4, suggesting an impairment in GR nuclear translocation. Furthermore, it was shown that RSD increased the expression of micro RNAs (miRNA), which are small non-coding RNAs that are known to play a role in the degradation of mRNA. Specifically, it was found that miRNA-340 and miRNA-29b were negatively correlated with GR mRNA expression, and that overexpressing these miRNAs in cell culture reduced the expression of GR mRNA. Collectively, these findings suggests that RSD induces the proliferation of primed, GC insensitive monocytes out from the bone marrow and into organs, such as the spleen and brain, which are insensitive to the anti-inflammatory effects of corticosterone.

**Stress and the Neuro-Immune axis**

The data discussed above clearly suggest that exposure to chronic stress is able to alter the functioning of the immune and endocrine system. These alterations lead to the behavioral phenotypes observed following chronic stress in rodent models and in the human clinical population. Although the brain was once considered to be an “immune-privileged” organ, recent research has demonstrated that the inflammatory processes discussed above may have a direct impact on brain function and structure. In this section, I will outline the mechanisms by which this occurs in the context of chronic stress.

*Chronic Stress Induces Monocyte/Macrophage Trafficking to the Brain*
Research using the RSD model has demonstrated that GC insensitive monocytes are able to mobilize to the perivascular space (blood vessels) and parenchyma (tissue) of the brain (Wohleb et al 2013). This was achieved through the use of bone marrow (BM) chimera mice. In this paradigm, the BM of recipient mice was reconstituted with cells from the BM of donor mice that had been tagged with GFP, thereby distinguishing BM-derived monocytes from all other immune cells. Interestingly, these cells were recruited to the brain in a region-specific manner. Cells which were positive for GFP were seen in the perivascular space and parenchyma of brain regions associated with mood, anxiety, and threat appraisal, such as the amygdala (AYMG), bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the hypothalamus (PVN), nucleus accumbens (NAc) and the pre-frontal cortex (PFC).

Although this finding is compelling, it should be noted that other studies have failed to detect the presence of peripheral monocytes in the brain parenchyma. In a recent study the \( \text{Ccr2}^{RFP} : \text{Cx3cr1}^{GFP} \) transgenic mouse was used in the CSDS model (Menard et al 2017). In this mouse line, RFP expression is under the control of the Ccr2 promoter, a chemokine receptor exclusively found in peripheral monocytes, while GFP expression is under the control the of the Cx3cr1 promoter, which is exclusively found in tissue resident microglia. Although CSDS increased the expression of Ccr2 in the brains of defeated mice, RFP expression was restricted to the perivascular space. Nonetheless, defeated mice showed an increase in the expression of IL-6 in the parenchyma of the NAc. The increase in the expression of IL-6 in the NAc was associated with a decrease in the expression of the tight junction protein Cldn5 within the blood brain barrier (BBB) of the NAc.

There are two possible reasons for the discrepancy in these findings. The first is the severity of the stressor. In the RSD model, an aggressive CD-1 is placed into the home cage of a
group of C57BL/6J mice for two hours a day over the course of six days. In contrast, CSDS consists of 5-10 minute physical encounters over the course of ten days. Due to the large amount of time spent interacting in the RSD model, the likelihood of the resident mice being wounded is largely increased. Thus, the physical wounding combined with the psychosocial stress may engage the peripheral immune system to a greater degree. The second potential explanation is the use of bone marrow chimeras as opposed to transgenic mice. Studies using bone marrow chimeras require the animals to undergo irradiation, which can degrade the integrity of the BBB and consequently allow for the passage of peripheral monocytes (Kaya et al 2004, Yuan et al 2003). Nonetheless, these findings suggest that monocytes are able reach the perivascular space and release pro-inflammatory cytokines into the brain parenchyma, possibly acting on neuronal circuits responsible for anxiety- and depressive-like behavior.

Central Cytokines Promote Depressive-Like Behavior

While cytokines are able to cross the BBB and as a result alter neural circuit function, microglia are another source of pro-inflammatory cytokines. Microglia are the main source of immune defense in the brain and are capable of releasing cytokines in response to infected and/or damaged cells. For instance, microglia isolated from stress-susceptible mice produced higher levels of cytokines following stimulation with LPS than isolated microglia from non-stressed mice (Wohleb et al 2012). Recent studies suggest that the cytosolic pattern recognition receptor NLPR3 found in microglia is involved in this pro-inflammatory response (Iwata et al 2013). NLPR3 is activated by ATP or binding of toll-like receptor 4. Activation of this complex leads to the cleavage of pro-IL-1β to IL-1β. The release of IL-1β by the activated microglial NLRP3 inflammasome is associated with depressive-like behavior that is not observed in NLRP3 null mutant mice (Iwata et al 2016). It remains unclear, however, how the cytokines exert their effects.
on neural circuits. One potential mechanism by which this may occur is through activation of the IKK- NFκB signal transduction pathway, which is activated by pro-inflammatory cytokines. Activation of this pathway has been shown to play a role in synaptic plasticity, alter the morphology of dendritic spines and induce depressive-like behavior when constitutively active in the NAc (Christoffel et al 2012). Indeed, a previous study demonstrated that CSDS increased the expression of IKK, which was associated with an increase in p-IKB in the NAc (Christoffel et al 2011). It is not known, however, if the activity of the NLRP3 inflammasome in the NAC is driving the activity of this pathway. Future studies are needed to test this hypothesis.

**Clinical Implications**

Many of the phenotypes observed in models of chronic psychosocial stress are also seen in the human clinical population. In this section I will first discuss the symptomatic overlap observed in individuals with MDD or PTSD and mice exposed to chronic stress and will then discuss the potential for anti-inflammatory compounds for treating these disorders.

*Dysregulation of the Immune and Endocrine Systems in MDD and PTSD*

The first piece of evidence suggesting a link between inflammation and psychiatric disorders came from the finding that as many as 50% of individuals who were undergoing treatment with interferon-α for chronic viral hepatitis developed symptoms of depression (Renault et al 1987). Further supporting the link between peripheral inflammation and MDD, numerous studies have observed significant elevations of various pro-inflammatory markers, such as IL-6, TNFα, C-reactive protein (CRP) and IL-1β in individuals who are diagnosed with MDD. These changes correlate with symptom severity (Alesci et al 2005, Dowlati et al 2010, Liu et al 2012, Maes 1995, Motivala et al 2005).
As in MDD, PTSD is associated with a pro-inflammatory state. For instance, it has been demonstrated that individuals who go on to develop PTSD have an increase in gene transcripts that code for inflammatory-related proteins prior to and following military combat compared to healthy combat-exposed controls (Breen et al 2015, Daskalakis et al 2016). Of these inflammatory molecules, IL-6, TNFα and IL-1β have been shown to be the most consistently up-regulated in patients with PTSD (Lehrner et al 2016, Passos et al 2015). Furthermore, individuals with PTSD as a result of childhood trauma display heightened NFκB activity in monocytes, which was inversely correlated with GC sensitivity (Pace et al 2012). In stark contrast to this finding, others have reported an increase in GC sensitivity in the monocytes of patients with PTSD who experienced trauma as an adult (Rohleder et al 2004).

MDD and PTSD are also associated with alterations in the HPA-axis. Circulating cortisol, the end product of HPA-axis activity has been shown to be elevated in two-thirds of patients (Stetler & Miller 2011) with MDD. Although the majority of patients display an increase in circulating cortisol, it has been demonstrated that the immune system of these patients develop GC resistance (Raison & Miller 2003). This has been demonstrated through the dexamethasone suppression test. Dexamethasone is a potent GR agonist which decreases the amount of circulating cortisol following oral administration. This is achieved through negative feedback mechanisms at the level of the pituitary and adrenal glands. In healthy subjects, oral administration of dexamethasone results in a profound decrease in the amount of circulating cortisol the following morning. Patients with MDD however, fail to suppress cortisol production following dexamethasone treatment (Maletic & Raison 2017). This finding suggests that although patients with MDD have higher levels of circulating cortisol, their bodies are less responsive to its anti-inflammatory effects. In contrast to MDD, PTSD is usually associated with
lower levels of circulating cortisol, however some individuals show an increase in cortisol production. Furthermore, it is not yet understood if hypocortisolemia and other potential biomarkers are a result of trauma exposure, or coping with PTSD (Lehrner et al 2016). For instance, individuals with PTSD as a result of childhood trauma have been shown to display heightened NFκB activity in monocytes, which was inversely correlated with GC sensitivity (Pace et al 2012). In contrast, others have reported an increase in GC sensitivity in the monocytes of patients with PTSD who experienced trauma as an adult (Rohleder et al 2004). Thus, it may be important to consider the timing of the trauma that led to PTSD when interpreting monocyte sensitivity to glucocorticoids in patients with PTSD.

**Current Treatment Options**

Currently, approved pharmacological treatments for anxiety and mood disorders are mainly designed to target the serotonergic and/or noradrenergic neurotransmitter systems. Although these medications have been somewhat successful, there are numerous documented side effects and up to 50% of patients do not respond to any type of treatment (Berger et al 2009, Rush 2006). Importantly, studies on the effects of SSRIs on circulating cytokines in patients have yielded mixed results. Studies have reported reductions, no effect, or even an increase in circulating pro-inflammatory cytokines following treatment (Hannestad et al 2011, Maes 1995). For instance, classic antidepressants have been shown to increase plasma IL-6 in response to LPS stimulation in depressed and healthy individuals (Hodes et al 2016). Furthermore, recent studies have demonstrated that resistance to treatment is associated with high levels of inflammatory markers, such as IL-6 (Haroon et al 2018, Kiraly et al 2017). In light of these findings, there have been recent efforts to evaluate the efficacy of anti-inflammatory medications in reducing symptoms of depression. A recent meta-analysis of 10 studies evaluating non-
steroidal anti-inflammatory drugs (NSAIDs) and cytokine inhibitors concluded that these drugs were more effective than placebo in treating patients with depression (Köhler et al 2014). Although encouraging, more studies are needed to support the idea that anti-inflammatory compounds represent a potential treatment option.

