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Neuroinflammatory Regulation of Stress Behavior and Physiology

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Neuroinflammatory Regulation of Stress Behavior and Physiology

Abstract
Neuropsychiatric diseases represent a major public health burden worldwide; due to gaps in our understanding of the pathogenic mechanisms of disease, approximately 30% of patients are refractory to treatment. Activation of neuroinflammatory signaling cascades has shown promise as a contributing factor for disease development. This hypothesis is driven in part by an intriguing overlap of symptoms in patients with neuropsychiatric and immunological disorders. Patients with depression, bipolar disorder, schizophrenia, and autism commonly present with immune dysfunction, while patients with multiple sclerosis, lupus, and rheumatoid arthritis often experience severe mood disturbances. Diversity in presentation of symptoms, however, has posed a research challenge to our mechanistic understanding of this link. In contrast to the complexity of modeling specific diseases, altered sensitivity to stress is a well-documented vulnerability marker across neuropsychiatric disorders. Of relevance to clinical advancement, aspects of stress behavior and physiology can be modeled and measured in animals, where core components of the stress axis are conserved in humans and rodents. Thus, we performed an examination of the neuroinflammatory regulation of stress behavior and physiology. Using a genetic model of stress sensitivity, we report the discovery that anti-inflammatory treatment ameliorates hypothalamic-pituitary-adrenal axis dysregulation, identifying the dorsal raphe (DR) as a locus of heightened responsivity. We then demonstrated sex differences in this brain region in response to the stress neuropeptide, corticotropin-releasing factor, suggesting that differences in its responsivity may underlie sex differences in vulnerability to stress-related disorders. Finally, we used a transgenic approach to show that neuroinflammation localized specifically to the DR results in dysregulated stress behavior and physiology through interactions with the serotonergic neurotransmitter system. Overall, this work demonstrates that hyper- or hypo-function of the DR, based on genetic susceptibility, sex, or neuroinflammatory insult, can result in altered stress physiology and behavior. Though the DR has previously been identified as a potential locus of dysregulation, here we establish the specific, mechanistic link between risk factors for stress-related disorders. We present evidence of quantitative changes to this brain region and its functional output, and demonstrate that differences in responsivity of the DR may underlie vulnerability to stress-related disorders.

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NEUROINFLAMMATORY REGULATION OF STRESS BEHAVIOR AND PHYSIOLOGY

Alexis Rose Howerton

A DISSERTATION

in

Neuroscience

Presented to the Faculties of the University of Pennsylvania

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This work is dedicated to the loving memory of my Grandpa Hank. Through life’s highs and lows, he saw the best in everyone around him. His eternal optimism was and always will be a great source of inspiration for us all.
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ABSTRACT

NEUROINFLAMMATORY REGULATION OF
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Alexis Rose Howerton
Tracy L. Bale

Neuropsychiatric diseases represent a major public health burden worldwide; due to gaps in our understanding of the pathogenic mechanisms of disease, approximately 30% of patients are refractory to treatment. Activation of neuroinflammatory signaling cascades has shown promise as a contributing factor for disease development. This hypothesis is driven in part by an intriguing overlap of symptoms in patients with neuropsychiatric and immunological disorders. Patients with depression, bipolar disorder, schizophrenia, and autism commonly present with immune dysfunction, while patients with multiple sclerosis, lupus, and rheumatoid arthritis often experience severe mood disturbances. Diversity in presentation of symptoms, however, has posed a research challenge to our mechanistic understanding of this link.

In contrast to the complexity of modeling specific diseases, altered sensitivity to stress is a well-documented vulnerability marker across neuropsychiatric disorders. Of relevance to clinical advancement, aspects of stress behavior and physiology can be modeled and measured in animals, where core components of the stress axis are conserved in humans and rodents.

Thus, we performed an examination of the neuroinflammatory regulation of stress
behavior and physiology. Using a genetic model of stress sensitivity, we report the
discovery that anti-inflammatory treatment ameliorates hypothalamic-pituitary-adrenal
axis dysregulation, identifying the dorsal raphe (DR) as a locus of heightened
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Finally, we used a transgenic approach to show that neuroinflammation localized
specifically to the DR results in dysregulated stress behavior and physiology through
interactions with the serotonergic neurotransmitter system.

Overall, this work demonstrates that hyper- or hypo-function of the DR, based on
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locus of dysregulation, here we establish the specific, mechanistic link between risk
factors for stress-related disorders. We present evidence of quantitative changes to this
brain region and its functional output, and demonstrate that differences in responsivity of
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CHAPTER 1: INTRODUCTION

The goal of this dissertation is to understand the contribution of neuroinflammation to the regulation of stress related behavior and physiology, with the hope of providing insight into specific targetable, pathogenic mechanisms of stress-related disease. In this dissertation I focus on the bidirectional relationship between stress exposure/coping behavior, and neuroinflammation, both brain-wide and in potentially susceptible brain regions. I have specifically focused on the contribution of the serotonergic dorsal raphe nucleus (DR) as a locus of vulnerability. Using a genetic model of stress sensitivity, I report the discovery that anti-inflammatory treatment ameliorates hypothalamic-pituitary-adrenal (HPA) stress axis dysregulation. I then characterize sex differences in the DR response to the stress neuropeptide, corticotropin-releasing factor (CRF), demonstrating that sex differences in responsivity may contribute to differences in vulnerability to stress-related disorders. Finally, I use a transgenic approach to show that neuroinflammation localized specifically to the DR results in dysregulated stress behavior and physiology through interactions with the serotonergic neurotransmitter system. This work has helped gained insight into the potential mechanism underlying the clinical overlap of neuroimmune and neuropsychiatric symptomology.

The following introductory chapter describes the challenges associated with the advancement of research in neuropsychiatric disease, providing background of how, by utilizing different models of vulnerability, one might learn about this family of stress-related disorders. This includes a general description of psychiatric disease symptom presentation, background on the stress response, and a primer on the neuroimmune system. As the majority of this dissertation describes experiments examining the specific
contribution of the serotonergic DR, I provide extensive background on the known role for this brain region and neurotransmitter system to respond to, and affect, stress related behavior and physiology. The introduction then focuses on how the relationship between these factors has been studied in humans with disease and in animal models. Finally, I present an overview of the goals of this dissertation, and the potential implications of the findings presented herein.

**Neuropsychiatric disease: a major unmet medical need**

Neuropsychiatric diseases represent a major public health burden worldwide, accounting for 13% of the global burden of disease (Collins et al., 2011). In the United States, it is estimated that 26.2% of adults suffer from a diagnosable mental disorder every year (Kessler et al., 2005b; Wang et al., 2005).

The complex clinical manifestations of neuropsychiatric disorders generally fall in an overlap of domains, where examples include: sadness, confused thinking or reduced ability to concentrate, excessive fears or worries, extreme mood changes of highs and lows, withdrawal from friends and activities, significant tiredness or low energy, problems sleeping, detachment from reality (delusions), paranoia or hallucinations, extreme feelings of guilt, alcohol or drug abuse, major changes in eating habits, changes in sex drive, excessive anger or hostility or violence, suicidal thinking, and an inability to cope with daily problems or stress (MayoClinic, 2012). However, even within a particular diagnosis, illnesses vary with respect to symptom number, type, intensity, and duration.

This heterogeneity in disease development, presentation, and trajectory is likely
due to the complex nature of risk factors, which include environment, genetics and gender. This has lead to significant diagnostic and treatment challenges; despite treatment being fairly common, gaps in our understanding of the pathogenic mechanisms contributing to disease have left up to 30-50% of patients refractory to treatment (Souery et al., 2006).

Stress dysregulation as a vulnerability marker for neuropsychiatric disease

Responding to stress is a normal, homeostatic response of the body and brain to a perceived threat or challenge. It is vital to an organism’s survival, allowing appropriate allocation of energy and attention. However, an inability to cope with stress exposure is a well-documented vulnerability marker of neuropsychiatric disease, where patients often present with altered basal stress hormones, inappropriate feedback following stress exposure, and a failure to produce adaptive stress coping responses (Breslau and Davis, 1986; Koenig et al., 2002; Nestler et al., 2002). Indeed, in addition to subjective measurement of behavioral outcome on scales such as the Hamilton Rating Scale for Depression (HRSD) or the Young Mania Rating Scale (YMRS), the amelioration of stress hormone dysregulation is an increasingly common readout for the success of clinical trials in the domain of neuropsychiatry.

Of relevance to clinical advancement, aspects of stress behavior and physiology can be measured with some reliably in animal models, where core components of the stress axis are conserved in humans and rodents. Thus, the examination of stress coping behavior and physiology is a tractable target for an investigation into the etiology and potential treatment of these diseases. This section outlines the role of the corticotropin-
releasing factor (CRF) and serotonin (5-HT) systems in stress responsivity, and the biochemical changes that occur with chronic stress exposure.

**Corticotropin-releasing factor (CRF)**

CRF represents an important link between stress and mood regulation (Vale et al., 1981; Sutton et al., 1982; Stenzel-Poore et al., 1994; Groenink et al., 2002). The classical neuroendocrine stress response - activation of the hypothalamic-pituitary-adrenal (HPA) stress axis - is initiated upon stress-induced release of CRF from the paraventricular nucleus of the hypothalamus (PVN). In parallel to its hypothalamic release, extra-hypothalamic CRF serves as an important neuromodulator to orchestrate stress responsivity within the brain (Bale and Vale, 2004).

Originating largely from the bed nucleus of the stria terminalis (BNST) and central amygdala (CeA), in addition to the PVN, CRF acts on two G-protein coupled receptor (GPCR) subtypes, CRFr1, and CRFr2. Characterization of transgenic mice that have been constructed to lack either CRFr1 or CRFr2 have aided in our understanding of the role of these receptors in stress related behavior and physiology. CRFr1 /− mice have a blunted stress response and display reduced anxiety-like behavior (Smith et al., 1998; Timpl et al., 1998). Conversely, mice lacking CRFr2 are hypersensitive to stress exposure, displaying augmented HPA stress axis corticosterone levels, increased anxiety-like behavior, and reduced ability to mount appropriate coping responses to stress exposure (Bale et al., 2000; Bale and Vale, 2003; McEuen et al., 2008). Because of this, the two CRF receptors have been described as playing opposing roles as the “gas” and “breaks” of the stress response. However, as biology generally teaches us, their roles are
not strictly dichotomous. This is evidenced by the generation of mice deficient for both CRFr1 and CRFr2, which exhibit a more blunted stress response compared to CRFr1 null mice (Bale et al., 2002). Clearly, the cell type and brain region of localization plays a critical in the response of these receptors to CRF.

**Serotonin (5-HT)**

Serotonin (5-hydroxytryptamine; 5-HT), a monoaminergic neurotransmitter, is produced by almost every organ in the body, including the skin, gut, liver, and lungs. In the brain, its primary sources are the dorsal and medial divisions of the raphe nucleus (DR), which sits below the cerebral aqueduct at the junction of the midbrain and brainstem. Despite there being relatively few 5-HT producing neurons, they innervate much of the brain and regulate many aspects of behavior (Dahlström and Fuxe, 1964; Steinbusch, 1981; Azmitia and Gannon, 1982). The most studied roles of 5-HT are in the modulation of arousal, sleep, memory, and mood.

Because selective 5-HT reuptake inhibitors (SSRIs), which target the 5-HT transporter, SERT, have had some success for the treatment of depression, this neurotransmitter system has been a major focus of research on mood disorders. Serotonin turnover rates are reduced in major depression, and increase with recovery (Asberg et al., 1976; Asberg et al., 1984). Alterations in 5-HT signaling have also been identified in patients with depression, including reduced 5-HT metabolites in the CSF and postmortem tissue, and reduced SERT levels (Reddy et al., 1992; Owens and Nemeroff, 1994; Huang et al., 2010; Chatzittofis et al., 2013). Single nucleotide polymorphisms in the promotor regions encoding for tryptophan hydroxylase-2 (TPH2), the rate-limiting
enzyme in the synthesis of 5-HT, has also been found correlated with depression (Gao et al., 2012). Polymorphisms in the promoter region of the 5-HT transporter have also associated with increased stress-induced depressive symptoms (Ho et al., 2013). Interestingly, this SERT polymorphism appears to have a greater impact on the depressive symptoms of women compared to men (Sjoberg et al., 2006).

5-HT also plays an important role in the “manic” side of the behavioral spectrum, influencing arousal, impulsivity, hedonic and hyper-aggressive behaviors, all of which are central manifestations of mania (Shiah and Yatham, 2000; Crockett et al., 2010). Clinical studies of CSF, platelet assessments, and neuroendocrine challenge, and postmortem tissue provide evidence to support the hypothesis that serotonin deficiency is involved in mania, and that enhancement of serotonin neurotransmission may exert a mood-stabilizing effect (Shiah and Yatham, 2000).

Importantly, there are major sex differences in the 5-HT system, which are thought to contribute to sex differences in neuropsychiatric disease, including the aforementioned behaviors associated with both depression and mania. Human PET imaging has revealed a reduced rate of serotonin synthesis (Nishizawa et al., 1997) and differences in 5-HT binding sites throughout the brains of women compared to men (Jovanovic et al., 2008). Rodent studies have demonstrated that 5-HT-increasing pharmacological agents, which are known to activate the HPA axis, show a greater effect on glucocorticoid production in female rodents compared to males (Goel and Bale, 2010). Central 5-HT levels and CSF concentrations of the 5-HT metabolite, 5-HIAA, too, appear to be higher in females compared to males (Rosecrans, 1970).
**Biochemical changes following chronic stress**

The consequences of stress are dependent on specific stressor characteristics, including intensity, controllability, and predictability. Importantly, they are also heavily dependent on the duration of exposure, which is generally categorized as acute or chronic. Chronic stress enhances both basal HPA tone and stress responsivity, often resulting in hypersecretion of glucocorticoids and adrenal hypertrophy. Chronic stress also impacts central stress circuitry, downregulating glucocorticoid receptors and reducing glucocorticoid negative feedback efficacy [reviewed in (Herman, 2013)]. Major stress responsive brain regions such as the hippocampus, amygdala, and prefrontal cortex, all undergo structural remodeling following chronic stress exposure (Vyas et al., 2002). Stress-induced changes in dendritic morphology have been observed in the dentate gyrus of the hippocampus (Wood et al., 2004), amygdala (Vyas et al., 2003), and medial prefrontal cortex (Liston et al., 2006). Interestingly, cortical changes may be particularly plastic, as they display substantial recovery by 3 weeks after the termination of stress exposure (Radley et al., 2004). Similar changes occur in these brain regions with chronic corticosterone administration, suggesting the effects of chronic stress may be glucocorticoid-mediated. This possibility is supported by a study that found treatment with 3-β-hydroxysteroid dehydrogenase, which blocks stress-induced corticosterone, prevents stress-induced dendritic remodeling in the CA3 region of the hippocampus (Magarinos and McEwen, 1995).

Importantly, many of the effects of chronic stress in the brain are sex-specific; for example, chronically stressed males and females show different patterns of morphological changes in the hippocampus (Hillerer et al., 2013). Additionally, chronic
stress has been shown to decrease norepinephrine, dopamine, and 5-HT in the hippocampus of males whereas increased levels of these neurotransmitter levels have been observed in females (Sunanda et al., 2000; Beck and Luine, 2002). These findings are of particular relevance considering the dramatic sex differences in behavior that occur following chronic stress.

**Inflammation as a risk factor for neuropsychiatric diseases**

The CNS contains a blood-brain-barrier (BBB) consisting of endothelial cells and astrocytes, which regulates the permeability of entry into the brain. This includes the regulation of immune cellular and molecular infiltration from the periphery. The question of how the brain and peripheral immune system communicate is still largely under investigation. Since cytokine and chemokine receptors are expressed by both glia and neurons in the adult brain, a humoral route of communication from the periphery has been proposed. Additionally, autonomic nerve fibers provide a loop of communication between the brain and peripheral blood vessels, which contain circulating immune cells.

The brain also contains specialized cells capable of performing immune surveillance and repair [reviewed in (Ousman and Kubes, 2012)]. Microglia, astrocytes, and neurons all display some immune function through their ability to produce and respond to pro- and anti-inflammatory cytokines, express cytokine and chemokine receptors, and display inducible expression of major histocompatibility complex (MHC). Microglia are generally considered the “resident immune cell” of the brain, where they function to survey and clean-up (i.e. phagocytose) damaged or intrusive cells. Microglia can promote inflammation through secretion of soluble factors, including TNFα, IFNγ,
IL-1β, and several chemokines. Additionally, they can serve an anti-inflammatory role through production of IL-4, IL-10, and TGF-β, as well as BDNF and GDNF. Astrocytes can play a similar pro- or anti-inflammatory function, though their role as immune cells is far less well examined. However, posed at the intersection of the BBB, astrocytic release of cytokines and chemokines can play an important role in regulating BBB permeability and promoting infiltration of peripheral immune cells.

Activation of inflammatory signaling cascades have been a promising environmental risk factor for the development of a number of neuropsychiatric disorders. Schizophrenia, bipolar disorder, depression, and other psychotic illnesses commonly co-present with immune dysfunction (Muller et al., 2009; Modabbernia et al., 2013). Furthermore, there is significant co-presentation of primary immunological disorders with psychiatric manifestations. Notably, patients with synaptic autoimmune encephalitis are often first admitted into care on the basis of symptoms of mania (Rosenfeld and Dalmau, 2011), and the neuropsychiatric manifestations of multiple sclerosis, lupus, and rheumatoid arthritis include depressive episodes, irritability, mood swings, rapid speech, distractibility, and impulsivity, in addition to cognitive decline (Kwentus et al., 1986; Bruce, 2008; Meszaros et al., 2012). Consequently, many researchers have hypothesized that (neuro)immunological mechanisms drive aspects of neuropsychiatric symptomology [reviewed in (Najjar et al., 2013)]. However, the complex nature of disease presentation, and the difficulty in modeling these complex disorders in animals, has left advancement stalled; while there is substantial epidemiological evidence, the understanding of mechanisms underlying this link is still very much in its youth.
The bidirectional relationship between stress and neuroinflammation

The first observation that stress impacts immunity came when Hans Selye noted that chronic stress results in atrophy of the thymus (Selye, 1936). Since then, stress has historically been considered immunosuppressive. Indeed, glucocorticoids, the steroid hormone produced by the adrenal cortex in response to stress exposure, play a well-appreciated anti-inflammatory and immunosuppressive role throughout the body, acting through both genomic and non-genomic modes of action (Coutinho and Chapman, 2011) (Silverman and Sternberg, 2012).

Within the brain, however, these stress-induced neuropeptides and hormones can actually serve a pro-inflammatory role (Sorrells and Sapolsky, 2007; Sorrells et al., 2009). Acute stress has been shown to enhance NF-κB activity (Madrigal et al., 2001), increase IL-1β (Nguyen et al., 1998), activate TNF-α (Madrigal et al., 2002), and upregulate prostaglandins and cyclooxygenase-2 (Madrigal et al., 2003). Importantly, stress exposure has been clearly documented to increase inflammatory cytokine production in humans and in rodent models (Reyes et al., 2003; Deak et al., 2005; Hueston et al., 2011; You et al., 2011).

Conversely, pro-inflammatory cytokines also have the ability to modulate neurotransmitter systems in the brain to affect stress related behavior and physiology (Konsman et al., 2002; Johnson et al., 2004; Gadek-Michalska et al., 2013). Most notably, IL-1β, TNF-α, and IL-6 can all stimulate glucocorticoid release through HPA axis activation at the level of the hypothalamus. This sequence of events has the potential to create a dangerous positive feedback loop, whereby increased stress exposure sets a stage of vulnerability for an elevated neuroimmune response to stress, which, in turn,
increases stress responsivity. In a chicken or egg type of scenario, elevated basal neuroinflammation, due to environmental or genetic influence, could also predispose individuals to susceptibility for stress-related disorders.

**Brain-wide, and region-specific neuroinflammation**

Animal models have clearly demonstrated the ability of pro-inflammatory cytokines, such as interleukin (IL)-1β, interferon-γ, tumor necrosis factor-α, and lipopolysaccharide (LPS) to modulate arousal and coping behaviors when administered peripherally (Dantzer et al., 2008). Intriguingly, the same molecules alter discrete aspects of behavior when delivered intracerebroventricularly, or acutely into specific brain regions, suggesting that access to and action on particular brain areas may be central to their effects (Song et al., 2006; Huang et al., 2008; Barrientos et al., 2012; Hayley et al., 2013).

*Toxoplasma gondii* (*T. gondii*) is an intracellular obligate parasite with the ability to cross the blood-brain-barrier and induce neuroinflammation. In the chronic stage of infection, neuroinflammation is maintained, while peripheral infection is cleared. As such, *T. gondii* is a powerful tool to ask about the impact of brain-wide neuroinflammation on behavior, both in humans and rodents. What *T. gondii* infected individuals have taught us, is that neuroinflammation has a profound ability to increase impulsivity, incidence of schizophrenia, and risk of suicide (Alvarado-Esquível et al., 2013; Ingram et al., 2013; Webster et al., 2013). The hypothesis that the location of the parasite might influence the behavioral outcomes of infection is not new, and in fact, was recently reviewed by McConkey and colleagues (McConkey et al., 2013).
Observations of patient behavior in association with measured loci of insult has helped to greatly improve our understanding of the impact of brain region-specific neuroinflammation on behavior. For example, traumatic brain injury (TBI) and stroke both result in a focal neuroinflammatory response, and commonly present with a host of neuro-cognitive challenges. TBI, the result of mechanical force applied to the skull and transmitted to the brain, leads to psychiatric problems in as many as 40% (Kim et al., 2007) and secondary mania in close to 9% of patients. Post-stroke depression occurs in roughly 30% of survivors (Ayerbe et al., 2013). As such, it has been proposed that TBI and stroke impact behavior through disruptions in neurotransmission in relevant behavior-coordinating brain regions, termed, the “lesion location perspective.” However, data to support this theory remain inconclusive, largely due to the fact that neuroimaging methods are not yet able to assess the full extent of the TBI- and stroke-affected areas with the precision necessary to denote sufficient anatomical specificity. However, with the current rate of advancement in neuroimaging, it is likely that information will soon be available.

Clearly the brain is not a homogenous organ, therefore understanding loci of neural dysfunction may help in our understanding of disease etiology, presentation, and treatment. Recent studies have found evidence for the presence of focal neuroinflammation in a number of psychiatric diseases, including autism (Onore et al., 2012; Theoharides et al., 2013), depression (Hercher et al., 2009), schizophrenia (Cagnin et al., 2007), and bipolar disorder (Rao et al., 2010). These findings suggest that anatomical specificity may underlie the epidemiological evidence linking neuroimmune and neuropsychiatric disorders.
Goals of this dissertation

It is becoming clear that neuroinflammation can have a profound effect on behaviors associated with neuropsychiatric disease. Furthermore, there is mounting evidence that impact of neuroinflammation on behavior is driven by brain region specificity. However, the mechanism by which this occurs remains unknown, in part due to the complex presentation and trajectory of neuropsychiatric disease symptoms. With this in mind, the goal of this dissertation was to elucidate the role of neuroinflammation as a determinant of stress sensitivity. As there are major sex difference in incidence and presentation of both neuroimmune and neuropsychiatric diseases, this thesis also contains an examination of sex differences as an additional “model of vulnerability” towards stress dysregulation. The hope is that a better understanding of the intersection of neuroinflammation, sex, and stress physiology, at a brain region-specific level of investigation, may provide the framework to develop more appropriate targets for individualized therapeutics.
CHAPTER 2: ANTI-INFLAMMATORY TREATMENT AMELIORATES HPA STRESS AXIS DYSFUNCTION IN A MOUSE MODEL OF STRESS SENSIVITY

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ABSTRACT

Dysregulated stress responsivity is a hallmark of neuropsychiatric disease. The regulation of stress activation and recovery involves tight coordination between neuronal and glial networks. At a certain threshold of sensitivity, stress exposure can evoke a neuroimmune response. Astrocytes are potential mediators of these effects, as they are able to respond to neuroimmune effector molecules and regulate neuronal activity. Mice deficient in corticotropin-releasing factor (CRF) receptor-2 (CRF2\(^{-/-}\)) display increased stress sensitivity, and are therefore a useful model in which to examine the intersection of neuroimmune activation and stress pathway dysregulation. We hypothesized that a component of elevated stress reactivity may involve an engagement of neuroimmune effectors, including astrocytes. Therefore, we hypothesized that this phenotype may be rescued by concomitant non-steroidal anti-inflammatory drug (NSAID) treatment. To examine this, mice exposed to chronic stress were treated with NSAIDs in their drinking water, and changes in HPA stress axis function were examined. As a correlate of altered astrocyte function, levels of glial fibrillary acidic protein (GFAP) were measured. Supportive of our hypothesis, NSAID treatment rescued the HPA stress axis dysfunction in stress-sensitive CRF2\(^{-/-}\) mice, and also reversed the stress-induced increase in GFAP in stress-regulating brain regions including the paraventricular nucleus of the hypothalamus, ventral hippocampus, and prefrontal cortex. These findings support the local involvement of astrocytes in the exacerbation of stress pathway dysregulation. The specificity of these effects in a stress-sensitive genotype highlights the importance of utilizing a model of stress dysregulation in the examination of factors that may translate to neuropsychiatric disease.
INTRODUCTION

Stress pathway dysregulation is one of the most pervasive symptoms in neuropsychiatric disease. Patients with stress-related affective disorders, such as anxiety, major depressive disorder, and post-traumatic stress disorder often present with altered basal stress hormones, inappropriate feedback following stress exposure, and a failure to produce adaptive stress coping responses (Breslau and Davis, 1986; Koenig et al., 2002; Nestler et al., 2002). Thus, the ability to appropriately respond and adapt to stress at the physiological, molecular, and cellular levels are necessary to prevent dysfunction and disease. While complex regulatory mechanisms likely contribute to the development of neuropsychiatric disease, increasing evidence implicates inflammatory processes in their pathophysiology (Strous and Shoenfeld, 2006; Dowlati et al., 2010; Dantzer et al., 2011). Within the central nervous system, astrocytes function as immune effector cells capable of producing and responding to proinflammatory cytokines, and are intricately involved in the integration of signals within neuronal networks (Theodosis and Poulain, 1999; Ullian et al., 2004; Allen and Barres, 2005; Volterra and Meldolesi, 2005; Haydon and Carmignoto, 2006; Theodosis et al., 2008; Schwarz and Bilbo, 2012). However, how such inflammatory processes intersect with stress reactivity is unknown.

Stress dysregulation and elevated neuroimmune activation commonly co-present in psychiatric patient populations, including major depressive disorder and posttraumatic stress disorder (Pace and Heim, 2011; Raison and Miller, 2011). However, animal models relevant to neuropsychiatric disease rarely consider this dual phenotype. In healthy individuals, mild stress exposures do not typically produce neuropsychiatric disease symptoms, nor in healthy wildtype (WT) mice does mild stress result in substantial
neuroimmune activation (Munhoz et al., 2006). In susceptible individuals, however, stressful life events can both precipitate disease onset and exacerbate symptoms (MacMillan et al., 2009). Thus, a co-presentation of stress dysregulation and neuroimmune activation may only be present in susceptible individuals. Mice deficient in corticotropin-releasing factor (CRF) receptor-2 (CRF2−/−) are hypersensitive to stress exposure, displaying augmented HPA stress axis corticosterone levels, increased anxiety-like behavior, and reduced ability to mount appropriate coping responses to stress exposure (Bale et al., 2000; Bale and Vale, 2003; McEuen et al., 2008). These stress-sensitive mice are therefore a useful ‘susceptible’ population in which to examine the intersection of neuroimmune activation and stress pathway dysregulation.

Therefore, we hypothesized that stress dysregulation, a significant factor in disease susceptibility, involves activation of neuroimmune factors in stress modulating brain regions. In addition, the ability to detect such changes may require an appropriate stress-sensitive animal model in which stress engages a neuroimmune response involving local astrocytes. The current studies examined changes in the astrocyte cytoskeletal protein, glial fibrillary acidic protein (GFAP), associated with exposure to chronic stress in brain regions central to the regulation of stress responsivity: the paraventricular nucleus of the hypothalamus (PVN), hippocampus, and medial prefrontal cortex (Keller-Wood and Dallman, 1984; Jankord and Herman, 2008; Radley and Sawchenko, 2011). To determine if genotypic differences in stress responsivity were related to differences in inflammatory processes, concomitant NSAID treatment was assessed for its ability to ameliorate stress dysregulation, as evidenced by hypothalamic-pituitary-adrenal (HPA) stress axis production of corticosterone and parallel changes in astrocyte GFAP
immunoreactivity in key stress-regulatory brain regions.

METHODS

Animals

Corticotropin-releasing factor (CRF) receptor-2 deficient (CRF2−/−) mice were generated in-house on a mixed C57Bl/6:129J background as described (Bale et al., 2000). Male WT and CRF2−/− littermates (14-17 wks) bred from heterozygous crosses were group housed under a 12 h light, 12 h dark cycle (lights on at 0600 h), with food and water available ad libitum. WT and CRF2−/− mice were randomly assigned to control (CTL; N = 7-9 per genotype) or chronic variable stress (CVS; N = 8-9 per genotype) treatment groups. In order to determine if inflammation may be involved in the stress dysregulation found after exposure to chronic stress, an additional group received treatment with NSAID concomitant with CVS exposure (CVS + NSAID; N = 9 per genotype). All studies were performed in accordance with experimental protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Chronic Variable Stress

Chronic variable stress was comprised of seven mild stressors presented in a randomized order for 4 weeks beginning at 14-17 weeks of age. Stressors were specifically selected to not directly perturb thermogenic, metabolic, or pain pathways, as described (McEuen et al., 2008). Stressors included: overnight wet cage bedding (damp but without standing water), 15-min acute restraint, cage change (3 x in a single day), overnight exposure to novel objects (eight glass marbles) in cages, overnight novel noise using a white noise
generator (Brookstone), 15-min predator odor exposure (fox odor, Acros Organics, NJ; diluted 1:10,000), and 36-h constant light exposure. The HPA stress axis assessment was administered to all mice and replaced the final stressor. Mice were sacrificed 48 h following the HPA axis assessment, and brains were processed for immunofluorescence analysis of GFAP.

**Non-Steroidal Anti-inflammatory Drug (NSAID) Treatment**

Beginning one week prior to the onset of chronic variable stress and throughout the course of the study, CVS + NSAID WT and CRF2⁻/⁻ mice received acetylsalicylic acid (CVS Pharmacy) at 162 mg/L in their drinking water (available *ad lib*), resulting in a dosage of approximately 480 µg/mouse/day (Amateau and McCarthy, 2004). Acetylsalicylic acid has been shown to remain stable in water, by fluorescent polarization assay, for up to 48 h. Therefore, water (with or without acetylsalicylic acid) was replaced every 48 h (Bulckaen et al., 2008).

**Hypothalamic-pituitary-adrenal (HPA) axis assessment**

To examine the effects of chronic variable stress exposure and NSAID treatment on the HPA axis stress response, plasma corticosterone levels were determined following a 15-min restraint stress in male WT and CRF2⁻/⁻ mice (N = 7-9 per genotype/treatment group). Testing was administered between 0800–1100 h on the final day of CVS by placing mice in a 50 ml conical tube containing a 50 mm air hole. Tail blood samples (10 µL) were taken at onset and completion of restraint (0 and 15 min, respectively). To examine the stress recovery phase, additional samples were collected at 15 min and 75
min following the culmination of restraint (30 and 90 min, respectively). Samples were collected into 5 µL of 50 mM EDTA, centrifuged, and stored at -80°C until analysis. Plasma corticosterone concentration was measured in duplicate using a ¹²⁵I-corticosterone radioimmunoassay kit (MP Biomedicals, Orangeburg, NY). The minimum detection limit of the assay was 7.7 ng/ml and intra-assay coefficient of variation was 7.3%.

**Immunofluorescence and analyses**

Whole brains were removed from WT and CRF2⁻/⁻ mice (N = 4) and frozen on dry ice 48 h after the HPA axis assessment test. Brains were stored at -80°C until cryostat sectioning. Frozen tissue was cut on a cryostat into 10 µm coronal sections containing the medial prefrontal cortex (Bregma 1.78 to 1.42 mm), paraventricular nucleus of the hypothalamus (Bregma -0.58 to -0.94 mm), dorsal hippocampus (Bregma -1.46 to -1.70 mm), and ventral hippocampus (Bregma -2.80 to -3.16 mm). Every third section was thaw-mounted, air-dried on charged slides (Colorfrost® Plus, Fisher Scientific), and stored at -80°C. Sections were identified and anatomically matched using the Paxinos and Franklin mouse atlas (Paxinos and Franklin, 2003). Immunofluorescence for the astrocyte-specific cytoskeletal protein, GFAP, was performed on 3 sections per animal per brain region. Slide-mounted fresh frozen sections were fixed for 10 min in ice cold acetone, washed 3 times in PBS, and then incubated in 3% normal goat serum and 0.25% Triton X 100 in phosphate buffered saline (NGS-PBST) to block and permeabilize, respectively. Sections were incubated overnight in rat anti-GFAP polyclonal antibody (1:250; Invitrogen, Carlsbad, CA, cat. 130300) in NGS-PBST. Sections were then
washed and incubated for 1 h in goat anti-rat Alexa 568 fluorescent secondary antibody (1:500; Rockland Immunochemicals, Gilbertsville, PA; cat A-11077) in NGS-PBST. Slides were mounted with ProLong gold anti-fade reagent containing DAPI to stain nuclei (Molecular Probes/Invitrogen, Carlsbad, CA), and then cured at room temperature overnight before image acquisition. Control sections were processed in parallel omitting either the primary or secondary antibodies.

Regions of interest were captured using a 10 bit cooled QICam digital camera (QImaging) affixed to a Nikon Eclipse fluorescent microscope at 20X magnification (medial prefrontal cortex, hippocampus), or 10X (paraventricular nucleus of the hypothalamus). Slides from each brain region were captured at a uniform exposure time; however, baseline differences in GFAP between brain regions necessitated different exposure times for different brain regions in order to remain within linear dynamic range for semi-quantification. Semi-quantitative fluorescence measurements were made within a defined region of interest to yield a mean intensity value, using IPLabs for Macintosh software (BD Biosciences/Scanalytics).

**Serum TNFα**

Trunk blood was collected, mixed with 15 µL of 50 mM EDTA, and centrifuged at 5000 rpm for 15 min. Plasma was collected and frozen at -80 C. Plasma TNFα levels were quantified by sandwich enzyme linked immunosorbant assay (R&D, Minneapolis, MN) according the manufacturer’s instructions. The minimum detection limit of the assay was 7.21 pg/ml and intra-assay coefficient of variation was 3.8%.
**Statistical Analyses: Corticosterone Radioimmunoassay**

Corticosterone data were analyzed using a repeated-measures MANOVA (genotype x treatment x time) with time as a within-subjects repeated measure. To assess magnitude of the stress response, area under the curve (AUC) was calculated separately for stress response (0 min – 30 min) and recovery (30 min – 90 min) phases. This approach yielded an integration of peak response with duration of stressor. A 2-way ANOVA was performed on AUC of the response phase and of the recovery phase. Main effects and interactions were further explored with Tukey honestly significantly difference (HSD) *post hoc* test. Differences were identified at p < 0.05. Statistical analyses were performed with R software (R Foundation for Statistical Computing, Vienna, Austria). Data are reported as mean ± standard error of the mean.

**Statistical Analyses: Immunofluorescence**

In each brain region, the intensity of GFAP immunofluorescence within a defined region of interest was measured in 3 sections per animal. Models that included all possible combinations of predictor variables (genotype, treatment, animal) and interaction terms were fit to mean fluorescence intensity data and the best-fit model was determined according to Akaike’s Information Criterion (AIC). The best-fit model across each brain region was a linear mixed model with different intercepts for each mouse. Confidence intervals that did not bound zero were considered to hold a measurable effect. Statistical analyses were performed with R software (R Foundation for Statistical Computing, Vienna, Austria). P values were calculated using the pvalues.fnc package. Data were fit to the mixed models using lme4 package. Reported values are based upon parameter
estimates of the best-fit model. Error bars represent the observed standard deviation of the respective groups.

RESULTS

HPA axis responses to acute restraint stress

In order to assess HPA axis stress responsivity, corticosterone levels were measured following a 15-min restraint stress (Fig.1). A repeated measures MANOVA of corticosterone levels in response to an acute restraint stress revealed a main effect of genotype (CRF2/−/− > WT) [F_{(1,45)} = 5.654; p = 0.02]. There was a significant interaction between genotype and treatment [F_{(2,45)} = 3.273; p = 0.048]. Post hoc analysis revealed this effect to be driven by augmented corticosterone secretion by CVS-treated CRF2/−/− mice. NSAID treatment concomitant with CVS exposure prevented the augmented rise in corticosterone secretion. A 2-way ANOVA of the area under the curve (AUC) of the rise (0 min – 30 min) in corticosterone production revealed a main effect of genotype (CRF2/−/− > WT) [F_{(1,45)} = 6.869; p = 0.012] and treatment (CVS > Control; CVS > CVS + NSAID) [F_{(2,45)} = 4.166; p = 0.023]. Analysis of AUC during the recovery phase (30 min – 90 min) revealed effects of genotype (CRF2/−/− > WT) [F_{(1,45)} = 3.898; p = 0.056] and treatment (CVS > Control; CVS > CVS + NSAID) [F_{(2,45)} = 2.657; p = 0.083] that did not reach statistical significance. As per our a priori objective, AUC of corticosterone production during the recovery phase were compared between CVS and CVS + NSAID treatment groups. No significant effect was observed in WT mice; however, NSAID treatment significantly attenuated the augmented corticosterone response of CVS-exposed CRF2/−/− mice.
Glial fibrillary acidic protein (GFAP) immunofluorescence

Paraventricular nucleus of the hypothalamus (PVN): Immunofluorescence of GFAP was performed on sections from the caudal and rostral PVN (Fig. 2). In the caudal PVN, there was a significant interaction \(T = -2.185, p = 0.033\), whereby NSAID treatment decreased GFAP in CRF2\(^{-/-}\) mice and increased GFAP in WT mice. GFAP did not significantly differ between genotype or treatment groups in the rostral PVN.