Curcumin as a Novel Treatment Option for Psychiatric Disorders

The above discussion on current treatment options suggest there is an urgent need to develop novel therapeutics that target inflammatory processes. In recent years, plant polyphenol compounds have generated attention for their potential to treat/prevent various chronic diseases that are associated with inflammation. Of these compounds, curcumin is one of the most widely studied. Curcumin is a biologically active polyphenol compound found in the rhizome of the turmeric plant (Curcuma longa). Previous pre-clinical studies have demonstrated that curcumin reduces depressive-like behavior in rats following chronic unpredictable stress (Xu et al 2006, Xu et al 2009). Furthermore, recent randomized placebo controlled clinical trials have demonstrated that curcumin is effective in reducing symptoms of inflammatory disorders, such as depression (Lopresti et al 2014, Lopresti et al 2015), rheumatoid arthritis (Chandran & Goel 2012), and ulcerative colitis (Hanai et al 2006). Curcumin has also been shown to prevent the development of type 2 diabetes in at-risk populations (Kunnumakkara et al 2017). Importantly, curcumin has been shown to be well-tolerated in human patients with minimal side effects (Anand et al 2007). Although the exact molecular mechanisms by which curcumin can prevent/treat the aforementioned medical conditions remains an outstanding question, one signaling pathway that may be regulated is the IKK-NFκB pathway. As detailed above, NFκB enhances the transcription of numerous genes involved in inflammatory signaling cascades that are relevant to the pathology of both MDD and PTSD (Bhatt & Ghosh 2014, Matsusaka et al
Previous research suggests that there are numerous ways in which curcumin may be inhibiting this pathway. For instance, curcumin inhibits IL-1β-mediated expression of genes that are transcribed by NFκB and blocks cytokine-induced activation of IKK (Jobin et al 1999). Curcumin may also be inhibiting NFκB dependent gene transcription by preventing the recruitment of the coactivator P300/CBP (Marcu et al 2006), which is required for changes in the patterns of acetylation on κB response elements of pro-inflammatory genes. Finally, a more recent cell-culture study has demonstrated that curcumin directly binds to and inhibits the activity of dual-specificity tyrosine kinase 2, which positively regulates the proteasome. As a result, proteasomal activity is decreased (Banerjee et al 2018). This finding suggests that curcumin’s observed effects on NFκB are due to the accumulation of IκB due, which fails to be degraded by the proteasome.

**Rationale for Current Study**

While previous studies have demonstrated that curcumin is able treat depression in the clinic, it is unknown if curcumin is able to prevent depressive- and anxiety-like symptoms by promoting resilience to chronic social stress. This a relevant question due to the fact that social stressors impose the greatest risk of developing MDD or PTSD. Therefore, I will evaluate whether dietary curcumin can promote resilience to CSDS. Furthermore, given its anti-inflammatory properties, I will evaluate if curcumin is able to prevent the production of peripheral cytokines downstream of NFκB as a result of CSDS. Lastly, I will explore the relationship between baseline measures of social and anxiety-like behavior and treatment outcome.

**Specific Aims**

1. **Does curcumin promote resilience to CSDS?**

To address this question, mice will be placed on a diet of 1.5% curcumin or a control chow five
days prior to and throughout CSDS. All mice will be tested in the social interaction test the day after the last episode of CSDS. One week later, a cohort of mice will be tested in the elevated plus maze, and open field, two measures of anxiety-like behavior. A separate cohort of mice will be fear conditioned one week following CSDS to model the acquisition of a traumatic memory. Mice will be tested for short- and long-term memory.

2. Does curcumin block stress-induced inflammation in the periphery?

To address this question, mice on a diet of curcumin or chow will be sacrificed immediately following the social interaction test. Trunk blood will be collected to measure pro-inflammatory interleukins, and the spleen will be weighed to indirectly measure monocyte proliferation.

3. Do individual differences in baseline social and anxiety-like behavior relate to treatment outcome?

As demonstrated in chapters 2 and 3, there is variability in the response to curcumin, such that some mice are “responders” and others are “non-responders as defined by their behavior in the social interaction test. To determine if there is a relationship between baseline levels of social and/or anxiety-like behavior and treatment response, mice will be put in the open field and social interaction test prior to any experimental manipulations. Correlations will be conducted between time spent in the center of the open field or time spent interacting with a CD-1 prior to stress and time spent with a CD-1 post-stress in mice fed a curcumin or control diet.
Chapter 2:

A Diet Enriched With Curcumin Promotes Resilience to Chronic Social Defeat Stress
Mood and anxiety disorders are among the most prevalent of all psychiatric disorders (Kessler et al 2012). Numerous risk factors have been identified for developing these disorders, with chronic stress being the best characterized (Kendler et al 1999). Interestingly, not everyone exposed to chronic stress develops a psychiatric disorder, an observation that has triggered interest in understanding the mechanisms that underlie individual difference in response to stress. Preclinical studies involving chronic social defeat stress (CSDS) indicate that stress susceptibility and resilience are each associated with distinct changes in signal transduction pathways, gene transcription and electrophysiological activity across cortico-limbic circuits (Bagot et al 2016, Bagot et al 2015, Krishnan et al 2007). In the periphery, susceptible and resilient animals also display different inflammatory and metabolic responses to stress (Chuang et al 2010a, Chuang et al 2010b, Hodes et al 2014). One approach for promoting stress resilience has been to block mechanisms implicated in susceptibility using optogenetics or overexpression of transcription factors (Chaudhury et al 2013, Donahue et al 2014). However, this work has not yet led to effective treatments in the clinic.

One treatment that has shown promise in the clinical setting is curcumin. Curcumin is a polyphenol compound found in the rhizome of the turmeric plant with known chemopreventative, neuroprotective, and anti-inflammatory properties (Duvoix et al 2005, Jobin et al 1999, Meja et al 2008, Xu et al 2009). It has been used for the treatment of several diseases with an inflammatory component, including rheumatoid arthritis, ulcerative colitis, and diabetes (Chuengsamarn et al 2012, Daily et al 2016, Hanai et al 2006, McFadden et al 2015). Interestingly, curcumin may also be effective in treating psychiatric disorders, such as major depressive disorder. Recent double-blind placebo controlled studies indicate that the effects of curcumin are particularly pronounced in patients with atypical depression, which is characterized
by somatic symptoms and high levels of inflammatory cytokines (Lopresti & Drummond 2017, Lopresti et al 2014, Lopresti et al 2015). Similarly, preclinical studies show that curcumin administered during exposure to chronic stress in rodents reduces the development of depressive-like behavior and prevents stress-induced morphological and functional changes in the hippocampus (Hurley et al 2013, Xu et al 2006, Xu et al 2009).

Although the antidepressant effects of curcumin have been addressed in several studies, to our knowledge no studies have directly investigated the effects of curcumin on anxiety or tested curcumin in patients with anxiety disorders. In the present study, we examined the effects of curcumin on anxiety-like behavior induced by CSDS. Experiments were conducted in 129/SvEv mice, a strain that we show is highly susceptible to this type of stress. We found that curcumin administered in the diet blocked stress-induced social avoidance behavior and anxiety-like behavior in the elevated-plus maze and open field test. Our findings demonstrate that curcumin promotes stress resilience and suggest that it may safely and effectively prevent the development of psychiatric disorders characterized by symptoms of anxiety.

METHODS

Animals

Male 129Sv/Ev mice were purchased from Taconic Biosciences, Inc. (Germantown, NY) at 8 weeks of age and male C57BL/6J mice were purchased from the Jackson Laboratory. Retired male CD-1 breeders were purchased from Charles River (Wilmington, MA). Food and water were provided ad libitum throughout all experiments. All CD-1 mice used in social defeat experiments were pre-screened for aggressive behavior as previously described (Golden et al 2011).
Diet

Male 129 Sv/Ev mice were fed a global 18% protein chow diet (Envigo Tekland) or a global 18% protein chow diet made with 1.5% curcumin (Pfaultz & Bauer, 95% diferuloylmethane). This high concentration was chosen due to the known low bioavailability of the compound (Prasad et al 2014b). Mice on average consumed 4g of each diet (60mg of curcumin) per day with no difference between groups (Control Chow: 4.07 ± 3g; Curcumin Chow: 3.94 ± .14 g, p > 0.05).

Social Defeat Stress

Social defeat was performed as previously described (Golden et al 2011) with minor variations. Briefly, 129 Sv/Ev mice were placed into the home cage of a retired CD-1 male breeder that had been pre-screened for aggressive behavior. The two mice were allowed to interact for 5 minutes, during which time the CD-1 mouse attacked the intruder mouse. The two mice were then housed together in the same cage overnight separated by a perforated Plexiglas divider that prevented further physical contact, but allowed for continuous psychological stress from sensory (e.g. visual, olfactory, auditory) cues. This procedure was repeated for each of the 10 days of social defeat. Control mice were pair housed in the same way, but with one 129Sv/Ev mouse on each side of the perforated divider. Control mice were never in contact with a CD-1 mouse and were introduced into a new cage with a new 129 Sv/Ev mouse each day. After the final episode of social defeat all mice were singly housed.