Hippocampus: Immunofluorescence of GFAP was performed on sections from the dorsal and ventral CA1, CA3, and dentate gyrus of the hippocampus (Fig. 3). In both the CA1 and CA3 subregions of the ventral hippocampus there was a significant interaction \(T = 2.138 - 2.16, p = 0.034 - 0.037\), whereby CVS increased GFAP selectively in CRF2\(^{-/-}\) mice. The CVS-induced elevation in GFAP protein was prevented with concomitant NSAID treatment. No differences in GFAP were observed in WT animals across any treatment group in the ventral CA1, CA3, or dentate gyrus. GFAP did not significantly differ between genotype or treatment groups in any of the subregions of the dorsal hippocampus.

Medial prefrontal cortex: Immunofluorescence of GFAP was performed on sections from the medial prefrontal cortex (Fig. 4). There was a significant effect \(T = 2.048, p = 0.045\), whereby CVS increased GFAP selectively in CRF2\(^{-/-}\) mice. The CVS-induced elevation in GFAP protein was prevented with concomitant NSAID treatment. No differences in GFAP were observed in WT animals across any treatment group.

Serum TNF\(\alpha\)

All samples were at the minimal level of detection, between 0.056 pg/mL and 0.128
pg/mL (data not shown). No significant differences in TNFα were found between genotypes \( [F_{(1, 38)} = 0.02; p = 0.89] \) or prior exposure to chronic variable stress \( [F_{(2, 38)} = 0.16; p = 0.85] \).

**DISCUSSION**

The aim of the present study was to test the hypothesis that an underlying component of elevated stress responsivity involves an engagement of neuroimmune responses during chronic stress exposure. We propose that the ability to detect such changes requires an appropriately stress-sensitive model. Using CRF2\(^{-/-}\) mice as a model of stress dysregulation, we uncovered several novel findings: 1) NSAID treatment ameliorated augmented HPA axis corticosterone production characteristic of CRF2\(^{-/-}\) mice exposed to chronic stress; 2) In CRF2\(^{-/-}\) mice, GFAP-positive astrocytes increased with chronic stress exposure in stress coordinating brain regions, paralleling changes in HPA axis corticosterone; 3) NSAID treatment prevented stress-mediated changes in GFAP-positive astrocytes.

HPA stress axis activity is a physiological indicator of organismal stress state; exposure to stress ultimately results in the production of the primary stress hormone, corticosterone (Whitnall, 1993). Prior exposure to stress can modify HPA axis activity, augmenting or blunting responsivity to novel stressors (Pfister and King, 1976; Bhatnagar and Dallman, 1998). While it is necessary to maintain the ability to respond to a novel stressor, overactive HPA axis activity can be detrimental. We previously reported that exposure to chronic stress in CRF2\(^{-/-}\) mice augmented HPA stress axis corticosterone levels, evidencing their heightened stress sensitivity (McEuen et al., 2008). In our current
study, NSAID treatment during chronic stress completely ameliorated this effect in CRF2−/− mice. These findings suggest an underlying neuroimmune involvement in the dysregulated stress response, and may suggest a mechanism by which NSAID treatment may be efficacious in affective disorder treatment (Muller et al., 2005; Brunello et al., 2006; Mendlewicz et al., 2006; Muller et al., 2006).

Astrocytes are a promising mediator of the neuroimmune consequence of stress exposure. In select environments, they can both produce and respond to neuroimmune effector molecules (reviewed in (Volterra and Meldolesi, 2005)). While numerous end-feet leave them elegantly poised to respond to and integrate signals within neuronal networks, perturbations to astrocytes have the potential for widespread impact, including changes in extracellular ionic homeostasis, local neurotransmitter regulation, and remodeling of neural circuits (Theodosis and Poulain, 1999; Ullian et al., 2004; Allen and Barres, 2005; Haydon and Carmignoto, 2006; Schwarz and Bilbo, 2012). Indeed, alterations in astrocyte structural morphology or function have been associated with neuropsychiatric illnesses, including depression and schizophrenia (Rajkowska et al., 1999; Bowley et al., 2002; Toro et al., 2006). Thus, altered astrocyte-mediated plasticity may explain why CRF2−/− mice are unable to mount an adaptive coping response to stress exposure (McEuen et al., 2008). Therefore, we examined GFAP immunoreactivity as a correlate of altered astrocyte function in the PVN, ventral hippocampus, and prefrontal cortex, stress-coordinating brain regions. Consistent with our hypothesis, GFAP immunoreactivity increased with chronic stress in CRF2−/− mice across these three brain regions. These stress-mediated changes were also prevented with concomitant NSAID treatment. Interestingly, GFAP immunoreactivity in the dorsal
hippocampus was unaffected by stress exposure. As astrocytes are widely heterogeneous in structure and function, and play distinct roles across different brain regions, these findings point to a specificity of astrocyte changes that may parallel the specifics of dysregulation (Kimelberg, 2004; Lee et al., 2006; Cahoy et al., 2008).

The PVN is a primary regulator of corticosterone production and is innervated by multiple limbic forebrain structures, including the prefrontal cortex and ventral hippocampus (Herman et al., 2005). These circuits play important roles in determining stressor intensity and in negative feedback (Jankord and Herman, 2008; Radley and Sawchenko, 2011). A surprising finding in the PVN was the remarkable increase in GFAP positive astrocytes in the WT mice exposed to chronic stress and concomitant NSAID treatment. While the explanation for this effect is not currently known, it demonstrates important genotypic differences in astrocyte plasticity following stress, and may suggest a unique interaction of stress and neuroimmune activation in the normal healthy brain that may be beneficial in stress coping (Munhoz et al., 2006).

CRF receptor-2 is expressed on peripheral immune cells (Lovenberg et al., 1995; Baigent and Lowry, 2000). Therefore, to ensure genotype effects detected here were not resultant from inflammatory cytokines produced in the periphery, we measured serum tumor necrosis factor (TNF)-α in these mice. Levels were below the minimal level of assay detection (7.7 pg/ml) for all animals. These findings are consistent with studies using similar models of chronic stress exposure that also failed to evoke a significant peripheral immune response in the absence of additional immune challenge (d'Audiffret et al., 2010; Farooq et al., 2012). These data do not preclude the possibility of peripheral inflammation at earlier time points or by other cytokines, but do suggest that changes in
neuroimmune modulation have arisen centrally rather than indirectly through activation of the peripheral immune system.

Neuroimmune effectors have been clearly documented to activate the stress response, and cytokine treatment can induce symptoms of depression (Tsagarakis et al., 1989; van der Meer et al., 1996). Taken together, this suggests a potential role for immune effectors in stress dysregulation and related phenotypes found in neuropsychiatric disease. However, results of several investigations examining the neuroimmune response to stress exposure are conflicting; while in some models stress drives increases in cytokine production, other models show fewer changes (Bartolomucci et al., 2003; Reyes et al., 2003; Deak et al., 2005; Hueston et al., 2011; You et al., 2011). Our studies indicate that these differences may be driven by the sensitivity of the model utilized. In support of a neuroimmune contribution to the dysregulated stress state, NSAID treatment prevented dysregulation of HPA stress axis corticosterone secretion in a model of stress sensitivity. Further, chronic stress resulted in upregulation of GFAP by astrocytes in the PVN, ventral hippocampus and prefrontal cortex of CRF2−/− mice. The current studies provide evidence that not only may NSAID treatment be beneficial in the treatment of disorders associated with dysfunctional HPA stress axis, but that it may do so through either direct or indirect modification of astrocytes in brain regions involved in stress regulation. The specificity of these effects to stress-sensitive animals highlights the importance of utilizing a model of stress dysregulation in the examination of factors that may translate to human disease.
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FIGURES AND LEGENDS

Figure 2.1 Anti-inflammatory treatment ameliorates chronic stress-induced corticosterone elevation in stress-sensitive CRF2⁻/⁻ mice. Corticosterone levels were examined following a 15 min restraint stress (shaded column) in (A) WT and (B) CRF2⁻/⁻ mice. Exposure to chronic variable stress (CVS) enhanced corticosterone production in CRF2⁻/⁻ mice, and this effect was ameliorated by non-steroidal anti-inflammatory drug (NSAID) treatment. (C) Corticosterone production during the rise phase (0 min - 30 min) was higher in CRF2⁻/⁻ mice, and was further enhanced during CVS exposure. Concomitant NSAID treatment during CVS rescued this outcome. (D) NSAID treatment reversed CVS-induced total AUC of corticosterone production in CRF2⁻/⁻ mice. Data are presented as mean values ± SEM. (N = 7-9; *, P < 0.05).
Figure 2.2 Anti-inflammatory treatment prevents chronic stress-induced increases in GFAP in the PVN of stress-sensitive CRF2\(^{−/−}\) mice. (A-F) Representative immunofluorescence images (10X magnification) of the astrocyte-specific cytoskeletal protein, GFAP (red), counterstained with DAPI (nuclei, blue) in the caudal PVN of (A-C) WT and (D-F) CRF2\(^{−/−}\) mice for (A, D) control, (B, E) following exposure to CVS, or (C, F) CVS + NSAID. Bar graphs illustrate semi-quantitative analysis of GFAP immunofluorescence. (G) No differences between groups were detected with CVS on GFAP in the rostral PVN. (H) In the caudal PVN, NSAID treatment increased GFAP in WT, but decreased GFAP in CRF2\(^{−/−}\) mice. Atlas images illustrating the brain sections analyzed for (I) rostral and (J) caudal PVN, adapted from the mouse atlas (Paxinos and Franklin, 2003). Data are presented as parameter estimates of the best-fit model + observed standard deviation of respective groups. (N = 3-4; *, P < 0.05).
Figure 2.3 Anti-inflammatory treatment prevents chronic stress-induced increases in GFAP in the ventral hippocampus of stress-sensitive CRF2−/− mice. (A, B) Brain
atlas images illustrating the brain regions analyzed for (A) ventral and (B) dorsal hippocampus, adapted from the mouse atlas (Paxinos and Franklin, 2003). Boxes highlight region corresponding to image acquisition. (C-H) Representative immunofluorescence images (10X magnification) of GFAP (red) counterstained with DAPI (nuclei, blue) in (C, F) CA1, (D, G) CA3, and (E, H) dentate gyrus of the ventral hippocampus of CVS exposed WT and CRF2⁻/⁻ mice. (I-K) Bar graphs illustrate semi-quantitative analysis of GFAP immunofluorescence. CVS increased GFAP in both the (I) CA1 and (J) CA3 subregions of the ventral hippocampus selectively in stress-sensitive CRF2⁻/⁻ mice. (K) No differences were observed in the dentate gyrus. (L-Q) Representative immunofluorescence images of the (L, O) CA1, (M, P) CA3, and (N, Q) dentate gyrus of the dorsal hippocampus of CVS treated WT and CRF2⁻/⁻ mice. There were no significant effects of genotype or treatment on GFAP levels in the (R) CA1, (S) CA3, or (T) dentate gyrus subregions of the dorsal hippocampus. Data are presented as parameter estimates of the best-fit model + observed standard deviation of respective groups. (N = 3-4; *, P < 0.05).
Figure 2.4 Anti-inflammatory treatment prevents chronic stress-induced increases in GFAP in the medial prefrontal cortex of stress-sensitive CRF2−/− mice. (A-F) Representative immunofluorescence images (20X magnification) of GFAP (red) counterstained with DAPI (nuclei, blue) in the medial prefrontal cortex (mPFC) of (A-C) WT and (D-F) CRF2−/− mice for (A, D) control, (B, E) following exposure to CVS, or (C, F) CVS + NSAID. (G) Bar graph illustrates semi-quantitative analysis of GFAP immunofluorescence showing significantly elevated levels in the mPFC following CVS in CRF2−/− mice. Concomitant treatment with NSAID rescued this effect. (H) Atlas image illustrating brain section used for analysis, adapted from the mouse atlas (Paxinos and Franklin, 2003). Data are presented as parameter estimates of the best-fit model + observed standard deviation of respective groups (N = 3-4; *, P < 0.05).
ADDITIONAL DATA

Figure 2.5 NSAID treatment rescues chronic stress-induced increase in cFos in the DR in response to an acute restraint stress. cFos immunohistochemistry was performed on DR brain sections taken from mice 90 minutes after an acute 15 minute restraint stress. Data are presented as mean values ± SEM (N = 4-6). *, P < 0.05 following 3-way ANOVA of genotype x CVS x NSAID.
Figure 2.6 NSAID treatment rescues chronic stress-induced reduction in TPH+ neurons in the DR. TPH immunoreactivity was assessed in the DR of mice after 28 days of chronic variable stress. Data are presented as mean values ± SEM (N = 4-6). *, $P < 0.05$ following 3-way ANOVA of genotype x CVS x NSAID.
CHAPTER 3: SEX DIFFERENCES IN CORTICOTROPIN-RELEASING FACTOR RECEPTOR-1 ACTION WITHIN THE DORSAL RAPHE NUCLEUS IN STRESS RESPONSIVITY

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ABSTRACT

Women are twice as likely as men to suffer from stress-related affective disorders. Corticotropin-releasing factor (CRF) is an important link between stress and mood, in part through its signaling in the serotonergic dorsal raphe (DR). Development of CRF receptor-1 (CRF\textsubscript{1}) antagonists has been a focus of numerous clinical trials, but has not yet been proven efficacious. We hypothesized that sex differences in CRF\textsubscript{1} modulation of DR circuits may be key determinants in predicting therapeutic responses and affective disorder vulnerability. Male and female mice received DR infusions of the CRF\textsubscript{1} antagonist, NBI 35695, or CRF, and were evaluated for stress responsivity. Sex differences in indices of neural activation (cFos) and co-localization of CRF\textsubscript{1} throughout the DR were examined. Whole-cell patch-clamp electrophysiology assessed sex differences in serotonin neuron membrane characteristics and responsivity to CRF. Males showed robust behavioral and HPA axis responses to DR infusion of NBI 35695 and CRF, whereas females were minimally responsive. Sex differences were also found for both CRF induced DR cFos and CRF\textsubscript{1} co-localization throughout the DR. Electrophysiologically, female serotonergic neurons showed blunted membrane excitability, and divergent IPSC responses to CRF application. These studies demonstrate convincing sex differences in CRF\textsubscript{1} activity in the DR, where blunted female responses to NBI 35695 and CRF suggest unique stress modulation of the DR. These sex differences may underlie affective disorder vulnerability and differential sensitivity to pharmacologic treatments developed to target the CRF system, thereby contributing to a current lack of CRF\textsubscript{1} antagonist efficacy in clinical trials.
INTRODUCTION

Stress-mediated affective disorders such as depression and anxiety show a marked sex disparity, affecting women at nearly twice the rate of men (Kessler et al., 1994; Kessler et al., 2005a). Corticotropin-releasing factor (CRF) represents an important link between stress and mood regulation (Vale et al., 1981; Sutton et al., 1982; Stenzel-Poore et al., 1994; Groenink et al., 2002). Studies have suggested that stress-induced elevations in CRF contribute to neuropsychiatric disease development through excessive activation of its type 1 receptor, CRFr1 (Britton et al., 1986; Skutella et al., 1998; Smith et al., 1998; Timpl et al., 1998; Contarino et al., 1999; Habib et al., 2000; Keck et al., 2003; Muller et al., 2003; Gehlert et al., 2005). Consequently, CRFr1 has received considerable attention as a novel pharmaceutical target for the treatment of stress-related affective disorders; GlaxoSmithKline, Pfizer, Neurocrine Biosciences, DuPont/Bristol-Myers Squibb, and others have developed CRFr1 small molecule antagonists toward this end (recently reviewed in (Paez-Pereda et al., 2011)). However, despite compelling results for antidepressant-like and anxiolytic-like effects of these drugs in pre-clinical studies in rodents and nonhuman primates (Deak et al., 1999; Spina et al., 2000; Harro et al., 2001; Griebel et al., 2002; Ducottet et al., 2003; Lelas et al., 2004; Nielsen et al., 2004; Overstreet et al., 2004; Jutkiewicz et al., 2005), none of the CRFr1 antagonists brought to clinical trial over the past decade have successfully completed a Phase III trial (reviewed in (Koob and Zorrilla, 2012)).

Considerable evidence supports the involvement of CRFr1 in stress modulation of the serotonergic (5-HTergic) dorsal raphe nucleus (DR) in regulation of mood and affect (Price et al., 1998; Valentino et al., 2001; Oshima et al., 2003; Hale and Lowry, 2011).
Robust sex differences exist across the stress-serotonin system, where females exhibit greater corticosterone and behavioral (anxiogenic) responses to acute selective 5-HT reuptake inhibitor (SSRI) treatment (Carlsson and Carlsson, 1988; Nishizawa et al., 1997; McEuen et al., 2009; Goel and Bale, 2010). A disruption in the ability of CRF to regulate 5-HT circuits during chronic stress is implicated in affective disorder pathophysiology (Bale et al., 2002; Lowry, 2002; McEuen et al., 2008; Wood et al., 2013). Thus, we hypothesized that sex differences in CRFr1 activation within the DR may contribute, in part, to an increased female predisposition to stress-induced affective disorders, and may underlie disparities between predicted outcomes from preclinical studies and those in clinical trials for CRFr1 antagonists.

METHODS

Subjects

A total of 268 adult male and female littermate mice were used for all experiments. Mice were maintained under a 12-hour light/dark cycle with ad libitum access to food and water. For behavioral experiments and electrophysiological studies, C57Bl/6:129S/J F1 hybrid were obtained from the Jackson Laboratory or bred in house. For CRFr1 colocalization studies, mice with fluorescent-labeled CRFr1 containing neurons were generated as previously described (Justice et al., 2008). Mice were implanted between ages 7 and 8 weeks, allowed to recovery for at least one week, and behaviorally tested in age-matched cohorts at age 8 to 20 weeks. Mice were singly housed following cannulation to prevent disturbance of the cannulae. For electrophysiological experiments, slices were obtained from mice at 9 to 13 weeks of age. To mimic the housing conditions
of behavioral studies, mice were individually housed for 7 to 12 days prior to recording. All studies were conducted in accordance with experimental protocols approved by the University of Pennsylvania Institutional Animal Use and Care Committee, and where applicable, by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

**Stereotaxic surgery and placement verification**

Mice were anesthetized using isofluorane and implanted with a 26-gauge guide cannula (Plastics One, Roanoke, VA) using a stereotaxic instrument (Kopf, Tujunga, CA) positioned 1 mm from the DR using the following coordinates (from brain surface): AP - 4.36 mm, ML +1.5 mm, DV –2.0 mm, angled 26 degrees (Takahashi et al., 2010). At the end of each study, mice were transcardially perfused and cannula placement was verified based on the termination point of the injector as estimated from the location of scar tissue in 50 μm sections through the DR. Mice with incorrect cannulae placement were dropped from the statistical analysis. Group sizes reported represent the final group size after subjects with incorrect placements were omitted.

**Drugs and microinfusion**

All drugs were reconstituted in distilled water, aliquotted, and frozen until the day of use. Fresh aliquots were dissolved in ACSF (artificial cerebrospinal fluid, Tocris) immediately prior to behavioral testing. NBI 35695 (Tocris), a highly selective CRFr1 antagonist, was used at 0.44 ng, 1000 times the Ki (Martinez et al., 2004). Ovine CRF (Sigma) was used because of its higher affinity for CRFr1 (Sutton et al., 1995). 1 ng and
50 ng doses were selected based on previous studies of DR infusion of this peptide to preferentially target CRFr1 (Lukkes et al., 2008; Bangasser et al., 2010). Drug in 0.25 µL ACSF was infused over 1 min through a microinjector attached to polyethylene tubing connected to a 10 µL Hamilton syringe on an infusion pump (KD Scientific, Holliston, MA). 0.50 µL drug or ACSF was perfused through the microinjector to ensure patency between injections.

**Hypothalamic-pituitary-adrenal axis assessment**

Testing was performed during a 4-h period beginning 1-h after lights-on. 10uL tail blood was collected immediately prior to DR infusion, and at 30, 45, 60, and 120 min post injection. Between the 30 and 45 min collections, mice were restrained in a 50 mL conical tube with a 5-mm air hole. Samples were collected into tubes containing 5 uL 50 mM EDTA, centrifuged, and stored at -80°C. Corticosterone concentration was determined using a ¹²⁵I radioimmunoassay kit (MP Biomedicals, Solon, OH). All details were followed according to manufacturer’s instructions except volume of sample and all solutions were halved.

**Behavioral testing**

Behavioral testing was performed on separate cohorts of mice 30 min after drug or ACSF infusion. The tail suspension test was carried out as described previously (Steru et al., 1985; Morgan and Bale, 2011). Mice were secured to a rod by adhesive tape placed ~1 cm from the tip of the tail, and suspended 50 cm from the bench-top in a visually isolated, sound-attenuated room. Testing was performed between 0900 and 1400 h.
Immobility during the 6-min testing period was hand scored using AnyMaze software (Stoelting, Wood Dale, IL). An animal was considered immobile when there was lack of all movement except breathing and whisker movement. Immobile time and latency to first immobility were determined. The Light-Dark Box was performed as previously described (Bale et al., 2000; McEuen et al., 2008). Briefly, light intensity was 5 lux in the dark compartment and 175 lux in the light compartment. Test duration was 10 min and was performed from 2000 to 0200 h (beginning one hour after lights-off). Anymaze software was used to analyze total light time, latency to enter dark, transitions, and distance travelled in the light compartment.

**cFos immunohistochemistry**

To assess CRF-induced neuronal activation in the DR, double labeling immunohistochemistry for cFos and TPH was performed on DR sections. Methods were similar to those described previously (Faulconbridge et al., 2008; Goel et al., 2011). 90 minutes following CRF or ACSF infusion (n=8-9), animals were perfused, and brains were post-fixed and stored in sucrose cryoprotectant. Brains were frozen and coronal sections (30 um) through the DR were cut using a rotary microtome equipped with a freezing stage. Free floating sections (every third section from each mouse) were washed in Tris-buffered saline (TBS), pH 7.4, and incubated for 15 min in TBS containing 0.3% H₂O₂ before being washed again and incubated overnight at room temperature in rabbit anti-cFos (1:5,000; Calbiochem, San Diego, CA) diluted in TBS containing 0.2% Triton X-100 and 3% normal donkey serum. Sections then were washed in TBS and incubated for 2 hr with biotinylated donkey anti-rabbit IgG (1:1000; Jackson ImmunoResearch,
West Grove, PA). After a brief wash with TBS, sections were incubated for 1 hr in an avidin–biotin–peroxidase complex (1:333; Elite kit; Vector Laboratories, Burlingame, CA). Sections were washed again with TBS and then with 50 mM Tris, pH 7.4, before cFos-immunoreactivity was visualized by incubation for 5 min with 3,3-diaminobenzidine (0.2 mg/ml) and 0.025% H2O2 in 50 mM Tris with 25mg/ml nickel sulfate. The reaction was stopped with TBS washes, after which the sections were incubated in H2O2 to quench remaining peroxidase activity, washed briefly and incubated overnight at room temperature in mouse anti-TPH (Sigma). Sections then were washed before being incubated for 2 hr with biotinylated donkey anti-mouse IgG (1:1000; Jackson ImmunoResearch). Sections were washed and incubated in avidin-biotin-peroxidase complex (1:333; Vector Laboratories) before being washed and reacted with 3,3-diaminobenzidine (0.1 mg/ml) and 0.025% H2O2 in 50 mM Tris for 5 min. The protocol was developed to produce dark, blue-black nuclear cFos-immunoreactivity and light brown somatic TPH-immunoreactivity. Sections were floated onto Superfrost plus slides (Fisher, Pittsburgh, PA), dehydrated with increasing concentrations of alcohol followed by Hemo-De (Fisher Scientific, Pittsburgh, PA), and coverslipped with Permount (Fisher Scientific).

**Gene expression analysis**

Brains were collected from experimentally naive adult male and female mice (n=5 per sex), frozen on dry ice, and stored at -80C. Female brains were all collected in diestrus. Brain sections (300 µm) corresponding to figures 66-70 of the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2004) were made on a cryostat, and DR
micropunches (0.75 µm) were made using a hollow needle (Ted Pella, Redding, CA) and collected directly into TRIzol (Invitrogen, Carlsbad, CA) and stored at -80°C until RNA isolation. RNA was isolated, and cDNA was transcribed as described previously (McEuen et al., 2008). Gene expression of CRFr1 (CRFr1; NM_007762.4, Mm00432670_m1) CRF-R2 (Crfr2; NM_009953.3, Mm00438303_m1), CRF binding protein (Crhbp; NM_198408.3, Mm01283832_m1), TPH2 (Tph2; NM_173391.3, Mm00557715_m1), GABA receptor subunits alpha-2 (Gabra2; NM_008066.3, Mm00433435_m1), delta (Gabrd; NM_008072.2, Mm01266203_m1), and gamma-2 (Gabrg2; NM_008073.2, Mm00433489_m1) were determined by quantitative real-time PCR using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) (for probe information, see Supplementary Methods). Samples were run in triplicate for the target gene and for GAPDH as the endogenous control on the same 96-well plate using the Applied Biosystems 7500 fast real-time PCR system. For each sample, the cycle threshold (CT) value for GAPDH was subtracted from the target threshold value and converted to fold change by calculating $2^{-\Delta \text{CT}}$. For each gene, reported values are normalized to the average male value.

**Immunofluorescence and CRFr1 localization**

To determine whether sex differences exist in the localization of CRFr1 on 5-HTergic or GABAergic neurons within the DR, dual immunofluorescence was performed on paraformaldehyde-fixed male and female CRFr1-GFP DR. (Sztainberg et al., 2011). Parvalbumin (PV) was used to detect a subset of GABAergic neurons in the DR, as it has previously been shown to colocalize with most GABAergic neurons in this brain region.
(Shikanai et al., 2012). TPH, a precursor of 5-HT synthesis, was used as a marker of 5-HT neurons. Regions of interest were captured at 20x magnification for quantification. Representative confocal images were acquired at 40x magnification. Four serial sets of DR sections (25 µm) were collected into cryoprotectant (30% sucrose/30% glycerol in PBS) and kept at -20°C prior to immunofluorescent processing. One set was dual-labeled for GFP and parvalbumin, and a second for GFP and TPH following standard free-floating immunofluorescent protocol. Both sets were stained concurrently following the same procedure and using the same solutions when applicable. All incubations were done using 12 well plates (Fisher Scientific, Pittsburgh, PA) with mesh inserts (Ted Pella, Redding, CA) on a lab rotator at room temperature unless otherwise specified. Pretreatment included a 30 minute incubation in 0.1M glycine, followed by two 1x PBS rinses, and 10 minutes in 0.03% SDS. Blocking was done using 4% normal goat or normal donkey serum (Jackson ImmunoResearch Laboratory, West Grove, PA), for GFP/parvalbumin and GFP/TPH staining respectively, in 0.3% Triton X-100 in 1xPBS (0.3% PBST) for 1 hour. Rabbit anti-GFP Alexa Fluor® 488-conjugated antibody (1:500; Invitrogen, Carlsbad, CA) was was applied to tissue overnight at 4°C to enhance the GFP signal, and sections were simultaneously incubated with either guinea pig anti-parvalbumin (1:500; Synaptic Systems, Germany) or sheep anti-TPH (1:500; Millipore, Temecula, CA). Protein interaction was visualized by incubating sections for 1 hour in goat anti-guinea pig or donkey anti-sheep Alexa Fluor® 568 (1:200; Invitrogen, Carlsbad, CA). Primary and secondary antibodies were diluted in their appropriate blocking solution. Following secondary incubation, sections were rinsed in 1x PBS three times,, mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA),
and coverslipped using ProLong Gold Antifade Reagent containing 4,6-diamino-2-phenylindole (DAPI) to stain nuclei (Invitrogen, Carlsbad, CA). Slides were allowed to cure overnight prior to image acquisition.

GFP-immunoreactive (-ir) cells were quantified from six sections per animal from each set of tissue stained. Analysis was conducted on 8-bit grayscale microphotographs. The number of GFP-ir cells was measured with the aid of a CellProfiler pipeline (Lamprecht et al., 2007; Jones et al., 2008; Vokes and Carpenter, 2008). Cell Profiler classified objects were visually confirmed by investigator. The total count of GFP-ir cells in each region was averaged between the parvalbumin and TPH sets to produce a single value/region/animal. Co-localization counts were performed manually with the aid of Fiji software. GFP-ir cells were outlined, and those outlines were superimposed atop either parvalbumin or TPH stained images. A cell was considered colocalized if the boundary demarcating a GFP-ir cell clearly outlined a parvalbumin+ or TPH+ cell as well.

**Electrophysiology**

Immediately upon removal from their home cage, mice underwent cervical dislocation followed by decapitation, and the head was placed in a slush of ice-cold artificial cerebrospinal fluid (ACSF) in which sucrose (248 mM) was substituted for NaCl. This solution was bubbled with 95% O₂/5% CO₂, and brains were removed while remaining submerged in the solution and trimmed to isolate the brainstem region. Slices (200 µm) were cut throughout the rostro-caudal extent of the DR using a vibratome (Vibratome 3000 Plus), and immediately placed in a holding vial containing ACSF with l-tryptophan (50 µM) at 35°C bubbled with 95% O₂/5% CO₂ for 1 h. Thereafter, slices were
maintained in room temperature ACSF bubbled with 95% O$_2$/5% CO$_2$. The composition of the ACSF was as follows: 124 NaCl mM, 2.5 mM KCl, 2 mM NaH$_2$PO$_4$, 2.5 mM CaCl$_2$, 2 mM MgSO$_4$, 10 mM dextrose, and 26 mM NaHCO$_3$.

**Electrophysiology: recording**

Slices were transferred to a recording chamber (Warner Instruments) and continuously perfused with ACSF at 1.5–2.0 ml/min at 32–34°C maintained by an in-line solution heater (TC-324; Warner Instruments). One cell was recorded per brain slice. Raphe neurons were visualized using a Zeiss microscope fitted with a 40x water-immersion objective, differential interference contrast and infrared filter (Optical Apparatus). The image from the microscope was enhanced using a CCD camera and displayed on a television monitor. Whole-cell recording pipettes were fashioned on a P-97 micropipette puller (Sutter Instrument) using borosilicate glass capillary tubing (1.2 mm outer diameter, 0.69 mm inner diameter; Warner Instruments). The resistance of the electrodes was 4 – 8 MΩ when filled with an intracellular solution of the following (in mM): 70 K-gluconate, 70 KCl, 2 NaCl, 4 EGTA, 10 HEPES, 4 MgATP, 0.3 Na$_2$GTP, 0.1% biocytin, pH 7.3. Spontaneous inhibitory post-synaptic potential (IPSC) recordings were made in cells located in the dorsomedial subdivision of the DR at the midcaudal level, which contains dense clusters of 5-HT neurons, and wherein we observed sex differences in cFos in response to CRF infusion. A visualized cell was approached with the electrode, a GΩ seal established, and the cell membrane ruptured to obtain a whole-cell recording using a Multiclamp 700B patch-clamp amplifier (Molecular Devices). Series resistance was monitored throughout the experiment. If the series resistance was unstable or
exceeded four times the electrode resistance, the cell was discarded. Once the whole-cell recording was obtained, IPSCs were recorded in voltage-clamp mode (Vm = -70 mV). Cells were allowed to stabilize and solution dialyze for approximately five minutes while a stable baseline was achieved with >-50pA holding current. Signals were digitized by a Digidata 1440 series analog-to-digital converter and stored on-line using pClamp 10 software (Molecular Devices). Signals were filtered at 1 kHz and digitized at 10 kHz. The liquid junction potential was ~9 mV between the pipette solution and the ACSF and was not subtracted from the data obtained.

**Electrophysiology: drug administration protocol**

GABAergic IPSCs were isolated by addition of the non-NMDA receptor antagonist 6, 7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) (20 µM). After a baseline recording of spontaneous IPSCs (sIPSCs), GABAergic IPSCs were recorded for 10 min. Ovine CRF (30 nM) was then added to the perfusion bath and recorded for 15 min.

**Electrophysiology: immunohistochemical identification of 5-HT neurons following recording**

Standard immunofluorescence procedures were used to visualize the filled cell and tryptophan hydroxylase (TPH) content to identify 5-HTergic cells. Following recording, slices were postfixed in 4% paraformaldehyde at 4°C until staining. Sections were then blocked for 30 min in phosphate buffered saline (PBS) containing 0.5% Triton and bovine serum albumin, and incubated in mouse α-tryptophan hydroxylase antibody (1:500; Sigma-Aldrich) overnight at 4°C. Sections were then incubated in FITC-
conjugated donkey α–mouse secondary antiserum (1:200; Jackson ImmunoResearch) for TPH, and streptavidin-conjugated Alexa 647 (1:200; Invitrogen) for biocytin. Both were applied for 60 min at room temperature. Between incubations, slices were rinsed in PBS. Sections were mounted with ProLong Antifade (Invitrogen) onto charged slides (Superfrost Plus, Fisher Scientific). Images were captured with a Leica DMIRE2 confocal microscope (Leica Microsystems, Exton, PA).

**Electrophysiology: analysis**

Membrane characteristics were analyzed using Clampfit as previously demonstrated (Crawford et al., 2010; Calizo et al., 2011). Characteristics measured included resting membrane potential, membrane resistance, time constant (tau), action potential threshold, amplitude and duration, and the afterhyperpolarization (AHP) amplitude. MiniAnalysis software (Synaptosoft) was used to analyze mIPSC events on the basis of amplitude, duration, and area. Noise analysis was conducted for each cell and amplitude detection thresholds set to exceed noise values. Events were automatically selected, analyzed for double peaks, and then individually confirmed by visual inspection. Event amplitude histograms were compared with the noise histogram to ensure they did not overlap. Synaptic activity was analyzed for frequency, amplitude, baseline holding current, rise time (calculated from 10 to 90% of peak amplitude), and decay time (calculated by averaging 200 randomly selected events and fitting a double-exponential function from 10 to 90% of the decay phase). The double-exponential function for the decay phase generates an initial fast component and a subsequent slow component of the decay phase. Data were collected in 1 min bins during the 15 minute period after drug application,
taking into account a 2 min lag time from drug addition to initial drug effect attributable to recording chamber volume and perfusion rate. The postdrug steady-state value (200 events or 120 sec, whichever was smaller) was reported as the drug effect for each cell, typically occurring 9-12 min after drug application.

**Data analysis and statistics**

Total corticosterone was analyzed by multivariate ANOVA (drug x time). Behavioral measures were analyzed by two-way ANOVA (sex x drug). Subsequent one-way analyses were performed on data within sex, with Dunnett’s test used to identify significant post-hoc comparisons. Student’s t-test was used to compare gene expression between males and females. To determine CRFr1 counts, a generalized linear mixed model was employed to analyze GFP count x sex x subregion using a Poisson distribution. Assuming a binomial distribution, further analyses were made to predict the likelihood that a given GFP-immunoreactive (-ir) cell co-expressed parvalbumin or TPH. Data are reported as estimated effect size ± 95% confidence intervals. Significance was determined as p < 0.05, with 95% confidence intervals not bounding 0. Statistics were performed in R software. For electrophysiological studies, results of 5-HTergic neuron response to CRF were compared between males and females via two-way rmANOVA and post hoc Tukey tests employing sex and drug (DNQX vs. DNQX + CRF) as the independent variables. Statistics were performed using JMP8 (SAS) software; data are reported as mean ± SEM.
RESULTS

**DR infusions of NBI 35695 or CRF preferentially alter male corticosterone production**

5-HT output from the DR has modulatory activity on the HPA axis (Lowry, 2002; Goel and Bale, 2010). CRF regulation of DR neurons could therefore influence HPA responsiveness. Thus, we assessed the effect of CRFr1 antagonism within the DR on the corticosterone response to restraint stress (Fig. 1). NBI significantly blunted corticosterone levels in males ($F_{(1,9)} = 7.085, p = 0.026$). The effect of NBI was manifested as a reduction in the rise time from 0 to 30 min prior to restraint ($t_{(9)} = 3.191, p = 0.011$) and a reduction in total corticosterone produced throughout the course of the experiment (area under the curve, AUC) ($t_{(9)} = 2.794, p = 0.021$). In females, NBI did not significantly impact corticosterone production ($F_{(1,10)} = 0.1180, p = 0.7383$). We next tested the effect of CRF infusion on the HPA axis. In males, CRF significantly increased corticosterone ($F_{(1,17)} = 5.926, p = 0.026$). In females, CRF did not significantly affect corticosterone ($F_{(1,18)} = 1.28, p = 0.27$).