Social Interaction Test

The social interaction test was performed as previously described (Brachman et al 2016b). Briefly, one day after the last defeat session mice were placed in an open arena (25 x 48 cm) containing two wire-mesh enclosures for 5 minutes. On one side of the arena, one
enclosure contained a novel CD-1 mouse. On the opposite side of the arena, there was an identical enclosure that was empty. Time spent interacting with the empty enclosure and the enclosure containing the novel CD-1 mouse was manually recorded. The defeat index (DI) was calculated by dividing the difference in the time spent between the two enclosures by the total amount of time spent with the two enclosures. Mice on a curcumin diet with a DI of 0 or greater were defined as “responders” while mice that had a DI below 0 were defined as “non-responders”.

**Elevated Plus Maze (EPM)**

Mice were tested for 5 minutes in the elevated plus maze (EPM) under low light conditions (70 lux). The maze is a cross-shaped maze with two open arms and two closed arms 40.5 cm above the ground. Individual mice were placed in the center of the maze facing an open arm and an overhead camera recorded locomotor behavior. The number of entries and time spent on the open and closed arms were manually scored.

**Open Field (OF)**

Mice were placed in the corner of a novel arena (45 x 45 cm) and were allowed to freely explore for 30 minutes. The center was defined as a square half the size of the entire arena. The dependent measures were: total distance traveled (cm), number of entries into the center, and time spent in the center. An overhead camera recorded all locomotor behavior. The first ten minutes was quantified using ANY-maze software (Wood Dale, IL).

**Acute Restraint Stress**

At roughly 0900 hours, mice were physically restrained for 15 minutes in a decapicone (Braintree Scientific Inc., Braintree, MA). Mice were then removed from the decapicone and immediately sacrificed.
Blood Collection/Corticosterone Assay

Blood samples were collected at two time points: immediately after social interaction and immediately after restraint stress. Following decapitation, trunk blood was transferred to Eppendorf tubes containing 5 µl 0.5 M EDTA, placed on ice, and immediately spun down at 3,000RPM for 10 min to obtain plasma. Serum levels of corticosterone were analyzed with an ELISA kit (Enzo Life Sciences, Inc.; Farmingdale, NY). 10 µl of sample were plated in duplicate and assessed according to the manufacturer’s instructions. Plates were read in a BioPlex Bead Array Reader (BioRad; Hercules, CA, USA) at 450nm.

Statistical Analysis

All data are expressed as the mean ± SEM. Statistical significance was defined as p < 0.05. To evaluate mean differences in the SI test a 2 (object) x 2 (stress) x 2 (diet) or a 2 (object) x 2 (stress) x 2 (strain) ANOVA was performed. For EPM, OF and corticosterone analyses, a 2 (Stress) x 2 (Diet) ANOVA was performed. To evaluate mean differences for the fear conditioning experiments, a 3 (tone) x 2 (stress) x 2 (diet) ANOVA was performed for acquisition and STM. For LTM, a 5 (tone) x 2 (stress) x 2 (diet) was performed. All ANOVAs were followed by planned-comparisons. These comparisons included: Stressed/Chow vs. Stressed/Curcumin, Stressed/Chow vs. Non- Stressed/Chow, and Stressed/Curcumin vs. Non-Stressed/Curcumin. The Boneferroni method was used to correct for multiple comparisons. To evaluate mean differences between responders and non-responders boneferroni corrected unpaired t-tests were performed.

RESULTS

129/SvEv Mice Demonstrate Enhanced CSDS-Induced Social Avoidance Compared to C57BL/6J Mice
The 129/SvEv strain exhibits higher levels of anxiety-like behavior than the C57BL/6 strain (Holmes et al 2002). Although it has been suggested that they are more susceptible to CSDS than C57BL/6 mice (Dadomo et al., 2011), a direct comparison between the two strains using a standardized protocol has not been made. We tested the effects of CSDS in a large cohort of 129/SvEv mice (n= 50) and found that 92% of defeated mice avoid the CD-1 in the social interaction test. Using the same CSDS protocol, we found that only 33% of C57BL/6 mice avoided the social target, indicating that the 129/SvEv strain is significantly more susceptible to this type of chronic stressor ($\chi^2(1)= 28.3, p < 0.0001$) (Figure 1B,D). A two-way ANOVA on interaction time in defeated mice revealed a strain X object interaction ($F(1,72) = 83.75, p < 0.0001$). Defeated 129s spent more time with the empty enclosure and less time with the CD-1 than defeated C57s ($p < 0.0001$ for each post-hoc comparison). Within-group comparisons revealed that defeated 129s spent less time with the CD-1 than the empty enclosure ($p < 0.0001$), while defeated C57s spent approximately the same amount of time with both enclosures (Figure 1C).

**Curcumin Protects Against Stress-Induced Social Avoidance Behavior**

In our next experiment, we placed 129s on a diet of 1.5% curcumin 5 days prior to and throughout CSDS to examine whether curcumin is able to promote resilience. The social interaction test was 24 hours after the last defeat session. We observed an increase in the proportion of mice (60%) with a DI score above 0 on a curcumin diet compared to mice on a regular chow diet (6%; Figure 1B). Furthermore, a three-way ANOVA revealed a significant diet
x stress x object interaction
(F_{(1,112)} = 22.71, p < 0.0001).

The Defeated/Chow group was found to spend more time with the empty closure (p < 0.001) and less time with the CD-1 (p < 0.001) than defeated mice on a curcumin diet and non-stressed mice on a regular chow diet (Figure 1C). An additional

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Figure 2.1: 129Sv/Ev mice are highly susceptible to CSDS. A) Schematic of general behavioral procedure. (B) Out of 50 defeated mice, only 4 (9%) are resilient. (C) Mean ± SEM amount of time spent with either the empty enclosure or the enclosure containing a novel CD-1 for No Stress/129 (n= 50), No Stress/C57BL/6J (n= 12), Stress/129 (n= 50), and Defeated C57BL/6J (n = 24). Defeated 129 Sv/Ev mice spent significantly more time with the empty enclosure and less time with the CD-1 than non-stressed 129 mice (p< 0.0001) and defeated C57BL/6J mice (p< 0.0001). **p< 0.0001 Stress/129 vs. Defeated/129. ^^^p< 0.0001 Defeated/C57 vs. Defeated/129

analysis restricted to defeated mice on a curcumin diet revealed that the non-responders (DI < 0) spent significantly more time interacting with the empty enclosure (t_{13} =4.02, p = 0.001) and significantly less time with the CD-1 (t_{13} = 6.22, p< 0.001) than responders. (Figure 2.1D). Collectively, these findings suggest that a dietary regimen of curcumin is able to prevent social avoidance behavior in a subset (60%) of 129 mice.
In our next experiments, we examined whether curcumin can prevent the anxiogenic effects of CSDS on two measures of anxiety-like behavior (EPM & OF). We found that defeated mice on a regular chow diet spent less time on the open arms (stress x diet interaction: $F_{(1, 56)} = 14.73, p < 0.0001$; Figure 2.2A) and more time on the closed arms (stress x diet interaction: $F_{(1, 56)} = 6.583, p = 0.01$; Figure 2.2B) of the EPM compared to defeated mice on curcumin and non-stressed.
mice on a regular chow diet. Interestingly, an analysis restricted to defeated mice on a curcumin diet revealed no differences in the amount of time spent on the open or closed arms (Figure 2.2C). We also observed that defeated mice on a regular chow diet made less open arm entries (interaction: $F_{(1, 56)} = 15.11$, $p < 0.001$) and more closed arm entries (stress x diet interaction: $F_{(1, 56)} = 15.11$, $p < 0.001$) compared to non-stressed mice on a regular chow diet and defeated mice on curcumin (Figure 2.2D & 2.2E). Once again, there were no differences between responders and non-responders in the number of entries into the open or closed arms (Figure 2F).

**Figure 2.3:** Dietary curcumin protects against the anxiogenic effect of CSDS on the EPM. (A-C) Mean ± SEM percentage of time spent on the open/closed arms of the EPM. (A) Defeated mice on a regular chow diet spent less time on the open arms compared to non-stressed mice on a regular chow diet ($p < 0.0001$) and defeated mice on a curcumin diet ($p < 0.0001$). (B) Defeated mice on a regular chow diet spent more time on the closed arms compared to non-stressed mice on a regular chow diet ($p < 0.001$) and defeated mice on a curcumin diet ($p < 0.0001$). (C) No significant difference in the amount of time spent on either the open or closed arms of the EPM between Responders and Non-Responders. (D-F) Mean ± SEM percentage of entries made onto the open/closed arms of the EPM for all groups. D) Defeated mice on a regular chow diet made less entries onto the open arms compared to non-stressed mice on a regular chow diet ($p < 0.0001$) and defeated mice on a curcumin diet ($p < 0.0001$). (E) Defeated mice on a regular chow diet made more entries onto the closed arms compared to non-stressed mice on a regular chow diet ($p < 0.001$) and defeated mice on a curcumin diet ($p < 0.001$). (F) No significant difference in the amount of time spent on either the open or closed arms of the EPM between Responders and Non-Responders. **$p < 0.0001$, *$p < 0.05$ No Stress/Chow vs. Defeated/Chow. ^^^$p < 0.0001$ Defeated/Curcumin vs. Defeated/Chow
Similar results were obtained for open field behavior. Specifically, defeated mice on a chow diet spent less time in the center of the open field (stress x diet interaction: $F_{(1,36)} = 4.91, p < 0.05$; Figure 3A) and made less entries into the center zone (Figure 3C) than defeated mice on curcumin and non-stressed mice on a regular chow diet (stress x diet interaction: $F_{(1,56)} = 5.18, p < 0.05$; Figure 3C). Additionally, we did not observe any differences in total distance traveled (Figure 3E). As in the EPM, no differences were observed in open field behavior.