**Male-specific effects of DR infusion of NBI 35695 and CRF on stress coping and anxiety-like behaviors**

To assess the role of DR CRFr1 in modulating stress coping behavior of male and female mice, the TST was performed 30 min following infusion of NBI 35695 or vehicle (ACSF) (Fig 2). We detected a significant interaction of sex and NBI on latency to become immobile ($F_{(1,37)} = 6.654, p = 0.014$), where NBI increased latency in males ($p = 0.015$), but not in females ($p = 0.37$). There was a trend towards an interaction effect of NBI on immobility in the TST, where NBI reduced immobility in males and increased
immobility in females, though this effect failed to reach statistical significance ($F_{(1,40)} = 2.651, p = 0.11$).

The observed sex difference in response to the CRFr1 antagonist suggested potential sex differences in the response to CRF. To address this possibility, we next assessed the effect of two doses (1 ng, 50 ng) of CRF infusion on behavior in the TST. The 1 ng dose of CRF was ineffective to change immobility or latency to become immobile on either test, suggesting ineffective local concentrations were achieved. The 50 ng dose of CRF decreased immobility ($F_{(2,26)} = 3.467, p = 0.046$) and increased latency to become immobile ($F_{(2,26)} = 8.684, p = 0.001$) in males, and was without effect in females.

To assess anxiety-like behavior, mice were tested in the LD. NBI had no effect in either sex on any parameter. However, as with behavioral outcomes in the TST, where 1 ng dose CRF was without effect, 50 ng dose CRF had sex-specific effects on behavior, increasing latency to exit the light compartment ($F_{(2,26)} = 5.313, p = 0.011$), and total time spent in the light compartment ($F_{(2,26)} = 5.024, p = 0.014$) in males but not females. In males, 50 ng CRF also reduced the number of transitions between compartments ($F_{(2,26)} = 4.915, p = 0.016$), but did not affect distance traveled in the light (normalized to time spent in the light), indicating that the animals did not freeze while in the light compartment.

**DR infusion of CRF increases cFos in males but decreases cFos in females**

To determine if sex differences in behavioral responsiveness to CRF in the DR were associated with differential patterns of neuronal activation, we next assessed cFos
immunoreactivity following infusion of CRF or ACSF (Fig 3, Table 1). In males, CRF increased the number of cFos-positive cells across the DR ($F_{(1,65)} = 14.79$, $p < 0.001$), whereas in females, CRF reduced the number of cFos-positive cells ($F_{(1,56)} = 7.563$, $p = 0.008$). Subregion analysis indicated that this interaction was present in dorsomedial ($F_{(1,17)} = 6.216$, $p = 0.023$), ventromedial ($F_{(1,19)} = 5.590$, $p = 0.029$), and lateral wing subregions of the DR (rostral, $F_{(1,24)} = 7.300$, $p = 0.013$, caudal, $F_{(1,21)} = 7.359$, $p = 0.013$). CRF had a similar effect of increasing cFos-positive cells in both male and female rostral DR ($F_{(1,21)} = 12.38$, $p = 0.002$), and no significant main effects were found in the caudal DR.

**CRFr1 is expressed differentially throughout subdivisions of the DR in males and females**

We next assessed whether the observed sex differences in responsiveness the CRFr1 antagonist or CRF were due to differences in transcript levels of genes relevant to CRF and 5-HT signaling. We quantified the relative expression of CRFr1, CRFr2, and CRF-binding protein mRNA in DR micropunches from experimentally-naïve male and female mice (Fig 4a). We observed no difference in the mRNA levels of any of these relevant transcripts. As we predict that some of our observed behavioral and physiological differences may be GABA-mediated, we also quantified the relative expression of GABA receptor subunits alpha-1, alpha-2, delta, and gamma-2, which play important roles in receptor kinetics. We observed no sex difference in mRNA in any of these receptor subunits. As has been previously described, we detected differences in TPH2, with females expressing 1.62-fold higher levels relative to males ($t_{(1,8)} = 2.652$, $p = 0.029$)
After finding no differences in CRFr1 gene expression, we hypothesized that sex differences in responsivity to CRFr1 antagonist or CRF may be due to differences in the neurotransmitter cell type expressing CRFr1. As current available antibodies are unable to distinguish between CRFr1 and CRFr2, we utilized a CRFr1-GFP transgenic mouse in which GFP is transcribed under the control of the CRFr1 promoter to identify CRFr1 positive neurons in the DR (Fig 4b-n) (Justice et al., 2008). Sections throughout the DR from these mice were dual labeled for either GFP and parvalbumin to identify putative GABAergic neurons expressing CRFr1, or GFP and TPH to identify serotonergic neurons expressing CRFr1. In accordance with our CRFr1 mRNA data, there were no sex differences in overall number of GFP-ir cells (-0.542 ± -1.59, +0.053; p=0.0796). However, we observed a sex difference in co-localization of GFP-ir cells throughout regions of the DR. In females, the probability that a given GFP-ir cell co-expressed parvalbumin was lower than in males (-2.535 ± -4.819, -0.259; p=0.0291). The probability that a given GFP-ir cell co-expressed TPH was less than 25% in all subdivisions, regardless of sex, except within the rDR, where males displayed significantly more co-localization with TPH than females (-2.16 ± -3.736, -0.583; p=0.007).

**5-HT neurons in females demonstrate reduced excitability and a blunted response to CRF**

To investigate physiological differences in DR 5-HT neurons of males and females, whole-cell electrophysiological recordings were conducted using current-clamp and
voltage-clamp techniques (Fig 5, Table 2). Data from a total of 21 neurons, using current-clamp recordings, were analyzed (9 from 6 male mice, 12 from 7 female mice). Cellular characteristics that were measured included resting membrane potential, resistance, time constant (tau), after hyperpolarization, and action potential amplitude, width, and threshold. Characterization of male and female 5-HT neurons revealed females to have a significantly depolarized resting membrane potential \(t_{(13)} = 2.498, p = 0.027\) and a reduced tau \(t_{(12)} = 2.225, p = 0.046\) relative to males. No other membrane characteristics differed between males and females. However, a non-linear regression on the frequency-current (F-I) plot of male and female 5-HT neurons revealed male neurons to have a greater excitability (slope) compared to females \(F_{(1,158)} = 8.929, p = 0.003\), firing at 27.28 Hz vs. 14.83 Hz, to a maximal injected current of 160 pA.

Voltage-clamp recordings of GABAergic IPSCs, isolated by the addition of DNQX, revealed several functional differences between male and female 5-HT neurons. In response to the addition of CRF (30 nM), males and females showed differential changes in the initial first decay time \(t_{(10)} = 2.673, p = 0.023\), with males showing an increase \(+2.148 \pm 1.376\) and females showing a decrease \(-2.380 \pm 1.045\) compared to DNQX alone. Males and females also exhibited a divergent CRF-mediated change in rise time \(t_{(11)} = 2.802, p = 0.0187\), and half width \(t_{(11)} = 5.994, p < 0.0001\); in both measures males showed an increase, while females showed a decrease.

**DISCUSSION**

Stress-mediated affective disorders display significant sex differences in incidence and treatment efficacy (Kessler et al., 1994; Khan et al., 2005). CRFr1 is a key
mediator of neuroendocrine and behavioral stress responses, in part through signaling in the 5-HTergic DR (Price et al., 1998; Valentino et al., 2001; Oshima et al., 2003; Hale and Lowry, 2011). As there are known sex differences in the DR, dysregulation of 5-HTergic signaling may contribute to increased disease risk in females (Dominguez et al., 2003; Felton and Auerbach, 2004). Development of CRFr1 small molecule antagonists has been a major focus in clinical trials for more than a decade, but these compounds have been largely unsuccessful. While preclinical studies have predominantly been conducted in males, the majority of clinical trials have either focused on female patient populations, or have been underpowered to evaluate gender differences in study outcomes (Table 1). Therefore, we hypothesized that sex differences in CRFr1 regulation of DR circuits are a key determinant in affective disorder vulnerability, and may be important in predicting therapeutic outcomes.

In our studies, male and female mice received an infusion of the CRFr1 small molecule antagonist, NBI 35695, or one of two doses of CRF directly into the DR, and were evaluated for changes in physiological and behavioral stress responsiveness. NBI 35695 in the DR significantly blunted corticosterone levels in response to a restraint stress only in males. Similarly, CRF infused into the DR in the absence of restraint significantly elevated corticosterone production above the levels induced by intracranial injection only in males (Kim et al., 1998; Goel and Bale, 2010). The 5-HT system is a known activator of the HPA axis, where selective SSRIs and 5-HT agonists increase corticosterone production during and independent of stress (Heisler et al., 2007). While direct innervation of the PVN has been reported (Petrov et al., 1992, 1994; Williamson and Viau, 2007), 5-HTergic fibers from the DR also heavily innervate the GABAergic
neurons of the PVN-surround (Sawchenko et al., 1983). Therefore, CRF-mediated changes in 5-HT output could modulate this axis through a disinhibition of medial parvocellular neurons. We have previously demonstrated sex differences in this pathway, with females showing a greater corticosterone response to peripheral SSRI administration compared to males (Goel and Bale, 2010; Goel et al., 2011). Thus, a male-specific response to administration of NBI 35695 and CRF directly into the DR suggests a unique sex-specific mechanism upstream of the PVN, including potential differences in CRFr1 co-localization or signaling within the DR.

In our assessment of behavioral stress coping strategies, including the TST, only males again showed a significant effect of NBI 35695 to increase the latency to immobility, and CRF infusion to reduce the immobile time. While NBI 35695 infusion produced no significant changes in male or female mice in the LD, CRF at the higher dose again increased time spent in the light and escape latency only in males. These findings are consistent with behavioral effects reported in previous studies for systemically administered CRFr1 antagonists and CRFr1 gene deletion, implicating the DR as a key brain region mediating these outcomes (Smith et al., 1998; Timpl et al., 1998). Interestingly, in our current studies, both CRF and NBI 35695 infusions produced similar effects in the TST. It may be that interactions with CRFr2, which is also found in the DR, and/or alternate DR projections may account for differences in this behavior (Hammack et al., 2003; McEuen et al., 2008; Neufeld-Cohen et al., 2012). As the DR has a heterogeneous cell population involved in the complex regulation of 5-HT neurotransmission throughout the brain, the localization of CRF receptors on different cell types and their recruitment likely determines specificity in 5-HT release (Valentino et
al., 2001; Hale and Lowry, 2011). Similar paradoxical findings have been reported for other CNS receptor systems involved in complex behaviors (Dragatsis et al., 1995; Alleweireldt et al., 2002). In support of our hypothesis, these data demonstrate striking sex differences in stress responses to DR infusion of a CRF1 antagonist or CRF.

To test whether sex differences in neuronal activation by CRF may underlie sex differences we detected in physiological and behavioral stress responses, we analyzed numbers of cFos positive cells following infusion of the behaviorally effective 50 ng dose of CRF into the DR. No significant sex differences in basal cFos staining following vehicle infusion were detected, suggesting similar basal cellular activation in the DR. However, CRF infusion into the DR resulted in a dramatic increase in the number of cFos positive cells in males, and a paradoxical reduction in females. This lack of an appreciable effect of CRF infusion in females supports the specificity of CRF administration to the DR with minimal diffusion into the cerebral aqueduct, as the latter would be expected to augment corticosterone irrespective of sex (Dunn and Berridge, 1990; Goel and Bale, 2010). These sex differences were most apparent in the dorsomedial and lateral wings of the DR, regions previously implicated in uncontrollable stress and anxiety (Abrams et al., 2004; Abrams et al., 2005; Hale and Lowry, 2011). Immunohistochemical evidence supports a dense CRF innervation of these DR subregions, where CRF fibers primarily contact GABA-containing dendrites (Lowry et al., 2000; Valentino et al., 2001; Waselus et al., 2005). Thus, differences in the number of CRF-responsive GABAergic neurons in females could account for the observed reduction in activation detected in these mice.

Within the DR, there is a well-described topographical organization, where
GABAergic interneurons exhibit tight control over the tonic and activity-mediated release of 5-HT (Valentino et al., 2001; Allers and Sharp, 2003; Hale and Lowry, 2011). To test the hypothesis that divergent sex responses to CRF application within the DR may be due to differences in CRFr1 localization on functionally distinct neuronal populations, we used a CRFr1-driven GFP transgenic reporter mouse line to quantify sex differences in co-expression of CRFr1 (Justice et al., 2008). As the available antibodies for the CRF receptors are known to be of poor quality and lack sufficient specificity, this reporter mouse provided an excellent tool to identify CRFr1-positive neurons in the DR. We quantified sex differences in co-expression of GFP with TPH, a marker of 5-HTergic neurons, or with parvalbumin, a marker of a subset of GABAergic neurons. Parvalbumin was used to mark a GABAergic neurons as 87% of GAD67-ir neurons in the DR co-express parvalbumin (Shikanai et al., 2012). These neurons may display some functional differences compared to the broader population of GABAergic neurons, but were selected based on reliable somal immunoreactivity for co-expression analysis with CRFr1 (McKenna et al., 2013). Overall numbers of CRFr1 neurons did not differ between males and females, as indicated by similar numbers of GFP-positive neurons in the DR. This was also confirmed by similar expression levels of CRFr1 mRNA in the DR of males and females. As expected, few GFP-positive neurons were co-expressed with TPH, consistent with previous reports that demonstrate CRF primarily acts on GABAergic neurons, and that CRFr1 shows little expression overlap with 5-HT neurons (Kirby et al., 2008). Surprisingly, females had reduced GFP co-labeling with parvalbumin compared to males. This outcome supports a revised model; rather than females demonstrating greater CRFr1-mediated GABA tone, sex differences in CRF-induced neuronal activation may
be due to differences in CRFr1 intracellular signaling, trafficking, or receptor kinetics (Bangasser, 2013). Such sex differences in CRF signaling have recently been demonstrated in the locus coeruleus (Bangasser et al., 2010; Valentino et al., 2013). Alternatively, CRF may be activating a population of parvalbumin-negative GABA neurons in the female DR.

Based on our observed sex differences in CRFr1 co-expression, we hypothesized that DR 5-HT neurons in males and females may receive differential GABA input in response to CRF. To examine this, we used whole-cell electrophysiological recordings to measure GABAergic IPSCs in 5-HT neurons before and after application of CRF. We observed a striking sex difference in CRF responsivity, where CRF increased IPSC decay time in males, but decreased decay time in females. IPSC decay time can correlate with the number of activated axonal inputs during the IPSC, suggesting sex differences in presynaptic GABAergic input onto 5-HT neurons (Balakrishnan et al., 2009). This is consistent with the differential co-expression of CRFr1 on parvalbumin neurons between males and females. Further, the reduced IPSC half-width in response to CRF exhibited by females also supports this sex difference in the number of GABAergic release sites onto 5-HT neurons. These differences in IPSC kinetics constitute a significant functional difference between males and females that could alter 5-HT neuron excitability and neurotransmission, and thereby influence stress physiological and behavioral measures.

In gathering basal characteristics prior to assessing sex differences in 5-HTergic neuron responses to CRF, we uncovered an unexpected sex difference in 5-HT neuronal excitability. Using whole-cell patch clamp recordings from 5-HT neurons from male and female dmDR slices, we found reduced neuronal excitability in females in response to a
series of current injections. In addition to sex differences in CRFr1 localization and GABAergic responses to CRF, this suggests that 5-HT neurons in females require a greater depolarizing stimulus to generate neuronal firing and subsequent 5-HT release, even at baseline. Compared to males, this may translate to 5-HTergic hypofunction in females, an underlying risk factor for the development of affective disorders during stress exposure (Van de Kar, 1989; Kapitany et al., 1999).

Overall, these studies revealed intriguing sex differences in behavioral and physiological effects of CRFr1 antagonist and CRF in the DR, which were mechanistically associated with sex differences in receptor co-expression and divergent electrophysiological responses to CRF in 5-HTergic neurons. The blunted response of females points to a potential explanation for the lack of efficacy in CRFr1 antagonists in clinical trials, which have focused primarily on female participants due to their increased disease prevalence (Table 1). These findings support the importance of identifying sex differences in central stress pathways in order to understand the heightened predisposition of females toward these disorders, and in identifying sex-appropriate, and potentially sex-specific, pharmacological treatments.

ACKNOWLEDGEMENTS

This study was supported by National Institutes of Health Grant MH073030. We gratefully acknowledge the assistance of Drs. B. A. Rood and C. L. Howerton for their consultation in electrophysiological experiments and data analysis, respectively.
FIGURES AND LEGENDS

Figure 3.1 DR infusion of the CRFr1 small molecule antagonist NBI 35965 or 50 ng CRF had male-specific effects on HPA responsiveness. (A,C) Time course of corticosterone response to a 15 min restraint (indicated by shaded region) in males (A) and females (C). (B,D) Area under the curve (AUC) analysis demonstrated a significant reduction of corticosterone output by NBI 35965 in males (B), but not in females (D). (E-H) Corticosterone response to infusion of 50 ng CRF infusion in males (E,F) and females (G,H). AUC analysis demonstrated that CRF enhancement of corticosterone release was specific to males (F). Data are presented as mean values ± SEM (n=8). *, P < 0.05 in comparison to ACSF.
Figure 3.2 Males and females show divergent behavioral responses to CRFr1 antagonism or CRF infusion into the DR. (A,B) Effects of CRFr1 antagonist NBI 35965 on total immobile time (A) and latency to first bout of immobility (B) in the tail suspension test (TST). Males showed a greater latency to immobility. (C,D) CRFr1 antagonism had no effect in the light-dark box (LD) on measures of total time spent in the light compartment (C) or latency to first exit from the light compartment (D). (E-H) Behavioral effects of 1 ng and 50 ng CRF infusion demonstrated no effect of the 1 ng dose, but male-specific effects of the 50 ng dose. 50 ng CRF infusion reduced immobile time (E) and increased latency to immobility (F) in males in the TST, and increased both total light time (G) and latency to exit the light (H) in males in the LD. Data are presented as mean values ± SEM (N= 8). *, P < 0.05 in comparison to ACSF.
Figure 3.3 A 50 ng CRF infusion elicited differential patterns of neuronal activation in males and females across DR subregions. (A-D) Representative dorsomedial (dmDR) sections show cFos induction 90 min following CRF infusion is enhanced in males (A,B), but reduced in females (C,D). (E) Fos-positive cell counts from DR subregions demonstrate increased counts in males, but reduced counts in females, particularly in dmDR and rostral and caudal lateral wing (rLW, cLW) subregions. Values represent the difference in mean number of Fos-positive cells in sections from mice administered CRF or ASCF. Data represent the difference in averages from 7-9 mice per group. *, $P < 0.05$. 