**Figure 2.4: Dietary curcumin protects against the anxiogenic effect of CSDS in the OF.** Mean ± SEM percentage of time spent in the center (A-B), number of entries into the center (C-D), and total distance traveled (E-F) for No Stress/Chow (n=10), No Stress/Curcumin (n=10), Defeat/Chow (n=10), Defeat/Curcumin (n=10), Curcumin Responder (n=5), and Curcumin Non-Responders (n=5). Defeated mice on a regular chow diet spent less time in the center (A) and made less entries into the center (C) than non-stressed mice on a regular chow diet ($p<0.01$ for each comparison) and defeated mice on a curcumin diet ($p<0.05$ for each comparison) with no significant differences in total distance traveled. There were no significant differences between the responders and non-responders for time spent in the center (B), entries into the center (D), or total distance traveled (F). **$p<0.0001$, *$p<0.05$ No Stress/Chow vs. Defeated/Chow. ^$p<0.05$ Defeated/Curcumin vs. Defeated/Chow**
between responders and non-responders (Figures 3B, 3D and 3F). Collectively, the results of these experiments suggest curcumin prevents the development of anxiogenic behavior following CSDS and that the effects of curcumin on social behavior do not necessarily predict the effects of curcumin on anxiety-like behavior.

**Curcumin Responders Do Not Display Sensitization to a Novel Stressor**

Previous studies have demonstrated that chronic stress leads to adaptations within the hypothalamic-pituitary-adrenal (HPA) axis, such that the response to a novel, acute stressor is exaggerated (Herman 2013). Therefore, we tested whether dietary curcumin is able to prevent the heightened response to a novel stressor. To this end, trunk blood was collected immediately following two time points: after the social interaction test and after 15 minutes of restraint stress (Figure 2.5) to assess levels of the stress hormone corticosterone. Following the social interaction test, we did not observe an effect of stress or diet on plasma corticosterone (Figure 2.5B/C). There was also no relationship between plasma corticosterone and the DI score (Figure 2.5D). Following acute restraint stress however, we observed that defeated mice regardless of their diet displayed a significant increase in circulating corticosterone (main effect of stress; $F_{(1,36)} = 22.98, p < 0.0001$) with no effect of diet or diet x stress interaction. However, there was a significant difference between non-responders and responders ($t_{(8)} = 2.93, p = 0.01$; Figure 2.5F) with non-responders displaying higher levels of corticosterone than responders. There was also a significant negative correlation between restraint-induced corticosterone and DI in all three defeated groups ($r = -.65, p = .001$; Figure 2.5G).
Curcumin Has No Effect on Stress Enhanced Fear Memory Consolidation

In a separate cohort of mice, we examined whether CSDS enhances the consolidation of a Pavlovian fear memory and if this could be prevented by dietary curcumin. As reported above, dietary curcumin increased the proportion of mice (80%) with a DI score above 0 compared to mice on a regular chow diet (0%; Figure 2.6B). Once again, defeated mice on a regular chow diet...
spent more time with the empty enclosure (p < 0.0001) and less time with the CD-1 (p < 0.0001) than defeated mice on curcumin and non-stressed mice on a regular chow diet (stress x diet x object interaction, (F(1,72) = 72.61, p < 0.0001; Figure 2.6C). Non-responders spent more time with the empty enclosure (t(8) = 3.5, p < 0.05) and less time with the CD-1 (t8 = 4.59, p < 0.01) than responders (Figure 2.6D). One week following the social interaction test mice were fear conditioned and tested for short-term memory (STM). There was a main effect of tone (F(2,108)= 173.1, p < 0.0001) but no effect of stress (F(3,36)= .43, p > 0.05), diet (F(2,108) = 1.56, p > 0.05) or a stress x diet x tone interaction (F(2,195)= .39, p > 0.05) suggesting that all four groups learned the tone-shock association equally. We did not observe any significant main effects or a significant stress x diet x tone interaction in the STM test. The next day mice were returned to the conditioning chamber and tested for long-term memory (LTM). We observed a main effect of stress (F(1,180)= 24.69, p < 0.0001) with defeated mice freezing more than non-stressed mice regardless of diet (Figure 2.6G & 2.6H). We did not observe a main effect of tone or diet, nor did we observe a stress x diet x tone interaction. These findings suggest that CSDS selectively enhances the consolidation of a fear memory and curcumin is unable to prevent this phenotype.

**Discussion**

In the present study, I demonstrate that a diet enriched with curcumin promotes resilience to CSDS in the highly susceptible 129S6 mouse strain. In defeated mice, we show that dietary curcumin produces a 5-fold increase in the number of resilient mice compared to defeated mice on a regular chow diet. Furthermore, defeated mice fed curcumin showed a reduction in anxiogenic behavior on two well-established pre-clinical measures of anxiety.
We also observed that curcumin responders and non-responders differed in their response to a novel acute stressor, with non-responders showing greater activation of the HPA-axis. Finally, we show that dietary curcumin was unable to prevent stress-enhanced consolidation of a Pavlovian fear memory. Our findings collectively provide the first pre-clinical evidence that curcumin may be a beneficial compound in treating psychiatric disorders that are characterized by high anxiety/social avoidance.

Curcumin is a biologically active polyphenol compound found in the rhizome of the turmeric plant (Curcuma longa), which is commonly used as a spice in Indian and East Asian cuisines.

Although turmeric contains several different curcuminoids, the therapeutic effects of curcumin (diferuloylmethane) have been the most widely studied, with doses that range from 0.5g-
Curcumin has been shown to effectively treat rheumatoid arthritis and ulcerative colitis, and prevent the development of type 2 diabetes in at-risk populations (Chuengsamarn et al 2012, Daily et al 2016, Hanai et al 2006). It has also been extensively investigated for the prevention and treatment of a range of different cancers (Prasad et al 2014a). Furthermore, recent double-blind placebo controlled clinical trials indicate that curcumin reduces symptoms of depression, with effects that are comparable to selective serotonin reuptake inhibitors (Lopresti & Drummond 2017, Lopresti et al 2014, Sanmukhani et al 2014). Within this extensive body of clinical work, it has been reported that curcumin is well tolerated by humans with side-effects that do not differ from placebo (Anand et al 2007, Chandran & Goel 2012, Hanai et al 2006, Lopresti et al 2014). Consistent with this low side-effect profile, we found no behavioral or physiological effects of curcumin in non-stressed mice. In contrast, SSRIs, which...
are the most commonly prescribed treatment for mood and anxiety disorders, have many well-documented side effects (Cascade et al 2009).

Although to our knowledge curcumin has not been tested in patients with anxiety disorders, it has been found to improve State and Trait scores in the Spielberger State-Trait Anxiety Inventory (STAI) in people with major depressive disorder (Lopresti & Drummond 2017). It has also been shown in one study to block the anxiogenic effects of acute restraint stress in the elevated plus maze (Haider et al 2015). However, this group did not find an effect of acute stress on behavior in the open field test, precluding an analysis of curcumin on this measure of anxiety. Unlike the acute restraint stress procedure used in that study, CSDS used in our study is one of the most robust models of stress-related illness that reliably leads to the development of anxiety-like behavioral abnormalities, allowing us to fully establish the anxiolytic properties of curcumin. Other preclinical studies show that in the absence of stress, curcumin also impairs the formation of fear memories by blocking their consolidation and reconsolidation in rats (Monsey et al 2015). Like the study reported here, these preclinical studies administered curcumin prior to experimental manipulation and showed that it prevented the development of fear and anxiety. Collectively, these findings suggest that curcumin may be a promising therapeutic option for reducing fear and anxiety in patients, particularly if it is administered as a prophylactic.

Given that may of the diseases mentioned above have an inflammatory component, the anti-inflammatory properties of curcumin may be central in mediating its therapeutic effects. Curcumin is best known for inhibiting the IKK-NFκB signaling pathway, which is integral in regulating inflammatory processes and activating the immune response (Bhatt & Ghosh 2014, Kopp & Ghosh 1995). For example, curcumin has been shown to block cytokine-induced activation of IKK and inhibit the expression of pro-inflammatory genes transcribed by NFκB.
(Jobin et al 1999). Importantly, CSDS activates the IKK-NFκB signaling pathway in the periphery and brain, which in turn plays an essential role in the development of stress-induced depression and anxiety. In the periphery, upregulation of the downstream cytokine IL-6 has been shown to be necessary for social avoidance behavior following CSDS (Hodes et al 2014). In the nucleus accumbens, CSDS upregulates the expression of IKK (Christoffel et al 2011), the overexpression of which has been shown to increase anxiety- and depressive-like behavior in the absence of stress (Christoffel et al 2012). A relationship between the IKK-NFκB pathway and depression is further supported by the finding that NFκB-dependent genes are upregulated in the ventral dentate gyrus of rats with endogenous depression (Bigio et al 2016). Activation of this pathway in the hippocampus has also been shown to play an integral role in downregulating adult neurogenesis following CSDS (Koo et al 2010). Collectively, these studies suggest that the anxiolytic effects of curcumin described in our study may be attributed to inhibition of the IKK-NFκB signaling pathway in the periphery and/or brain. However, future studies are required to test this hypothesis.

Similar to the heterogeneous response to antidepressant treatment found in human populations, we found two distinct treatment responses to curcumin. While the majority of defeated mice on curcumin spent more time with the CD-1 than the empty enclosure during the social interaction test (responders), the other mice in that group showed social avoidance behavior that was comparable to non-treated defeated mice (non-responders). In contrast to non-responders, responders failed to show sensitization of the HPA axis when it was later activated by acute restraint stress, indicating that treatment response may result from differential effects of curcumin on the HPA axis. Given the role of glucocorticoid receptors (GRs) in terminating the activity of the HPA-axis, the upregulation and/or increased sensitivity of these receptors may
account for the attenuated levels of plasma corticosterone seen in curcumin responders. In support of this idea, cell culture studies show that curcumin restores sensitivity of GRs compromised by exposure to inflammatory stimuli (Meja et al 2008). Interestingly, GRs have also been shown to inhibit NFκB signaling by preventing its translocation into the nucleus and its transcriptional activity (De Bosscher et al 2000, De Bosscher et al 1997), which is consistent with a role for the GRs in mediating some of the effects of curcumin on this signaling pathway. Further evidence implicating the involvement of the IKK-NFκB pathway in treatment response is found in clinical studies showing that the antidepressant effects of ketamine and SSRIs are associated with a decrease in plasma IL-6, which was not found in patients who were characterized as treatment resistant (Kiraly et al 2017, Yoshimura et al 2009). Based on these studies, it appears likely that alterations in this inflammatory pathway contribute to the behavioral effects of curcumin in responders, but it is still unclear why some animals did not respond. Future studies would benefit greatly from characterizing the baseline differences between responders and non-responders at the behavioral and molecular level. Such findings may lead to the identification of clinical populations who would benefit most from curcumin treatment.

Interestingly, the effects of curcumin on social avoidance did not predict treatment response in tests of anxiety. That is, defeated animals on curcumin that avoided the CD-1 in the social interaction test (non-responders) displayed the same decrease in anxiety in the elevated plus maze and open field test as those that did not avoid the CD-1 (responders). This is consistent with previous studies showing that C57BL/6J mice characterized as resilient or susceptible based on their social interaction behavior did not differ when tested in the elevated plus maze (Krishnan et al 2007). Numerous CSDS studies have used the social interaction test to model
social withdrawal, which is commonly reported in patients with depression and is therefore interpreted to be a measure of depression-like behavior (Bagot et al. 2017, Berton et al. 2006, Krishnan et al. 2007, Nestler & Hyman 2010). This interpretation is supported by the finding that C57BL/6J mice not only avoid the CD-1, but also avoid littermates. Therefore, we might be getting different behavioral effects in the social interaction test and our other behavioral tasks, because the former is measuring depression-like behavior and the latter are measuring anxiety-like behavior. However, it has previously been shown that defeated 129/SvEv mice do not avoid mice of the same strain (Brachman et al., 2016). Therefore, avoidance of the CD-1 mice by the 129/SvEv strain may be an adaptive response that had been acquired during the 10 days of defeat. Even though we used a novel CD-1 during each defeat session, avoidance of another unfamiliar CD-1 during the social interaction test could reflect generalization of learned fear. Regardless of the motivation for avoiding the CD-1, our data suggest that there are differences in the neural circuits underlying social avoidance and more typical measures of anxiety-like behavior that are differentially affected by curcumin treatment.
Chapter 3:

Dietary Curcumin Alters Peripheral Immune Responses to Chronic Social Defeat Stress
Psychiatric disorders such as MDD and PTSD are commonly referred to as “brain diseases”. While it is certainly true that the brain plays a central role in the pathogenesis of these disorders, focusing solely on the brain runs the risk of ignoring interactions between the brain and other biological systems that are involved in the development and maintenance of these disorders (McEwen 2017). Numerous studies have demonstrated that chronic exposure to psychosocial stress in humans and rodents results in the elevation of numerous systemic biomarkers related to inflammation (Hodes et al 2014, Powell et al 2013, Segerstrom & Miller 2004, Weber et al 2017, Wohleb et al 2012, Wohleb et al 2011). Furthermore, co-morbidity between MDD and inflammatory diseases such as heart disease, rheumatoid arthritis, and metabolic syndrome are high (Barth et al 2004, Dickens et al 2002, Dunbar et al 2008).

Currently, selective serotonin re-uptake inhibitors (SSRIs) are the only currently approved pharmacological treatment for anxiety and mood disorders. Although this approach has been successful, there are numerous documented side effects, and up to 50% of patients do not respond to treatment (Berger et al 2009, Rush 2006). Studies on the ability of current anti-depressants to reduce circulating cytokines in patients have yielded mixed results with studies reporting reductions, no effect, or even an increase following treatment. (Castanon et al 2002, Hannestad et al 2011, Kenis & Maes 2002, Maes 1995). Importantly, treatment resistance to is associated with heightened levels of inflammation (Haroon et al 2018, Kiraly et al 2017, O’Brien et al 2007). Thus, there is an urgent need to develop novel therapeutics that target inflammatory processes.

In recent years, plant polyphenol compounds have gained interest for their potential to treat/prevent various chronic diseases that are associated with inflammation (Guo et al 2009, Lopresti et al 2012, Tangney & Rasmussen 2013). Of these compounds, curcumin is one of the
most widely studied and is known for its strong anti-oxidant and anti-inflammatory properties (Gupta et al 2012). Previous studies suggest that curcumin may be a promising pharmacological agent for treating MDD. Oral administration of curcumin reduces symptoms of depression and co-morbid diseases, such as metabolic syndrome (Jiang et al 2013, Lopresti et al 2014, Panahi et al 2015, Sanmukhani et al 2014, Xu et al 2006, Xu et al 2009). Although these studies suggest that curcumin may be an effective alternative and/or adjunctive treatment option to traditional antidepressant medications, curcumin’s effects on peripheral biomarkers that are associated with stress-related psychiatric disorders are largely unknown. Therefore, the aim of this study was to examine whether curcumin’s pro-resiliency effects are associated with downregulation of inflammatory biomarkers. In this chapter I show that dietary curcumin prevented the increase of IL-6 and IL-1β in responders. Curcumin did not, however, prevent the induction of splenomegaly in mice that were responders. These results suggest that reducing levels of cytokines are needed for treatment response.

**METHODS**

**Animals**

Male 129/SvEv mice were purchased from Taconic Biosciences, Inc. (Germantown, NY) at 8 weeks of age. Retired male CD-1 breeders were purchased from Charles River (Wilmington, MA). Food and water were provided *ad libitum* throughout all experiments. All CD-1 mice used in social defeat experiments were pre-screened for aggressive behavior as previously described (Golden et al 2011). Mice were fed a global 18% protein chow diet (Envigo Tekland) or a global 18% protein chow diet made with 1.5% curcumin (Pfaultz & Bauer, 95% diferuloylmethane).

**Social Defeat Stress**
Social defeat was performed as previously described. Briefly, 129Sv/Ev mice were placed into the home cage of an aggressive retired CD-1 male breeder. The two mice were allowed to physically interact for 5 minutes and were then housed together in the same cage overnight separated by a perforated Plexiglas divider. This procedure was repeated for each of the 10 days of social defeat. Control mice were pair housed in the same way, but with one 129Sv/Ev mouse on each side of the perforated divider. After the final episode of social defeat all mice were singly housed.

Social Interaction Test

The social interaction test was performed as described in Chapter 2. One day after the last defeat session mice were placed in an open arena containing two enclosures for 5 minutes. One enclosure contained a novel CD-1 mouse and an identical enclosure that was empty on the opposite side of the arena. Time spent interacting with the empty enclosure and the enclosure containing the novel CD-1 mouse was manually recorded. The defeat index (DI) was computed by dividing the difference in the time spent between the two enclosures by the total amount of time spent with the two enclosures. Mice on a curcumin diet with a DI of 0 or greater were defined as “responders” while mice that had a DI below 0 were defined as “non-responders”.

Organ/Blood Collection.

Mice were sacrificed immediately following the social interaction test. The spleen was immediately extracted and weighed. Trunk blood was collected in Eppendorf tubes containing 5 µl 0.5 M EDTA, placed on ice, and centrifuged at 3,000RPM for 10 min to obtain plasma. Serum levels of IL-6 and IL-1 β were analyzed with ELISA kits (Enzo Life Sciences, Inc.; Farmingdale, NY). Samples were plated in duplicate and assessed according to manufacturer’s
instructions. Plates were read in a BioPlex Bead Array Reader (BioRad; Hercules, CA, USA) at 450nm.

Results

Curcumin Promotes Resiliency to CSDS

The effects of curcumin on social avoidance described in Chapter 2 were replicated in this study. A larger proportion of mice on a curcumin diet (60%) displayed a DI score above 0 compared to mice on a regular chow diet (12%; Figure 3.1B). A three-way ANOVA revealed a significant diet x stress x object interaction ($F_{(1,192)} = 28.84, p < 0.001$) in the social interaction test.

The Defeated/Chow group spent more time with the empty closure ($p < 0.0001$) and less time with the CD-1 ($p < 0.0001$) than defeated mice on a curcumin diet and non-stressed mice on a regular chow diet (Figure 1C). An additional analysis restricted to defeated mice on curcumin revealed that the non-responders spent significantly more time interacting with the empty
enclosure \(t_{(23)} = 6.34, p < 0.0001\) and significantly less time with the CD-1 \(t_{(23)} = 7.10, p < 0.0001\) than responders (Figure 3.1D).