65
<table>
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<th>Region</th>
<th>Sex</th>
<th>ACSF  (50 ng)</th>
<th>CRF (50 ng)</th>
<th>Delta (CRF-ACSF)</th>
<th>Sex Effect</th>
<th>CRF Effect</th>
<th>Interaction</th>
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<td>.002</td>
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<td>0233</td>
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<tr>
<td>Ventromedial</td>
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<td>.2026</td>
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<td></td>
<td>Female (6.5)</td>
<td>51.500 ± 9.258</td>
<td>63.000 ± 11.000</td>
<td>11,5</td>
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<td>.1472</td>
<td>0125</td>
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<td></td>
<td>Female (6.8)</td>
<td>254.162 ± 38.640</td>
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<td>CLW</td>
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<td>.9231</td>
<td>013</td>
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<td></td>
<td>Female (4.7)</td>
<td>204.750 ± 52.744</td>
<td>119.143 ± 25.842</td>
<td>−86,607</td>
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Sample sizes vary because some sections were excluded from data analysis due to poor tissue quality.

ACSF: artificial cerebrospinal fluid; CLW: caudal lateral wing; CRF: corticotropin-releasing factor; RLW: rostral lateral wing.

Table 3.1 Quantification of dorsal raphe cFos response to ACSF or CRF infusion
Figure 3.4 CRFr1 is expressed on different cell populations in males and females. (A) Expression of genes relevant to CRF and serotonergic signaling in the dorsal raphe. There were no sex differences in mRNA levels of CRFr1, CRFr2, CRF-binding protein.
(CRF-BP), GABA receptor (GABR) subunits alpha-1 (α1), α2, delta (δ), or gamma-2 (γ2); however TPH2 was significantly higher in females. Data are presented as fold change relative to the mean value in males ± SEM (n=5). *, P < 0.05 compared to males. (B) Regional, but no sex differences in number of GFP-ir cells throughout subregions of the DR. (C, D) Brain atlas diagrams depict the region viewed in the representative images of (C) the rostral lateral wings (rLW) and (D) the dorsomedial (dm) DR. (E-L) Representative, split channel confocal images from the rLW (E, G) and the dmDR (I, K) highlight these regional differences in GFP expression. Sections were dual-labeled with anti-parvalbumin (PV), (F, G) and anti-tryptophan hydroxylase (TPH) (J, K) to identify the phenotype of GFP+ cells in the DR. Arrows denote location of GFP-ir cells enlarged to illustrate co-localization (single arrow, GFP only; double arrow, co-localized). Examples of GFP-ir cell bodies with (left) or without (right) co-localization of PV (H) and TPH (L) show that GFP is transcribed by a subset of both types. (M, N) Probability that a given GFP-ir cell colocalizes with PV (M) or TPH (N). Females presented with an overall reduction in the probability that a given GFP-ir cell colocalizes with PV (N), and reduced likelihood that GFP-ir cells within the rostral DR (rDR) colocalize with TPH. Data are presented as an average probability ± SEM. *, P < 0.05 in comparison to male.
Figure 3.5 Males and females exhibit differences in baseline characteristics, and divergent responses to bath applied CRF. (A) Representative IPSC trace. (B-C) Scaled IPSC averaged from a single male (B) and female (C) illustrating the effect of DNQX and CRF on half-width. Insets (B’,C’) illustrate sex difference in effect of CRF on rise time. (D-F) CRF increases rise (D), half-width (E) and decay time (F) in males, and decreases these measures in females. (G) Males exhibit increased excitability compared to females to a series of current injections. Values represent the difference in response of CRF – DNQX. (N = 7-9). *, P < 0.05.
Table 3.2 Active and passive cell characteristics of 5-HT neurons

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<tr>
<th>Sex (n)</th>
<th>$V_{\text{m rest}}$ (mV)</th>
<th>$R$ (mW)</th>
<th>Decay (msec)</th>
<th>AP Threshold (mV)</th>
<th>AP Amp (mV)</th>
<th>AHP Amp (mV)</th>
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</thead>
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<tr>
<td>M (7–9)</td>
<td>$-68.61 \pm 3.60$</td>
<td>$-663.01 \pm 102.1$</td>
<td>$28.18 \pm 3.93$</td>
<td>$-33.57 \pm 1.75$</td>
<td>$53.67 \pm 5.59$</td>
<td>$26.80 \pm 2.82$</td>
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<tr>
<td>F (5–10)</td>
<td>$-54.78 \pm 3.50$</td>
<td>$-629.89 \pm 78.89$</td>
<td>$31.33 \pm 2.95$</td>
<td>$-35.02 \pm 1.81$</td>
<td>$60.97 \pm 7.40$</td>
<td>$25.71 \pm 2.55$</td>
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### Active Cell Characteristics

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<th>Amplitude (pA)</th>
<th>Frequency (Hz)</th>
<th>AUC (pA)</th>
<th>Rise (msec)</th>
<th>Half-Width (msec)</th>
<th>Decay (msec)</th>
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<td>$2.92 \pm .28$</td>
<td>$1.73 \pm .19$</td>
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<td>DNQX</td>
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<td>$7.03 \pm .51$</td>
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<td>Delta (CRF-DNQX)</td>
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<td>Female</td>
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<td>$-.16 \pm .10$</td>
<td>$-.14 \pm .11$</td>
<td>$-.14 \pm .15$</td>
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AHP, after hyperpolarization; AP, action potential; AUC, area under the curve; CRF, corticotropin-releasing factor; DNQX, 6,7-dinitroquinoxaline-2,3-dione.

$^a$Significant sex differences on the basis of $t$ test.
Table 3. Clinical Trials Targeting CRF1 for Treatment of Major Depression and Anxiety Disorders

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<tr>
<th>Compound</th>
<th>Trial Title</th>
<th>Indication</th>
<th>Sex</th>
<th>Total (M/F)</th>
<th>Phase</th>
<th>Status</th>
<th>Results</th>
<th>Identifier</th>
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<td>NBI-34041*</td>
<td>Elevated stress response</td>
<td>Male</td>
<td>24 (20/4)</td>
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<td>Success</td>
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<td>BMS-552086 (Paxil)</td>
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<tr>
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<td>Phase 2/3</td>
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<td>Phase 2</td>
<td>Completed</td>
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<td>GSK561179 (Eli Lilly)</td>
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CRF1, corticotropin-releasing hormone receptor 1; F, female; M, male; MDD, major depressive disorder; SAD, social anxiety disorder.

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CHAPTER 4: DORSAL RAPHE NEUROINFLAMMATION PROMOTES DRAMATIC BEHAVIORAL STRESS DYSREGULATION

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ABSTRACT

Impulsivity, risk-taking behavior, and elevated stress responsivity are prominent symptoms of mania, a behavioral state common to schizophrenia and bipolar disorder. Though inflammatory processes activated within the brain are involved in the pathophysiology of both disorders, the specific mechanisms by which neuroinflammation drives manic behavior are not well understood. Serotonin cell bodies originating within the dorsal raphe (DR) play a major role in the regulation of behavioral features characteristic of mania. Therefore, we hypothesized that the link between neuroinflammation and manic behavior may be mediated by actions on serotonergic neurocircuitry. To examine this, we induced local neuroinflammation in the DR by viral delivery of Cre recombinase into interleukin (IL)-1βXAT transgenic male and female mice, resulting in overexpressing of the proinflammatory cytokine, IL-1β. For assertion of brain region specificity of these outcomes, the prefrontal cortex (PFC), as a downstream target of DR serotonergic projections, was also infused. Inflammation within the DR, but not the PFC, resulted in a profound display of manic-like behavior, characterized by increased stress-induced locomotion and responsivity, and reduced risk-aversion. Microarray analysis of the DR revealed a dramatic increase in immune-related genes, and dysregulation of genes important in GABAergic, glutamatergic, and serotonergic neurotransmission. Behavioral and physiological changes were driven by a loss of serotonergic neurons and reduced output as measured by HPLC, demonstrating inflammation-induced serotonergic hypofunction. Behavioral changes were rescued by acute SSRI treatment, supporting the hypothesis that serotonin dysregulation stemming from neuroinflammation in the DR underlies manic-like behaviors.
INTRODUCTION

Schizophrenia, bipolar disorder, and other psychotic illnesses commonly co-occur with immune dysfunction, where patients exhibit altered cytokine profiles, and postmortem tissue indicates neuroimmunological abnormalities (Muller et al., 2009; Modabbernia et al., 2013). Brain-wide neuroinflammation can impact behavior, including those that are altered in disease. Chronic brain infection with the parasite *Toxoplasma gondii*, for example, has a profound ability to increase impulsivity, as well as the incidence of schizophrenia and suicide, even following parasite clearance (Alvarado-Esquivel et al., 2013; Ingram et al., 2013; Webster et al., 2013). Furthermore, there is significant co-presentation of primary immunological disorders with psychiatric symptoms. Notably, patients with synaptic autoimmune encephalitis are commonly first treated following presentation with manic behavior (Rosenfeld and Dalmau, 2011), and the neuropsychiatric manifestations of multiple sclerosis, lupus, and rheumatoid arthritis include irritability, mood swings, distractibility, and impulsivity (Kwentus et al., 1986; Bruce, 2008; Meszaros et al., 2012). Recent studies have highlighted the important role of neuroinflammation as a contributing factor in neuropsychiatric disease [reviewed in (Najjar et al., 2013)].

Animal models have clearly demonstrated the ability of proinflammatory cytokines, such as interleukin (IL)-1β, interferon-gamma, tumor necrosis factor-α, and lipopolysaccharide to modulate arousal and coping behaviors when administered peripherally (Dantzer et al., 2008). These same molecules alter discrete aspects of behavior when delivered intracerebroventricularly, or acutely into specific brain regions, suggesting that access to and action on particular brain areas may be central to their
effects (Song et al., 2006; Huang et al., 2008; Barrientos et al., 2012; Hayley et al., 2013). Brain regions adjacent to sources of cerebrospinal fluid (CSF), which carries circulating immune cells, may be particularly susceptible to the effects of inflammation (de Vries et al., 1997).

The serotonergic dorsal raphe (DR) is located in apposition to the cerebral aqueduct, making it a potentially vulnerable site for inflammatory insult. Serotonin plays a central role in the modulation of arousal, impulsivity, hedonic, and hyper-aggressive behaviors, as well as stress responsivity, all of which are central manifestations of mania (Shiah and Yatham, 2000; Crockett et al., 2010). In vitro, neuroinflammatory signaling molecules, including IL-1β, alter serotonergic neuron excitability and neurotransmitter release (Manfridi et al., 2003; Zhu et al., 2006). Clinical studies of CSF, platelet assessments, and neuroendocrine challenge, and postmortem tissue provide evidence to support the hypothesis that serotonin deficiency is involved in mania, and that enhancement of serotonin neurotransmission may exert a mood-stabilizing effect (Shiah and Yatham, 2000). Largely due to the challenge of experimental induction of a relevant inflammatory trigger, however, the mechanism and anatomical specificity by which this occurs remains unknown.

Here, we used an IL-1βXAT model to induce neuroinflammation with temporal and brain region specificity (Shaftel et al., 2007a; Shaftel et al., 2007b; Hein et al., 2010). This model has the advantages of sustained IL-1β overexpression, allowing continuous cytokine production, and of regional specificity, as release in neuronal projection regions or sites of microglial trafficking is prevented through astrocytic-specific production.
Using this approach, we tested the hypothesis that neuroinflammation in the serotonergic DR can drive manic-like behavior.

**METHODS**

*Animals*

The construction and characterization of the IL-1β<sup>XAT</sup> line has been described previously (Shaftel et al., 2007a,b). In our studies, IL-1β<sup>XAT</sup> male mice on a C57BL/6 background were bred with 129S/J females to create F1 hybrid mice carrying the IL-1β<sup>XAT</sup> transgene. Justification for using a mixed background strain in these studies is related to stress responsive phenotypes and physiology. C57Bl/6 are extremely low stress responders making them a less desirable choice for stress studies. 129S/J mice are more robust stress responders, but frequently lack a fully formed corpus collusum and can be poor performers in behavioral tests. However, the combination of these two strains produces a hybrid vigor that serves stress research well with consistent responses and behavioral outcomes (McEuen et al., 2008; McEuen et al., 2009; Gerber and Bale, 2012). Both WT and transgenic littermates were used in initial behavioral studies. Mechanistic follow-up experiments were conducted in transgenic mice only. Because we observed no sex differences in behavior or gene expression, immunofluorescence of the DR was conducted on brain sections from male mice only. Mice were maintained under a 12-hour light/dark cycle with *ad libitum* access to food and water. All studies were conducted in accordance with experimental protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee.
Viruses and Stereotaxic Surgery

Adeno-associated virus (AAV) vectors were produced and purchased from the University of Pennsylvania Vector Core (Philadelphia, PA) and injected into 8-10 week old WT or IL-1βXAT transgenic littermates. Two viruses were used in this study: AAV2/5 containing a transgene for green fluorescent protein (AAV-GFP; AAV2.5.CMV.GFP.WPRE.SV40) and AAV2/5 containing transgenes for both GFP and Cre recombinase (AAV-Cre; AAV2.5.CMV.GFP.Cre.WPRE.SV40). AAV2/5 was used for its ability transduce glial cells (Koerber et al., 2009), which we confirmed in comparison to AAV2/1 and AAV2/9 in pilot studies. Correct infusion placement was verified by visualization of GFP in 300 μM sections containing the DR. For viral infusions, mice were anesthetized using isoflurane, before a 33-gauge needle attached to a 10 μL Hamilton syringe was positioned within the DR using a stereotaxic instrument (Kopf, Tujunga, CA), according to the following coordinates (from brain surface): AP -4.36 mm, ML + 1.5 mm, DV 3.625 mm, angled 26 degrees (Takahashi et al., 2010). 0.25uL of virus diluted to 1.0e10 GC with sterile artificial cerebrospinal fluid was administered over the course of 1 min. Based on pilot testing of the time course of viral gene expression, and to allow for adequate recovery time, behavioral testing began 5 weeks after. All males were infused within a 3-day time window, followed by all females, again within a 3-day time window, one week later.

Immunofluorescence

Six weeks after surgery, animals were perfused with ice-cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Whole brains were removed, post-fixed in 4%
paraformaldehyde overnight, and transferred to 30% sucrose solution for 48 h. Brains were then removed from solution and stored at -80°C until cryostat sectioning. Tissue was cut on a cryostat into 4 serial sets of 30 µm coronal sections containing the DR, and stored in cryoprotectant (30% sucrose, 30% glycerol, in PBS) at -20°C until staining. Sections were identified and anatomically matched using the Mouse Brain Atlas (Paxinos and Franklin, 2001). 1 set of DR sections from each animal was stained in each antibody. TPH-stained sections were first pre-treated with a 30 min incubation in 0.1M glycine followed by a 10 min incubation in 0.03% SDS. All sections were then washed 3 times in PBS, and incubated in 3% normal goat serum and 0.25% Triton-X 100 in phosphate buffered saline (NGS-PBST) to block and permeabilize, respectively. Sections were incubated overnight in the appropriate primary antibody (rat anti-GFAP at 1:250, Invitrogen, Carlsbad, CA, cat. 130300; sheep anti-TPH at 1:500, Millipore, Temecula, CA, cat. AB1541; mouse anti-NeuN at 1:500, Chemicon, Billerica, MA cat MAB377B) in NGS-PBST. Sections were then washed and incubated for 1 h in goat anti-rat Alexa 568 fluorescent secondary antibody (1:500; Rockland Immunochemicals, Gilbertsville, PA, cat. A-11077) in NGS-PBST. Slides were mounted with ProLong gold anti-fade reagent containing DAPI (Molecular Probes/Invitrogen, Carlsbad, CA) to stain nuclei, and then cured at room temperature overnight before image acquisition. Control sections were processed in parallel omitting the primary antibodies. A separate cohort of animals was sacrificed without perfusion to better identify immunological markers, and brains were immediately frozen on dry ice and stored at -80°C until cryostat sectioning. These sections were cut at 10 µm directly onto slides, air dried overnight, and then post-fixed for 20 min in ice-cold acetone. Sections were processed for the immunological markers
rat anti-CD11b (M1/70, 1:500, eBiosciences, San Diego, CA, cat. 14-0112-81) and rat anti-IDO (1:500; BioLegend, San Diego, CA, cat. 122402) as above, omitting any pre-incubation steps.

Regions of interest were captured using a 10-bit cooled QICam digital camera (QImaging, Surrey, British Columbia) affixed to a Nikon Eclipse fluorescent microscope at 20X magnification. Slides for each antibody were captured at a uniform exposure time; however, baseline differences in immunofluorescence between antibodies necessitated different exposure times for different antibodies in order to remain within linear dynamic range for semi-quantification. Semi-quantitative fluorescence measurements were made within a defined region of interest to yield a mean intensity value, using IPLabs for Macintosh software (BD Biosciences/Scanalytics, San Jose, CA). Cell counts for TPH-ir cells were performed with the aid of CellProfiler, as previously described (Howerton et al., 2013).

**LacZ detection**

4 sets of four 30 µm sections were made from the DR of six animals, which were perfused 8 weeks following AAV-Cre infusion, and three animals following AAV-GFP infusion. One set of each was used to assess LacZ as an indicator of transgene expression (Shaftel et al., 2007b). Sections were collected into cryoprotectant (30% sucrose, 30% glycerol in PBS) and stored at 4°C until processing. Sections were transferred to PBS the day before LacZ staining, and washed on a rotator overnight. Sections were then transferred to a solution containing 0.1% X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), 2 mM MgCl₂, 5 mM potassium ferricyanide, and 5 mM potassium
ferrocyanide for 24 h at 37°C. Sections were washed twice in PBS, successively dehydrated in 75%, 95%, and 100% ethanol, and left in Citrisolv prior to coverslipping. Sections were mounted with Permount, and allowed to dry overnight before image acquisition. Images were captured using a 10-bit cooled QICam digital camera (QImaging, Surrey, British Columbia) at 4X and 40X. Sections were manually assessed for LacZ-positive cells through visualization of punctate blue staining.

**Behavioral testing**

Behavioral testing was performed beginning 5 weeks after surgery targeting the DR in the following order: elevated plus maze, light-dark box, hypothalamic-pituitary-adrenal (HPA) axis stress test. A separate cohort was used in the locobox. Behavioral testing on animals infused with virus into the prefrontal cortex were tested in the elevated plus maze and light-dark box followed by an HPA axis stress test. All behavioral testing was performed by an experimenter blinded to mouse genotype and treatment. Males and females were tested in separate cohorts (males, followed by females) to allow testing at equal days post-surgery, and to prevent the influence of female odor on male behavior. All behavioral data are therefore presented as within-sex comparisons.

**Elevated plus maze**

Mice were placed in the center of the maze facing an open arm at the beginning of the 5 min test. Light intensity in the open arms was 6 lux. All testing occurred 2-5 h after lights off. Distance traveled and time spent in each arm was analyzed by AnyMaze software. If a mouse fell off the open arm, the test was paused and the animal was placed back in the
center of the maze. If the animal fell off the maze more than once, this was noted and they were excluded from analysis.