**Dietary curcumin attenuates stress-induced increases in plasma cytokines**

Next, I tested whether curcumin is able to prevent stress-induced increases in the pro-inflammatory cytokines IL-6 and IL-1β. For IL-6 we observed a main effect of stress \((F_{(1,36)} = 33.16, p < 0.0001)\), diet \((F_{(1,36)} = 5.69, p < 0.05)\) and a stress x diet interaction \((F_{(1,36)} = 7.20, p < 0.01)\). Planned comparisons revealed that the Deceived/Chow group had significantly higher levels of plasma IL-6 than the Non-Stressed/Chow group \((p < 0.0001)\) and the Deceived/Curcumin group \((p <

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**Figure 3.2 Curcumin prevents CSDS induced increases in IL-6.** (A) Mean ± SEM for IL-6. Defeated mice on a control chow diet display a significant elevated in IL-6 compared to non-stressed mice on a control diet and defeated mice on a curcumin diet. (B) Mean ± SEM for IL-1β. There was a main effect of stress on IL-1β \((p < 0.05)\) but no stress x diet interaction \((p > 0.05)\). There were significant correlations between both IL-6 (C) and IL-1β (D) and the DI score of defeated mice.
0.001; Figure 2A). For IL-1β, there was a main effect of stress \( (F_{(1,36)} = 5.55, p < 0.05) \) with none of the planned comparisons reaching significance (Figure 2D). Furthermore, circulating levels of IL-6 \( (r = -.61, p < 0.001, \text{Figure 2C}) \) and IL-1β \( (r = -.52, p = 0.01) \) correlated with the DI scores of defeated mice.

**Dietary curcumin does not prevent splenomegaly**

![Diagram showing the effect of curcumin vs. control on spleen size.](image)

*Figure 3.3 CSDS increases spleen size. There was a main effect of stress on spleen size \( (p < 0.0001) \) but no stress x diet interaction.*

Previous studies have demonstrated that social defeat induces the proliferation of monocytes from the bone marrow into inflamed organs, such as the spleen, resulting in splenomegaly. We therefore compared spleen weights to indirectly measure monocyte proliferation.

We found a main effect of stress \( (F_{(1,32)} = 44.78, p < 0.0001; \text{Figure 3A}) \) with no main effect of diet or diet by stress interaction.

**Responders and non-responders display distinct levels of plasma cytokines**

Given that a proportion of mice on a curcumin diet continue to display social avoidance behavior, we compared the levels of IL-6 and IL-1β between responders and non-responders.

Boneferroni-corrected t-tests revealed that non-responders had higher circulating levels of IL-6 \( (t_{(11)} = 3.06, p = 0.01; \text{Figure 2B}) \) and IL-1β \( (t_{(8)} = 3.10, p = 0.01; \text{Figure 2D}) \). Interestingly, there was no difference in spleen weight between the responders and non-responders \( (t_{(10)} = .59, p > 0.05) \)

**Discussion**
The present study indicates that dietary curcumin may prevent stress-induced social avoidance behavior, at least in part, by diminishing the production of pro-inflammatory cytokines. Mice on a curcumin diet that did not display social avoidance had lower levels of circulating IL-6 compared to mice on a control diet and mice that did not respond to curcumin.

Furthermore, responders had lower levels of IL-1β than non-responders, which is consistent with a previous study in the C57 strain demonstrating that susceptible mice had higher levels of IL-1β than resilient but not control mice (Hodes et al 2014).

In recent years, it has become increasingly clear that dysregulation of the immune system plays a major role in the pathology of psychiatric disorders, such as MDD and PTSD (Michopoulos et al 2017, Miller et al 2009, Raison et al 2006). Recent meta-analyses suggest that of the numerous pro-inflammatory cytokines that are increased in individuals with psychiatric disorders, IL-6 is the most consistently elevated cytokine (Dowlati et al 2010). As a result, there has been a surge in the number of studies that have tested the ability of various compounds to prevent the increase in IL-6 induced by CSDS (Guimaraes et al 2018, Hodes et al 2014, Ramirez et al 2015, Ramirez & Sheridan 2016, Wang et al 2018). For instance, a daily injection of a
monoclonal antibody against IL-6 promotes resilience to CSDS (Hodes et al 2014). Furthermore, pre-treatment with a dietary polyphenol preparation or the antidepressant imipramine also reduces social avoidance behavior and plasma levels of IL-6 (Ramirez & Sheridan 2016, Wang et al 2018). Future studies should begin to administer these compounds following exposure to stress to more accurately reflect a clinical setting. While the current study suggests that curcumin promotes resiliency by preventing an increase in peripheral inflammatory signaling, it remains unknown how this occurs.

Previous research suggests that curcumin inhibits the NFκB pathway and its downstream gene products in vitro. For instance, curcumin inhibited IL-1β-mediated expression of genes which are transcribed by NFκB and blocked cytokine-induced activation of IKK (Jobin et al 1999). Curcumin may also be inhibiting NFκB dependent gene transcription by preventing the recruitment of the coactivator P300/CBP (Marcu et al 2006), which is required for changes in the patterns of acetylation on κB response elements of pro-inflammatory genes. Finally, a more recent cell-culture study has demonstrated that curcumin directly binds to and inhibits the activity of dual-specificity tyrosine kinase 2, which positively regulates the proteasome. As a result, proteasomal activity was decreased (Banerjee et al 2018). This finding suggests that curcumin’s observed effects on NFκB is due to the accumulation of IκB that results when it is not degraded by the proteasome. Although curcumin has been shown to modulate the NFκB pathway in vitro and has shown some promise in treating/preventing inflammatory disorders, there are some causes for concern regarding its translational utility. A recent perspective discussing curcumin’s chemical properties points to confusing results in molecular drug screens making it difficult to determine specific compound/target interactions (Nelson et al 2017). Although this may be true it has been argued that focusing on molecular targets as opposed to
effective compounds may preclude the examination of promising drug candidates (Heger 2017). Another concern of Nelson and colleagues is curcumin’s poor systemic bioavailability. Due to its instability in neutral and basic pH values, curcumin is rapidly degraded when orally administered in humans (Anand et al 2007). While these are legitimate causes for concern, there is evidence that orally administered curcumin accumulates in the gastrointestinal tract at a high level (Irving et al 2013). Interestingly there has been a recent surge of interest in the role that the gut microbiome plays in psychiatric disorders, such as anxiety and depression (Borre et al 2014, Kelly et al 2015). Individuals with these disorders have been shown to have a “leaky gut” (Maes 2008, Maes et al 2012). This term refers to the deterioration of the epithelial lining of the intestines, allowing for bacteria to translocate into circulation. Gram-negative bacteria which contain the endotoxin LPS can activate intracellular signaling molecules, such NF-κB, which in turn activates the production of pro-inflammatory cytokines. Interestingly, serum from individuals with symptoms associated with atypical depression, such as fatigue, altered appetite, and chronic pain, demonstrate high levels of the antibodies IgA and IgM in response to LPS stimulation. (Maes et al 2012). The activation of IgA, an antibody produced in the mucosa membrane, suggests that bacteria containing LPS have translocated from mucosal membranes into circulation. This is due to the fact that under healthy conditions, the systemic immune system is separated from mucosal defenses and as a result does not normally mount an antibiotic response to gram negative bacteria. In light of these studies, it is possible that curcumin is preventing inflammation by restoring the epithelial lining of the gut as previously demonstrated (Ma et al 2004). Therefore, future studies examining the therapeutic effects of curcumin in pre-clinical models should evaluate the efficacy of curcumin in preventing stress-induced alterations in the epithelial lining and the microbiota of the gut.
Chapter 4:

Baseline Social Behavior Predicts Post-Stress Phenotypes in Mice on a Diet Enriched with Curcumin
It is well-known that individual differences emerge throughout development in genetically identical twins, as reflected in differences in their behavior, physiology, and disease susceptibility. This is thought to be due to differences in DNA methylation, which emerge as a result of non-shared life experiences (Haque et al 2009, Kaminsky et al 2008, Ouellet-Morin et al 2013). In laboratory rodents, numerous studies have documented differences in behavior, physiology and susceptibility to stress amongst genetically identical mouse strains. For instance, 65-70% of C57 mice are “susceptible” to CSDS and exhibit social avoidance and anhedonia while the remaining mice are “resilient” and do not exhibit such behavior (Krishnan et al 2007). Furthermore, it has also been demonstrated that genetically identical mice living in an enriched environment display individual differences in exploratory behavior which correlate with individual differences in hippocampal neurogenesis (Freund et al 2013).

Recently, studies have begun to explore baseline differences in behavior and physiology in an effort uncover phenotypes which may confer vulnerability to chronic stress (Weger & Sandi 2018). For instance, mice that go on to develop a susceptible phenotype as a result of CSDS have a higher number of circulating leukocytes than mice that do not develop the susceptible phenotype. Cells from to-be susceptible mice also released higher amounts of IL-6 following stimulation with LPS than cells from to-be resilient mice (Hodes et al 2014). In addition, mice which display high levels of anxiety-like behavior in the light-dark test are more likely to develop depression-like behavior following chronic unpredictable stress (Nasca et al 2015).

While progress has been made with regards to understanding the traits that may play a role in the development of a stress-susceptible phenotype, it is still largely unknown if these traits play a role in response to treatment. Individuals who do not respond to available treatment
often display the same constellation of symptoms as individuals who respond to antidepressant treatment and very little is known about what distinguishes individuals who don’t respond from those who do (Akil et al 2017). Therefore, it is of extreme importance to study whether individual differences can predict treatment outcome in animal models, as this may aid in the development of precise treatments targeted for distinct patient populations. In this study, it was found that individual differences in baseline social behavior (social interaction test) or anxiety-like behavior (open field) did not correlate with post-stress behavior in mice fed a regular chow diet. In mice fed curcumin, however, it was found that mice that responded spent significantly more time in the center of an open field and more time interacting with a CD-1 pre-stress than mice that did not respond to treatment.

Methods

Animals

Male 129/SvEv mice were purchased from Taconic Biosciences (Germantown, NY) and male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) at 8 weeks of age. Mice were group housed (4/cage) upon arrival with mice of the same strain. Retired male CD-1 breeders were purchased from Charles River Laboratories (Wilmington, MA) and were individually housed upon arrival. All mice were acclimated to the colony room for a minimum of 1 week and maintained on a 12h (05:00-17:00) light-dark schedule with free access to food and water. Experiments were conducted in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of Hunter College, City University of New York.

Open Field (OF)
Mice were placed in the corner of a novel square chamber (45 x 45 cm) and monitored for 5 minutes. An overhead camera recorded all behavior, which was later analyzed using ANY-maze software (Stoelting, Wood Dale, IL) that tracked the animal and calculated total distance traveled (cm), number of entries into the center, and time spent in the center. The center was defined as the square area occupying the center half of the total arena.

**Social Interaction Test**

As previously described (Brachman et al. 2016b), mice were placed in the middle of an open field arena (25 cm x 48 cm) containing two identical wire-mesh enclosures for 5 minutes. An enclosure located in one end of the arena contained a CD-1 mouse and an enclosure located on the opposite side of the arena and was empty. Sessions were videotaped and an observer blind to treatment quantified the amount of time mice spent interacting with each enclosure. A defeat index (DI) was calculated by dividing the difference in the time spent with each enclosure (time with CD-1 enclosure minus time with empty enclosure) by total time spent with both enclosures post-defeat. Mice on a curcumin diet with DI values greater than 0 were defined as “responders” while those with DI values below 0 were defined as “non-responders”.

**Curcumin Diet**

Standard laboratory chow was replaced with either a global 18% protein chow diet (Control chow; Envigo Teklad) or a global 18% protein chow diet commercially made with 1.5% curcumin (Curcumin chow; Pfaltz & Bauer, 95% diferuloylmethane) one day after the first social interaction test. This high concentration of curcumin was chosen based on the known low bioavailability of the compound (Prasad et al. 2014b).

**Social Defeat Stress**
Chronic social defeat stress was performed as previously described (Golden et al 2011) with minor modifications. Retired CD-1 breeders were individually housed in large plastic cages (30.8 cm x 30.8 cm x 14.29 cm) (Thoren Caging Systems, Hazleton, PA) modified to accommodate a divider, where they remained throughout the duration of social defeat stress. Screening for aggressive CD-1 mice started the next day and lasted for three consecutive days. Social defeat began 24 hours later, which involved placing 9-10 week old experimental mice (129/SvEv) into the home cage of the CD-1 aggressor and allowing the mice to interact for 5 minutes. Experimental mice were then placed on the opposite side of the aggressor’s home cage behind a clear perforated Plexiglas divider that prevented further physical contact, but allowed for continuous psychological stress from sensory (e.g. visual, olfactory, auditory) cues for 24 hours. This procedure was repeated for 10 consecutive days, with each experimental mouse being exposed to a novel CD-1 aggressor mouse each day. Control mice were treated the same way, but were never exposed to a CD-1 aggressor. Control mice were in contact with a new mouse of the same strain each day for 5 minutes and remained housed with that mouse on opposite sides of the perforated divider for 24 hours. After the final social defeat session, all mice were removed from the large cages and individually housed in standard mouse cages (19.56 cm x 30.91 cm x 13.4 cm) (Thoren Caging Systems, Hazleton, PA).

**Results**

**Baseline Anxiety-Like Behavior Does Not Predict Treatment Outcome**

To examine if baseline anxiety-like behavior predicts treatment outcome, mice were placed in an open field for five minutes and time spent in the center was quantified. Mice were counterbalanced across experimental conditions with no group differences prior to stress exposure ($F_{(3,56)} = .59, p > 0.05$). Analysis of the mice in the Defeat/Curcumin group revealed
that mice that responded to curcumin spent significantly more time in the center of the open field at baseline than mice that did not respond to curcumin ($t_{(13)} = 2.42, p < 0.05$). Although responders displayed significantly more time in the center, this measure did not correlate with time spent with the CD-1 after CSDS in curcumin fed mice ($r = .18, p > 0.05$) or in mice fed a control diet ($r = -.19, p > 0.05$).

**Baseline Social Behavior Predicts Treatment Outcome**

To examine if baseline social behavior predicts treatment outcome, mice were placed in the social interaction test for five minutes and time spent interacting with the CD-1 was quantified.

![Figure 4.1 Responders spend more time in the center of an open field.](image)

- **A** Time spent in the center of an open field for all four groups. No differences were observed between any of the groups.
- **B** Time spent in the center for responders/non-responders. Mice that were to become responders spent significantly more time in the center than mice that did not respond.
- **C&D** There was no correlation between time spent in the center and time spent with the CD-1 following CSDS in mice fed a control diet or a diet enriched with curcumin.
There were no differences between the mice that were to be placed into the four experimental groups ($F_{(3,75)} = 1.38, p > 0.05$). Analysis of mice in the Defeat/Curcumin group revealed that mice which went on to be responders spent significantly more time interacting with the CD-1 prior to stress exposure than mice that did not respond to curcumin ($t_{(28)} = 3.98, p < 0.001$).

Furthermore, time spent with the CD-1 pre-defeat was correlated with time spent with the CD-1 post-defeat in curcumin fed mice ($r = .57, p > 0.001$) but not in mice fed a control diet ($r = .13, p > 0.05$).

**Figure 4.2** Responders spend more time interacting with a CD-1. (A) Time spent interacting with a CD-1 for all four groups. No differences were observed between any of the groups. (B) Time spent interacting with a CD-1 for responders/non-responders. Mice that were to become responders spent significantly more time interacting with a CD-1 than mice that did not respond. (C&D) There was no correlation between time spent with the CD-1 pre- and post-CSDS in mice fed a control diet (C) but there was in mice fed a diet enriched with curcumin (D).
**Discussion**

Exposure to the same event or medication affects people differently, even when they share the same genetic make-up. The results presented in this chapter demonstrate that differences in social and anxiety-like behavior predict treatment outcome in an in-bred mouse strain. Specifically, it was found that mice which went on to respond to the curcumin diet spent more time in the center of an open field and more time interacting with a CD-1 mouse prior to stress. Interestingly, these baseline behaviors were not associated with post-stress behavior in mice fed a control diet. The fact that there was no relationship likely reflects a floor effect. The 129Sv/Ev strain displays high levels of baseline anxiety as a whole and do not display a large amount of variability in their post-stress behavior. Nonetheless, the fact that 129Sv/Ev mice are highly susceptible to CSDS supports the hypothesis that high anxiety poses a major risk for the development of depression.

Recently, pre-clinical studies have begun to focus on baseline behaviors to uncover certain traits that may predict vulnerability/resiliency to stress. Of these traits, it has been recognized that high levels of anxiety renders individuals vulnerable to chronic stress (Weger & Sandi 2018). For instance, mice that avoid the light side of the light-dark apparatus prior to stress exposure display depressive-like behaviors to a larger degree post-stress than mice that explore the light-side of the apparatus (Nasca et al 2015). Furthermore, rats bred to display high levels of anxiety spend more time frozen and emit more ultrasonic vocalizations during social defeat than rats bred to display low-levels of anxiety (Frank et al 2006, Landgraf & Wigger 2002). Importantly, anxiety has also been implicated as a risk factor for the development of depression in humans. Recent genome-wide association studies have revealed that genes which are associated with trait neuroticism also confer a higher risk of developing depression (Kendler &
Myers 2010, Lo et al 2017). However, it is still largely unknown which traits are associated with treatment resistant depression.

Although multiple modalities of treatment for depression currently exist, a significant proportion of individuals with depression do not respond to any treatment. Furthermore, non-responders often display the same symptoms as responders, making it difficult to determine what distinguishes individuals who don’t respond from those who do. This pre-clinical study represents a first-step toward understanding what differentiates responders from non-responders prior to experimental manipulation. One outstanding question is how these individual differences in an inbred mice strain may emerge. Current evidence supports a role for ‘non-shared’ experiences early in life. Previous work has demonstrated that mother-infant interactions are critical for cognitive and emotional development. Pups that receive poor maternal care demonstrate increased anxiety-like behavior and decreased sociability in adulthood (Weaver et al 2006). One mechanism by which early life stress may increase anxiety-like behavior is through epigenetic modification of genes involved in the stress response (Liu et al 1997, Weaver et al 2004, Weaver et al 2006). In support of this notion are numerous studies demonstrating that animals with high anxiety exhibit enhanced activation of the HPA-axis when exposed to environmental stressors (Castro et al 2012, Landgraf & Wigger 2003). Interestingly, this effect was seen in chapter 2, with non-responders exhibiting higher activation of the HPA-axis in response to a novel stressor. In humans, patients with a history of early life stress are less likely to respond to currently available antidepressant treatments further suggesting that individual differences in early life stress exposure may play a role in treatment outcome (Nanni et al 2012, Williams et al 2016).
In summary, this study indicates that measures of social behavior may be a useful screening method to identify mice that are not likely to respond to currently available and potential antidepressants. Identifying this population *a priori* will greatly assist in the generation of novel animal models where the efficacy of treatment is low. This will allow for an exploration of the underlying mechanisms of depression not addressed by current or novel treatments. Knowledge of these mechanisms may lead to the identification of biomarkers that are associated with resistance to treatment, which may ultimately lead to patients spending less time on antidepressants which are not working for them.
Chapter 5:

General Discussion
Summary

There has been considerable progress in delineating the mechanisms by which chronic social stress affects numerous biological systems implicated in the etiology of disease pathology (Ménard et al 2017, Russo & Nestler 2013). Although the ultimate goal of this research is to discover novel pharmacological compounds that could treat psychiatric disorders, very few compounds have emerged that are both effective and safe. The present dissertation provides preclinical evidence that curcumin as a possible adjunctive/alternative treatment for MDD or PTSD.