**Light-dark box**

Mice were placed in the light compartment at the beginning of the 10 min test session. Light intensity was set at 175 lux in the light compartment. All testing occurred 2-5 h after lights off. Total distance traveled, time spent in the light compartment, and transitions between the light and dark compartments were analyzed with ANY-maze v4.75 software (Stoelting). Methods were similar to those described previously (McEuen et al., 2009).

**LocoBox**

The locobox consists of a two-compartment apparatus connected by a 6 cm throughway. Mice were placed in the center of the wide compartment (25 cm x 27 cm), built to the size of the light compartment of the light-dark box, facing the narrow entry at the beginning of the 10 min test. The narrow compartment (8 cm x 84.375 cm) lay adjacent to the wide compartment, giving equal floor space to traverse (675 cm²) but in a confined space, similar to the closed arm of the elevated plus maze. Light intensity in the wide compartment was set at 300 lux. This novel test was employed following testing on the light-dark box and elevated plus maze for several reasons. First, it was used as an additional measure of stress-induced locomotion. Additionally, as equal floor space was given in both the open and closed compartments, it prevented the potential confounding interpretation of choice of side preference (as in the light-dark box). Lastly, it tested the
possibility that the animals experienced a claustrophobic reaction to the closed compartment of the elevated plus maze, which would explain their preference for the open arm, rather than differences in risk-taking, general arousal, or anxiety-like behavior. All testing occurred 4-9 h after lights on. Distance traveled and time spent in each zone were recorded, tracked, and analyzed by AnyMaze software.

**Hypothalamic-pituitary-adrenal (HPA) axis assessment**

Testing was performed over a 4-h period beginning 1-h after lights-on. 10uL tail blood was collected at 0, 15, 30, and 90 min. Between 0 and 15 min time points, mice were restrained in a 50 mL conical tube with a 5-mm air hole. Corticosterone was determined by radioimmunoassay (MP Biomedicals, Orangeburg, NY) using 3 µL plasma with a variance coefficient of \( R^2 = 0.9985 \), as previously described (Gerber and Bale, 2012).

**Gene expression microarray**

Whole brains from behaviorally tested animals were cryosectioned at -20°C. Using a hollow needle (Ted Pella, Redding, CA), three 0.75 mm punches containing the DR from 300 µM slices were micropunched according to the Mouse Brain Atlas (Paxinos and Franklin, 2001). Punches were bath sonicated for 2.5 min (30 s on/off cycles) in TRIzol reagent (Invitrogen). RNA was isolated by RNeasy kit (Qiagen) and suspended in RNase-free water. Total RNA was sent to the University of Pennsylvania Path BioResources Molecular Profiling Core for Affimetrix GeneChip Mouse Gene 1.0 ST analysis.
Quantification of biogenic amines

Levels of monoamine neurotransmitters were determined using high performance liquid chromatography (HPLC) as described previously (Stein et al., 2006) with modifications. Animals were sacrificed by rapid cervical dislocation, and brains were dissected and immediately frozen on dry ice to be stored at -80°C until processing. Thereafter, eight 1 mm punches containing the medial prefrontal cortex or three 0.75 mm punches containing the DR from 300 µm slices were collected from AAV-GFP- and AAV-Cre-infused animals into homogenization buffer (22 mM sodium acetate, 75 mM glacial acetic acid, 0.05 mM EDTA, pH 4.95) containing 100 nM 3,4-dihydroxybenzylamine (DHBA) as an internal standard, and stored at -80°C. On the day of the HPLC analysis, samples were thawed on ice, sonicated in a bath sonicator (3 5-sec pulses) and centrifuged at 21,000 x g for 20 min at 0°C. 30 µL of tissue sample was combined with 5 µL of ascorbate oxidase (0.3 mg/ml), and 20 µl of this mixture was injected. Samples were injected onto a C18 reverse-phase column (ODS, 150 × 4.6 mm; 5 µm particles; Thermo Electron Corporation, Waltham, MA) by a Waters (Milford, MA) 717 Plus Autosampler, eluted using an isocratic system with a mobile phase (MD-TM; ESA, Bedford, MA) containing 1.0 mM 1-octanesulfonic acid, and pumped by a Waters 515 HPLC pump. Under these conditions, norepinephrine (NE), serotonin (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were clearly resolved. External standards ranging from 0 to 2 pM were interspersed within the run and the 2 pM standard was injected twice (first and last) to determine whether there had been any drift during the analysis. A Coulochem III electrochemical detector equipped with a 5011A analytical
cell (ESA) was used to detect the biogenic amines. Data were captured using the Empower 2 Software system (Waters).

**Drug treatment**

To examine the hypothesis that serotonergic hypofunction is a causal factor in the behavioral dysfunction observed following DR neuroinflammation, an acute dose of citalopram (30 mg/kg, i.p.; Abcam, Cambridge, United Kingdom), a selective serotonin reuptake inhibitor, was administered 32 min prior to testing on the Elevated Plus Maze. This dose and time course was selected based on the ability to acutely modulate serotonin levels and affect serotonin-mediated behaviors (Griebel et al., 1994; McEuen et al., 2009; Mombereau et al., 2010), while having no affect on locomotion (Umehara et al., 2013). Our choice of timing was slightly adjusted from the literature (32 minutes instead of 30 min prior to testing) to stagger mice appropriately, allowing for the testing of all animals of one sex to be performed within one test session. Drug was prepared fresh and distributed into syringes prior to the first injection.

**Data analysis and statistics**

Behavioral measures were analyzed within sex by two-way ANOVA (genotype x virus treatment), with Tukey’s test used to identify significant post-hoc comparisons. Total corticosterone was analyzed within sex by multivariate ANOVA (genotype x drug treatment x time). Statistics were performed using JMP8 (SAS) software; data are reported as mean ± SEM. Microarray data were modeled comparing AAV-GFP vs. AAV-Cre in accordance with behavioral and physiological data using R (version 2.14.2) and
the packages arm, bbmle, and limma to fit optical density data to linear models, and estimates for main and inter-action effects were determined from these models. Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional annotation clustering was used for determination of gene clusters that significantly differed between groups. The top five gene clusters are shown in the figure. Heat maps and hierarchical clustering were performed using MultiExperiment Viewer (TM4.org) with Pearson’s correlation. HPLC analysis was performed by t-test, comparing IL-1β infused with AAV-GFP vs. AAV-Cre. Repeated testing on the elevated plus maze during the citalopram rescue experiment was conducted within sex by two-way ANOVA (treatment x trial), with post-hoc testing comparing citalopram treatment to each of the saline treatments. Significance was set at p<0.05.

**RESULTS**

*Interleukin-1β overexpression within the dorsal raphe produces a local neuroinflammatory response*

We utilized an IL-1β<sup>XTAT</sup> mouse (generously provided by M.K. O’Banion, University of Rochester), where chronic IL-1β overexpression is inducible under the control of a glial fibrillary associated protein (GFAP) promoter upon delivery of Cre recombinase. This model allows both spatial and temporal control of IL-1β-mediated neuroinflammation. These mice have been previously described as displaying a robust, long-lasting neuroinflammatory response characterized by increased GFAP expression, microglial ramification, and MHCII expression (Shaftel et al., 2007a; Shaftel et al., 2007b). Here we show that with a small viral delivery (0.25uL), we are able to induce a
neuroinflammatory response that is contained within the DR (Fig. 1). Stereotaxic infusion of an adeno-associated virus (AAV) 2.5 containing Cre recombinase and GFP (AAV-Cre) into the DR of IL-1βXAT mice (Fig. 1A) led to spatially restricted LacZ expression (Fig. 1B), and GFP expression (Fig. 1C), indicating viral transduction within the DR. This resulted in a 1.7 log-fold increase in the IL-β transcript within the DR (Fig. 1D) ($F_{(1,24)} = 182.2, p < 0.0001$). Neither AAV-GFP infusion into IL-1βXAT mice nor AAV-Cre or AAV-GFP infusion into WT littermates resulted in detectable LacZ expression (data not shown). In AAV-Cre IL-1βXAT mice we observed robust microglial activation (Fig. 1E), as indicated by CD11b expression and ramified morphology of CD11b-ir cells. We also observed differences in neuronal morphology (NeuN) in AAV-GFP infused animals (Fig. 1F) compared to AAV-Cre infused animals (Fig. 1J), and profound glial activation, as indicated by robust GFAP and aquaporin-4 (AQP4) immunoreactivity in animals infused with AAV-Cre (Fig. 1K, L) compared to animals infused with AAV-GFP (Fig. 1G, H). Interestingly, we observed a notable nuclear localization of the tryptophan catabolic enzyme indoleamine 2,3-dioxygenase (IDO) expression in mice infused with AAV-Cre (Fig. 1M), whereas IDO was undetectable in AAV-GFP animals (Fig. 1I). Importantly, these DR changes were not associated with an increase in circulating IL-1β, as measured by enzyme-linked immunosorbant assay ($F_{(1,29)} = 0.4631, p = 0.5016$) (Fig. 1N).

*Raphe neuroinflammation promotes dysregulated stress behavior and physiology*

To test the hypothesis that the serotonergic DR is responsible for aspects of neuroinflammation-mediated behavioral dysfunction, animals were behaviorally tested on
the elevated plus maze, light-dark box, and locobox beginning 5 weeks after viral infusion (Fig. 2). In the elevated plus maze (Fig. 2A-F), we detected a significant interaction of genotype (WT vs. IL-1β) and virus (AAV-GFP vs. AAV-Cre), where both male (Fig. 2A-C) and female (Fig. 2D-F) IL-1β mice infused with AAV-Cre spent more time in the open arms ($F_{(1,30)} = 27.12, p < 0.0001$; $F_{(1,24)} = 23.08, p < 0.0001$) (Fig. 2B, E). In the males, there was a significant effect of genotype ($F_{(1,30)} = 5.439, p = 0.0266$) and virus ($F_{(1,30)} = 11.44, p = 0.002$) on total distance traveled, where IL-1β mice infused with AAV-Cre traveled a greater distance throughout the test than the other groups (Fig. 2A). In females this presented as a significant interaction ($F_{(1,24)} = 8.376, p = 0.008$) (Fig. 2D). Intriguingly, transgenic IL-1β mice infused with AAV-Cre spent a substantial amount of time at the distal ends of the open arms (males: $34.73 \pm 6.8$ sec, females: $48.40 \pm 15.981$ sec), whereas few of the WT mice or AAV-GFP infused mice ever entered the distal ends of the open arms (Fig. 2C, F). In the light-dark box (Fig. 2G-L), we detected a significant interaction effect of genotype and virus, where both male (Fig. 2G-I) and female (Fig. 2J-L) IL-1β mice infused with AAV-Cre traveled a greater distance when normalized to time spent in light compartment ($F_{(1,27)} = 7.525, p = 0.011$; $F_{(1,25)} = 13.49, p = 0.001$) (Fig. 2G, J) and had a faster speed of travel ($F_{(1,27)} = 7.607, p = 0.010$; $F_{(1,25)} = 12.99, p = 0.0014$) (Fig. 2H-N), and spent more time in the light compartment of the box (Fig. 2I, L).

We performed additional testing of novelty-induced locomotion on an additional cohort of IL-1β mice infused with AAV-GFP or AAV-Cre in the locobox (Fig. 2M-T). In this task, where animals were given equal floor space to traverse in an open or narrow compartment, we recapitulated genotype by treatment effect on locomotive arousal in
both sexes, where males (Fig. 2M-P) showed increased distance traveled ($t_{(1,8)} = 4.127$, $p = 0.003$) (Fig. 2M) and females (Fig. 2Q-T) showed the same pattern ($t_{(1,9)} = 1.909$, $p = 0.088$). Both males and females infused with AAV-Cre displayed increased speed ($t_{(1,8)} = 4.122$, $p = 0.003$; $t_{(1,9)} = 1.915$, $p = 0.089$), while showing no preference towards either the open or narrow compartments.

In our assessment of neuroendocrine stress responsivity, as measured by corticosterone production in response to an acute restraint stress, we found that male IL-1β mice infused with AAV-Cre produced significantly more corticosterone compared to any of the other control groups, displaying a significant interaction effect of genotype by treatment by time ($F_{(2,18)} = 8.1046$, $p = 0.0014$) (Fig. 3A). Females showed the expected higher corticosterone curve compared to males ($F_{(1,40)} = 71.345$, $p < 0.0001$), however there was no significant interaction of genotype by treatment by time to further increase their corticosterone levels ($F_{(2,18)} = 0.1243$, $p = 0.9445$) (Fig. 3B).

**Acute IL-1β infusion affects only locomotion**

We next assessed the impact of an acute IL-1β infusion within the DR to better elucidate the difference between the effects of acute IL-1β, and the consequences of chronic IL-1β-mediated neuroinflammation, the latter of which being more disease relevant. We found that an acute infusion of IL-1β directly into the DR increased locomotion (Fig. 4A), as measured by distance traveled throughout the elevated plus maze ($F_{(1,22)} = 4.649$, $p = 0.043$), but had no significant effect on time spent in the open arm (Fig. 4B) ($F_{(1,21)} = 0.7403$, $p = 0.340$). Further, acute IL-1β failed to elicit the risk-taking behavior of exploration onto the distal ends of the open arms where none of the animals entered the
distal ends during the test (Fig. 4C). Of note, all animals in this study had reduced locomotor activity compared to the animals in the transgenic study, likely due to the stress of intracranial infusion 30 min prior to behavioral testing, and as expected the magnitude of locomotor effects are much smaller than those seen with IL-1β<sup>XAT</sup> mice, and none of the mice in this study ventured into the distal ends of the open arms.

The behavioral consequences of neuroinflammation are brain region-specific

To assess whether the impact of neuroinflammation on behavior is brain region-specific, we performed parallel experiments to induce IL-1β overexpression in the prefrontal cortex, a region which receives dense serotonergic input, is involved in relevant behaviors, and is also implicated in bipolar disease and schizophrenia (Lewis, 2012; Price and Drevets, 2012; Mann, 2013). When we assessed the behavioral consequences of neuroinflammation in this brain region, we observed no significant effect on behavior or physiology (Fig. 4). Animals performed similarly in the elevated plus maze (Fig. 4A-C) and light-dark box (Fig. 4D-E), and exhibited no difference in corticosterone response to an acute restraint stress (Fig. 4F-G).

Changes in gene expression following raphe neuroinflammation

Given the observed behavioral results, we undertook an investigation of the molecular mechanisms that may lead to the increased manic behavior seen with DR neuroinflammation. We carried out gene expression profiling studies using microarray to evaluate global change in basal gene expression in the DR of males and females following AAV-GFP or AAV-Cre infusion two weeks after the culmination of behavioral
testing (Fig. 5). We detected robust changes in gene expression, with 4,873 genes showing significant differences, as determined by an FDR of 0.01 (Fig. 5A). Functional annotation clustering of these affected genes with a fold change of at least 1.1 revealed a significant enrichment for 177 gene clusters (Fig. 5B), which were primarily involved in inflammatory signaling. This analysis also identified enrichment for the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway for apoptosis, suggesting some degree of cell death in this brain region. 66 gene clusters were identified as significantly down-regulated (Fig. 5C), the top 20 of which were found to be involved in neurotransmission, ion transport, axon/dendrite morphology and function, and steroid biosynthesis. In order to identify transcriptional changes that would be most relevant to our hypothesis that neuroinflammation mediates behavioral changes via its effects on neurosignaling within the DR, we conducted a further sub-analysis on genes annotated with any gene ontology (GO) term containing “neur*” to identify genes specifically related to neuron/signaling. This analysis revealed a list of 26 genes corresponding primarily to serotonin, GABA, glutamate, and potassium signaling and transport (Fig. 5D).

**Dorsal raphe neuroinflammation results in serotonergic hypofunction**

The DR is home to the majority of serotonergic neurons, and we observed changes in behavior indicative of serotonergic dysfunction. Therefore we investigated the potential changes in the neurochemistry following IL-1β overexpression in a separate cohort of male mice (Fig. 6). We first quantified tryptophan hydroxylase-immunoreactive (TPH-ir) neurons within the DR following AAV-GFP or AAV-Cre infusion to assess whether
neuroinflammation has the potential to broadly change the population of serotonergic neurons. AAV-Cre infused animals had significantly fewer TPH-ir cells in the dorsomedial DR ($t_{(1,10)} = 4.787, p = 0.0007$) compared to AAV-GFP infused animals 8 weeks following viral infusion (Fig. 6A-C). No differences in TPH-ir were detected in the lateral wing subregions of the DR ($t_{(1,10)} = 0.434, p = 0.670$), indicating local specificity of these effects. To assess functional consequences, the concentration of norepinephrine (NE), dopamine (DA), and 5-HT and their major metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) were measured in the PFC and DR. Within the DR, there was a substantial reduction in NE ($t_{(1,12)} = 6.766, p < 0.0001$) (Fig. 6E), with no difference in DA ($t_{(1,12)} = 0.492, p = 0.632$) (Fig. 6F). In the PFC, a major DR output region, there was less 5-HT ($t_{(1,11)} = 2.232, p = 0.047$) (Fig. 6H), more 5-HIAA ($t_{(1,11)} = 2.748, p = 0.019$) (Fig. 7I), and greater turnover ($t_{(1,11)} = 6.368, p < 0.0001$) (Fig. 7J). No significant differences were found in NE or DA in the PFC (data not shown).

**Acute citalopram treatment ameliorates behavioral dysfunction following IL-1β-induced neuroinflammation in the DR**

To test the hypothesis that the observed behavioral dysfunction was the result of an inadequate pool of available 5-HT during the acute behavioral testing, we next assessed whether pharmacological elevation in 5-HT would rescue the behavioral phenotype caused by DR neuroinflammation. To do this, male and female mice were repeatedly tested on the elevated plus maze following i.p. injection of 30 mg/kg citalopram to acutely elevate 5-HT levels (Fig. 7). As observed in previous cohorts, saline-injected
AAV-Cre-infused animals had an increase in total distance traveled throughout the test \((F_{(1,7)} = 14.13, p = 0.0071)\) (Fig. 7A, C), and spent significantly more time exploring the open arms of the maze \((F_{(1,7)} = 26.87, p = 0.0013)\) (Fig. 7B, E) and the distal ends of the open arms \((F_{(1,7)} = 18.18, p = 0.0037)\) (Fig. 7C, F). Repeated measures ANOVA revealed a significant interaction effect of citalopram treatment to ameliorate open arm time \((F_{(2,14)} = 4.380, p = 0.0333)\), distance traveled \((F_{(2,14)} = 8.557, p = 0.0037)\), and end of open arm entries \((F_{(2,14)} = 5.168, p = 0.0186)\) in AAV-Cre-infused male IL-1β mice (Fig. 7A-C).