In Chapter 2, I investigated the role of curcumin in promoting resiliency to CSDS in the stress-susceptible 129/SvEv mouse strain. In contrast to previous studies demonstrating that 30-40% of C57 are resilient to CSDS, out of the 75 mice tested on a control-chow diet, only 6 (8%) were resilient. In contrast, of the 25 mice that were tested on a curcumin diet, 17 (68%) were resilient (responders). Furthermore, when taking the data presented in chapter 3 into account, I find that of 100 mice tested on a regular chow diet only 9 were resilient (9%). In contrast, of the 50 mice tested on a curcumin diet, 32 were resilient (64%). Unlike the effects found in the SI test, we found that curcumin prevented the emergence of anxiety-like behavior as measured by the EPM and OF in both responders and non-responders. The absence of phenotypic differences in the elevated plus maze and open field tasks in defeated mice on curcumin suggest that social avoidance behavior occurs independently of anxiety-like behavior. This is in line with previous reports examining natural resiliency in C57 mice demonstrating that susceptible and resilient mice do not differ in measures of anxiety-like behavior in the elevated plus maze (Krishnan et al 2007).
Interestingly, we did observe differences between responders and non-responders in the activation of the HPA axis to a novel stressor. Furthermore, the amount of corticosterone released into circulation correlated with social avoidance behavior, but not anxiety-like behavior. Lastly, in contrast to previous studies, we did not see an effect of curcumin on the consolidation of Pavlovian fear memory. One reason for this discrepancy is a possible floor effect. 129Sv/Ev mice only freeze ~40-50% of time at the beginning of testing, while rats often freeze ~80% of time (Hefner et al 2008, Monsey et al 2015). The lack of an effect in the defeated mice may represent a treatment-resistant phenotype, despite the fact that 80% of the mice were responders as defined by their DI score. Of note is a previous study, which demonstrated that non-stressed 129 mice chronically treated with an SSRI fail to extinguish a previously learned CS-US association (Camp et al 2012). Thus, it is likely that stress-induced enhancements in the consolidation of fear memories in this strain are unable to be ameliorated by currently available compounds, although this remains to be tested.

In chapter 3, I replicated the effects of curcumin in promoting resiliency to CSDS and demonstrated that this behavioral finding is associated with a reduction in the pro-inflammatory interleukins IL-6 and IL-1β in responders. This is consistent with a multitude of in vitro studies demonstrating that curcumin prevents the activation of the NFκB pathway, thereby preventing the transcription of numerous pro-inflammatory genes (Jobin et al 1999). Furthermore, it was found that non-responders displayed elevations of these two cytokines compared to responders. This is consistent with previous research demonstrating that patients with MDD and PTSD who do not respond to treatment display elevated levels of cytokines, such as IL-6, than patients who do respond (Haroon et al 2018, Kiraly et al 2017).
In light of the findings in chapters 2 and 3 that defeated mice fed curcumin can be broken down into two phenotypes based on their post-stress behavior (SI) and systemic biology (HPA activation/cytokine expression) I examined differences in pre-stress behavior in an attempt to better understand why some mice respond while others don’t. I found significant differences in both anxiety-like and social behavior in mice that went on to become responders versus non-responders. Specifically, I found that mice that went on to respond to curcumin spent more time interacting with a CD-1 and in the center of an open field than mice that did not respond. Interestingly, time spent with the CD-1 press stress correlated with time spent with a novel CD-1 post-stress in curcumin fed mice, but not mice on a control diet. These results suggest that social behavior at baseline may be a useful tool for predicting treatment outcome in preclinical research.

Collectively, the findings of this dissertation are the first to demonstrate that curcumin promotes resiliency to an ecologically valid social stressor. It is also the first to demonstrate that curcumin is able to prevent the production of systemic pro-inflammatory cytokines as a result of stress. Future studies (discussed below) should attempt to pinpoint exactly how curcumin is able to produce these effects in vivo despite its low systemic bioavailability.

**Future Directions**

In the present dissertation, I present overwhelming evidence that 129Sv/Ev mice are highly susceptible to CSDS and that curcumin enhances resiliency. However, it is unknown if curcumin following CSDS, or any compound, can reverse the effects of stress in this mouse strain. Only one previous study has used this strain in the standardized CSDS model. It was found that ketamine, but not fluoxetine was able to promote resilience to CSDS prior to stress.
However, ketamine was unable to ameliorate depressive-like symptoms following CSDS (Brachman et al 2016a). This is in contrast to previous studies in C57 mice demonstrating that ketamine is effective in reversing social avoidance and anhedonia following CSDS (Bagot et al 2017, Donahue et al 2014, Iniguez et al 2014). It is also in contrast to previous studies demonstrating that pre-treatment with currently available antidepressants such as imipramine are able to prevent social avoidance in C57 mice (Ramirez & Sheridan 2016). In light of these findings, future studies should attempt to develop a model of treatment resistance using this mouse strain in numerous chronic stress paradigms. This would likely lead to a model where the effect of current antidepressant treatment is low to begin with, allowing for a more in-depth understanding of the molecular mechanisms underlying treatment resistance. Furthermore, such a model will be useful for screening novel pharmacological compounds that may ultimately work in individuals with treatment resistant depression.

While this dissertation provides evidence that the anti-inflammatory properties of curcumin result in resilience to CSDS, future studies should attempt to study exactly how curcumin is producing these effects. One promising avenue of research may be found in studying curcumin’s ability to prevent alterations in the microbiome and the epithelial lining of the intestinal tract (Ma et al 2004, McFadden et al 2015). For instance, in a mouse model of colon cancer, it was found that diet of .5% curcumin increased survival, bacterial diversity, and increased the abundance of the Lactobacillales genus, which prevent stress-induced permeability of the intestines (McFadden et al 2015). Interestingly, rodents exposed to chronic stress exhibit alterations in gut microbiota, which have been shown to be necessary for the development of a depressive-like phenotype (Ait-Belgnaoui et al 2012, Foster & Neufeld 2013). Furthermore, chronic stress increases intestinal permeability, thus giving bacteria an opportunity to translocate
across the intestinal mucosa and influence the activity of immune cells. As a result, these bacteria can activate intracellular signaling molecules, such as NF-κB, which in turn activates the production of pro-inflammatory cytokines (Maes 2008). Thus, future studies should evaluate whether curcumin is reducing the activity of NF-κB directly or by preventing the translocation of bacteria across the intestinal mucosa.

There has been a recent surge of interest in the relationship between baseline measures of anxiety and susceptibility to chronic stress-induced depressive-like phenotypes. These studies have revealed that mice that exhibit anxiety-like behavior at baseline are more susceptible to chronic stress than mice that don’t exhibit high levels of baseline anxiety. However, there is little known about the role these behaviors play in determining treatment outcome. This dissertation is the first to draw a link between treatment outcome and baseline levels of social behavior. There are numerous questions which arise as a result of this finding. For instance, would baseline behavior prior to stress predict treatment outcomes to currently available antidepressants? If they do, it is important to ask whether these behaviors would predict treatment outcome following chronic stress, as this is a better model of what occurs in a clinical setting.

Research using these behavioral tests to identify susceptible sub-populations of mice has revealed biomarkers that are associated with this phenotype. For instance, mice which went on to develop a susceptible phenotype following CSDS had a higher number of circulating leukocytes which released higher levels of IL-6 when stimulated with LPS than resilient mice prior to CSDS (Hodes et al 2014). Additionally, it has been found that mice which avoid the light side of the light-dark apparatus display lower levels of mGlu2 receptors and higher levels of MRs within the hippocampus (Nasca et al 2015). This phenotype is indicative of aberrant glutamate transmission and is seen in mice following exposure to chronic stress. However, it remains unknown if these
behavioral tests will lead to the identification of biomarkers which would be able to predict whether or not a particular treatment is likely to be effective. Thus, future studies should incorporate these baseline tasks in studies evaluating current or potential antidepressants.

Overall Conclusions

The findings of the present dissertation clearly suggest that curcumin alters behavioral, endocrine, and immune responses to chronic social stress. Importantly, these effects are found in a mouse strain that is highly susceptible to chronic stress. This is also the first study to show that dietary curcumin leads to the reduction in the pro-inflammatory interleukins IL-6 and IL-1β in response to chronic stress. This suggests that future studies should examine biomarkers associated the IKK-NFκB pathway in order to try and predict treatment response to curcumin.
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