Females showed the same pattern in all measures, with significance reached in measures of open arm time \((F_{(2,12)} = 6.359, p = 0.0131)\) (Fig. 7D-F). Post-hoc testing in these parameters indicated significant difference of saline treatment (injection 1 and 3) from citalopram treatment (injection 2), indicating the citalopram effect was not one of repeated testing. IL-1β mice infused with AAV-GFP animals showed decreased exploration over repeated testing, as expected, with no effect of citalopram administration.

**DISCUSSION**

Impulsivity, risk-taking behavior, and elevated stress responsivity are prominent symptoms of mania, a state of hyper-arousal common to schizophrenia, bipolar disorder, and other psychotic illnesses. Epidemiological evidence suggests that inflammation within the brain plays a role in the pathophysiology of these diseases (Najjar et al., 2013). However, the mechanistic link between neuroinflammation and the development of manic-like behavior remains unknown, though likely involves serotonin, which plays a central role in modulating relevant behaviors. Through the use of an IL-1β\textsuperscript{XAT} transgenic
model of brain region-specific neuroinflammation, we tested the hypothesis that neuroinflammation within the serotonergic DR is able to recapitulate aspects of manic-like behavior.

The manic-like phenotypes that we observed following IL-1β-mediated neuroinflammation in the DR were striking, reminiscent of recently defined examples of manic-like behavior in mouse models (Han et al., 2013; Kirshenbaum et al., 2013; Saul et al., 2013). Across several behavioral paradigms, animals displayed increased novelty-induced locomotion and a disinhibited exploration of adverse environments (e.g., distal ends of the open arm in the elevated plus maze). These features, coupled with the observed increase in neuroendocrine stress responsivity, closely resemble the behavioral and physiological dysregulation observed in schizophrenia and bipolar disorder. Patients with schizophrenia, for example, exhibit emotional over-arousal to neutral social scenes (Haralanova et al., 2012), engage in risky sexual behavior associated with a higher incidence of sexually transmitted infections (Ramrakha et al., 2000), and present with exacerbated symptoms in the face of stress exposure (Jones and Fernyhough, 2007). Patients with bipolar disorder, as well, show increased exploration and novelty seeking (Minassian et al., 2011), engage in high-risk behaviors such as gambling (Chandler et al., 2009), and exhibit a hyperactive neuroendocrine response to stress (Watson et al., 2004). The effects we observed appear to be mediated by the neuroinflammation following IL-1β, as an acute administration of IL-1β directly into the DR did not impact these aspects of manic-like behavior. Serotonergic cell bodies within the DR play a central role in the regulation of the behavioral features of mania (Homberg and Lesch, 2011; Bortolato et al., 2013). Thus, the observed changes in behavior are highly suggestive of a
recapitulation of neuroinflammation-induced mania in an animal model that may be driven by alterations to the serotonergic system.

We predicted an anatomical specificity to the effects, and tested this hypothesis by conducting parallel experiments targeting the PFC, which receives dense serotonergic input from the DR, and thus is an important downstream mediator of the behavioral effects of serotonin. Serotonin signaling within the PFC is critical for impulse control and cognitive flexibility, where depletion results in capricious and perseverative behavior (Clarke et al., 2004). Indeed, there is evidence for reduced serotonin in the prefrontal cortex of patients with schizophrenia (Sumiyoshi et al., 2013). Neuroinflammation localized to this key serotonergic output region was insufficient to alter behavior, suggesting that inflammatory signals alter serotonin-mediated behavior via effects on the serotonergic cell bodies at the level of the DR.

Therefore, we next evaluated the impact of our manipulations on gene expression within the DR, focusing both on broad programmatic changes, as well as those of particular relevance to neurotransmission and serotonergic signaling. The microarray revealed a predicted increase in inflammation-related genes, consistent with our immunofluorescent characterization of the model. More interestingly, genes related to serotonin, GABA, glutamate, and noradrenergic neurotransmitter systems were profoundly decreased in the DR, suggesting dramatically altered neuronal excitability, synaptic transmission, and ability to adapt to external input. This imbalance is consistent with the ability of IL-1β to directly inhibit spontaneous serotonergic neuron firing in vitro (Manfridi et al., 2003). Additionally, IL-1β has recently been shown to play a role in modulating glutamate transmission in the experimental autoimmune encephalomyelitis
(EAE) model of multiple sclerosis (Mandolesi et al., 2013), supporting the ability for IL-1β to modulate neurotransmission and regulate serotonergic neuron excitability and output.

Based on the directionality of transcriptional changes, we hypothesized that there may be fewer serotonin-producing neurons within the DR. This was confirmed immunohistochemically, where we observed a 30% reduction in overall TPH-ir neurons. This may be the result of a downregulation of TPH itself (i.e., reduced detection of TPH-ir), or to a loss of serotonergic neurons. In the same animals, HPLC analysis revealed reduced serotonin output to the PFC. While 5-HT was measured in the PFC as one readout of DR serotonergic output, this is likely predictive of a brain-wide reduction in 5-HT. Interestingly, DR lesions have previously been shown to increase exploration in the open arm of the elevated plus maze, consistent with our findings of serotonergic hypofunction and an increase in apoptosis-related gene expression in the DR (Briley et al., 1990). Therefore, the effect of neuroinflammation within the DR could be due to reduced serotonergic responsivity, neuron number, or ability to produce serotonin.

To further test this hypothesis, we employed a pharmacological strategy to rescue the phenotype of these mice by treatment with the selective serotonin reuptake inhibitor (SSRI), citalopram, to rapidly increase serotonin availability. We found that a single dose of citalopram ameliorated the behavioral dysfunction of IL-1β mice infused with AAV-Cre on the elevated plus maze. These animals spent less time in the open arm, had fewer visits to the end of the open arm, and had an overall reduction in hyperlocomotion compared to their original performance one week earlier. Importantly, when animals were re-tested again following vehicle treatment, the direction of their behavior returned
towards a dysregulated state. The transient rescue of behavior demonstrates that reduced serotonin does indeed play a causal role in the manic-like behavior observed following DR neuroinflammation. However, as behavior was not restored completely to control levels it is also possible that having fewer serotonin-producing neurons could have reduced the effectiveness of the drug to raise serotonin levels sufficiently and/or that an acute increase in available serotonin does not completely compensate for the chronic dysregulation within the DR.

One possible co-existing mechanism that could contribute to the observed behavioral dysfunction is an increase in lipocalin-2 (Lcn2), a suggestion that is supported by our microarray dataset in which Lcn2 displayed a 2.5 log-fold increase in expression. Lcn2, an iron-binding sideophore, is commonly increased in response to inflammatory insult due to its ability to deprive bacteria of necessary iron. Thus, it serves as an important protective role in the case of bacterial infiltration. Pathological consequences of Lcn2 upregulation, however, are substantial. Tryptophan hydroxylase (TPH), the rate-limiting enzyme in the synthesis of serotonin, requires iron for proper enzymatic activity (Nakata and Fujisawa, 1982a, b). Therefore, Lcn2-mediated deprivation of iron could result in an inability to synthesize serotonin (Bachman et al., 2009). Additionally, Lcn2 has the ability to reduce the bioavailability of catecholamines, as sequestration of iron occurs through binding available catechol groups (Bao et al., 2010; Miethke and Skerra, 2010). In the brain, maintaining appropriate levels of catecholamines, which include norepinephrine and dopamine, is vitally important to behavioral homeostasis. In the DR, specifically, noradrenergic input from the locus coeruleus exerts tonic control on 5-HT neurons to increase serotonergic tone. (Baraban and Aghajanian, 1980, 1981;
Vandermaelen and Aghajanian, 1983; Pan et al., 1994). Therefore, our measured reduction of norepinephrine in the DR could be indicative of insufficient norepinephrine, which has been shown to result in reduced serotonergic neuron firing and less 5-HT release in target brain regions (Pudovkina et al., 2002). Inflammation-induced serotonergic hypofunction could be partly explained by the observed elevation in Lcn2, and consequent reduced TPH functioning and/or norepinephrine in the DR.

Our findings of a 5-HT-mediated manic phenotype raise an important question: How can serotonergic hypofunction result in both manic and depressive-like behavior? Most reports of the association between neuroinflammation and neuropsychiatric disease have focused on the depressive aspects of disease, for example, “sickness behavior” following immune activation, or symptoms of depression in patients with rheumatoid arthritis, multiple sclerosis, or following IFNγ treatment. Furthermore, SSRIs, which increase 5-HT availability, treat symptoms of depression in many patients implicating reduced 5-HT as an aspect of their pathophysiology. However, substantial evidence also links the 5-HT system to manic behavior. 5-HT is known to regulate hedonic, aggressive, and risk-taking behavior, all aspects of human mania (Jones and Fernyhough, 2007; Chandler et al., 2009; Minassian et al., 2011; Haralanova et al., 2012). Interestingly, a recent study reported an association between immune activation and manic symptoms in patients with depressive disorders (Becking et al., 2013), helping to link the seemingly conflicting epidemiological evidence. Indeed, the prevalence of sub-threshold manic symptoms is ~40% in patients with unipolar depression (Zimmermann et al., 2009; Angst et al., 2010), suggesting that these disorders may be part of a spectrum with potentially overlapping etiology.
In summary, our findings demonstrate a profound ability for localized inflammation in the DR to induce manic-like symptoms, which were associated with neurotransmitter imbalance within the DR. These findings support the hypothesis that neuroinflammation may be a key etiological component in the development of mania, and further demonstrate that the DR is a critical brain region mediating these behavioral outcomes. These findings implicate dysregulation of serotonin signaling as important mediators of the effects of neuroinflammation on behavior.

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**Figure 4.1** IL-1β overexpression within the DR causes local neuroinflammation.

Location of the DR in the Mouse Brain Atlas (A) (Paxinos and Franklin, 2001). Representative images highlight spatially-restricted β-Gal (B) and GFP immunofluorescence (C) following AAV-Cre infusion. Insets (b’ and c’) represent higher magnification of subfield. AAV-Cre infusion significantly increases IL-1β mRNA in both males and females in the DR (D). Immunofluorescent characterization of microglia (E, CD11b) neurons (F, J NeuN), astrocytes (G, K GFAP, H, L AQP4) and indoleamine 2,3-dioxygenase (I, M IDO), an enzyme that catabolizes tryptophan in AAV-GFP infused mice (E-H) and AAV-Cre infused mice (I-L). Enzyme linked immunosorbannt assay (ELISA) of plasma samples reveals no effect of DR AAV-Cre infusion on circulating IL-1β (N). Data are presented as mean ± SEM, N = 6-7; **, P < 0.01 denotes main effect of treatment following two-way ANOVA comparing sex or genotype (WT vs. IL-1β) by treatment (AAV-GFP vs. AAV-Cre).
Figure 4.2 IL-1β-mediated neuroinflammation in the dorsal raphe causes manic-like behavior. In the elevated plus maze, both male (A-C) and female (D-F) IL-1βXAT mice infused with AAV-Cre display increased locomotion (A, D), time in the open arm (B, E),
and time in the distal ends of the open arm (C, F). In the light-dark box, both male (G-I) and female (J-L) IL-1β mice infused with AAV-Cre travel a greater distance in the light compartment (distance, normalized to time spent in light compartment) (G, J), transition more between light and dark compartments (H, K), and spend more time in the light compartment (I, L). In the locobox, male (P-S) IL-1β mice infused with AAV-Cre travel more throughout the test (M) at a greater speed (N), while transitioning fewer times between compartments (O) and having no significant preference for either compartment (P). Female (Q-T) mice showed a similar pattern as the males. Data are presented as mean ± SEM, N = 6-10; *, P < 0.05, **, P < 0.01 using within sex two-way ANOVA of genotype (WT vs. IL-1β) by treatment (AAV-GFP vs. AAV-Cre), followed by Tukey’s multiple comparison test.
Figure 4.3 IL-1β-mediated neuroinflammation in the dorsal raphe increases male stress responsivity. Male IL-1βXAT mice infused with AAV-Cre (A) display an increase in corticosterone production in response to a 15-min restraint stress (shaded), while exhibiting no difference in baseline or recovery. No significant effects were observed in females (B). Data are presented as mean ± SEM, N = 9-11, **, P < 0.001 using a within-sex two-way rmANOVA comparing genotype (WT vs. IL-1β) vs. treatment (AAV-GFP vs. AAV-Cre), with time as a repeated measure.
Figure 4.4 Acute microinfusion of IL-1β into the DR results in increased locomotion but no change in open arm exploration in the elevated plus maze. Male and female mice infused with IL-1β 30 min prior to testing on the elevated plus maze display increased locomotor activity (A), but no change in open arm exploration (B) or time spent in the distal arms (C). PFC neuroinflammation does not induce manic-like behavior or physiology. Male and female IL-1β<sup>XAT</sup> mice infused with AAV-Cre showed no difference in behavior on the elevated plus maze (D-F) or light-dark box (G, H). Neither males (I) nor females (J) display differences in corticosterone response to an acute 15 min restraint stress (shaded). Data are presented as mean ± SEM, N = 7-8. *, P < 0.05 denotes main effect of infusion following a two-way ANOVA comparing sex vs. treatment (AAV-GFP vs. AAV-Cre).
Figure 4.5 IL-1β-mediated neuroinflammation in the dorsal raphe results in broad changes in gene expression. Heat map illustrating significant differences in gene expression between AAV-GFP and AAV-Cre infused male and female DR RNA (A), where data are expressed as relative levels within each gene, and both animals and genes are organized by hierarchical clustering. Genes that were either significantly up-regulated (B) or significantly down-regulated (C), as determined by an FDR of 0.01, are ranked by enrichment score and organized by functional gene clusters identified using the DAVID bioinformatics database. A separate analysis was performed on genes annotated with the gene ontology (GO) term “neur*,” which identified 26 genes that are down-regulated in IL-1β animals infused with AAV-Cre (D).
**Figure 4.6 IL-1β-mediated neuroinflammation in the DR results in serotonergic hypofunction.** Immunofluorescence of TPH-immunoreactivity (A, B) was used to identify serotonergic neurons (red), counterstained with the nuclear stain, DAPI (blue), of AAV-GFP (A) and AAV-Cre (B)-infused DR 8 weeks following AAV infusion (n=4). Quantification revealed fewer TPH-ir cells in AAV-Cre infused animals (C). HPLC analysis of biogenic amines of the DR (D) less norepinephrine (E, NE) but no change in dopamine (F, DA). In the PFC (G), AAV-Cre infused animals had less serotonin (B, 5-HT), increased levels of the metabolite (C, 5HIAA), and an overall increase in neurotransmitter turnover (D, 5HIAA/5-HT ratio). Data are presented as mean ± SEM, N = 6-7; **, P < 0.01, ***, P < 0.0001 following t-test comparing AAV-GFP and AAV-Cre infusion.
Figure 4.7 Acute increase in serotonin ameliorates manic-like phenotype in the elevated plus maze. IL-1β mice were infused with AAV-GFP or AAV-Cre into the DR and tested for three consecutive weeks on the elevated plus maze 32 minutes after i.p. injection with saline (tests 1 and 3) or 30mg/kg citalopram (test 2, shaded). Male IL-1β mice infused with AAV-Cre (A-C) show a significant attenuation of distance traveled (A), open arm time (B) and distal open arm entries (C) following citalopram treatment. Females (D-F) show a similar pattern of behavior. Data are presented as mean ± SEM, N = 4-5, *, P < 0.05, **, P < 0.01 following two-way ANOVA of treatment by trial. Post-hoc test compared citalopram to saline treatments.
CHAPTER 5: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Neuropsychiatric diseases represent a major public health burden worldwide, and are the leading cause of disability in the United States. Treatments do exist for many of these diseases, yet gaps in the understanding of their causal mechanisms have left up to 30% of patients refractory to treatment (Souery et al., 2006). In light of this lack of efficacy, it is necessary to identify common mechanism and risk factors for neuropsychiatric disorders. Fortunately, epidemiological observations have identified activation of inflammatory signaling cascades as a risk factor for and stress dysregulation as a concomitant symptom of a number of neuropsychiatric disorders. Therefore, the goal of this dissertation was to elucidate the role of neuroinflammation as a determinant of stress sensitivity and perhaps ultimately of disease. As there are major sex difference in incidence and presentation, this body of work also includes an investigation into how sex differences in the stress-responsive dorsal raphe nucleus (DR) may contribute to differences in vulnerability to neuropsychiatric disease. The hope is that a better understanding of the intersection of neuroinflammation and stress physiology, at a brain region-specific level of investigation, may provide the framework to develop more appropriate targets for individualized therapeutics.

The work described in the previous chapters extends our knowledge of the contribution of both neuroinflammation and sex as representing specific vulnerabilities towards stress dysregulation. In Chapter 2 we utilized a genetic model of stress sensitivity to describe a role for inflammatory signaling pathways to regulate hypothalamic-pituitary-adrenal (HPA) stress axis function. In this model, the DR, and not
other stress responsive brain-areas, was identified as a locus of heightened responsivity. *Chapter 3* furthered this investigation of the stress-sensitive DR by describing sex differences in response to the stress neuromodulator, corticotropin-releasing factor (CRF). Together, these findings support a unique role for the DR as a site of potential vulnerability towards neuropsychiatric disease, whereby differences in responsivity may influence susceptibility to stress related disorders. In *Chapter 4*, we probed a role for DR neuroinflammation to affect stress sensitivity, showing that neuroinflammation localized to this brain region results in dysregulated stress behavior and physiology through interactions with the serotonergic neurotransmitter system.

Below I discuss the implications and important future directions supported by these studies, as well as the utility for using appropriate animal models to understand how vulnerabilities can impact stress behavior and physiology through discrete action in specific brain regions.

**Modeling stress dysregulation**

A variety of animal models have been developed to advance our understanding of the pathophysiology of stress-related disorders, with recent approaches emphasizing the modeling of symptoms, aspects, or endophenotypes, rather than specific disorders per se. Common approaches are the use of genetics, social and environmental manipulation, and pharmacology, each of which pose varying challenges with respect to construct, face, and predictive ability (Nestler and Hyman, 2010).

Work presented in this thesis began with the utilization of a genetic model of stress sensitivity, the corticotropin-releasing factor receptor 2-null mouse (CRFr2<sup>−/−</sup>), to
test the hypothesis that inflammation plays a causal role in the dysregulated stress state. Numerous studies have previously examined the impact of different stressor paradigms on pro- and anti-inflammatory cytokines in the brain and periphery. However, reports have been conflicting; in some models stress drives increases in cytokine production, while other models demonstrate an immunosuppressive response. For example, Reyes and colleagues identified a number of immune-related genes that were upregulated in the paraventricular nucleus of the hypothalamus of mice in response to restraint stress (Reyes et al., 2003). Similarly, inflammatory cytokines were increased and anti-inflammatory cytokines were decreased after a 4-day chronic mild stress paradigm in male Wistar rats (You et al., 2011). In contrast, a longer chronic mild stress paradigm in mice actually reduced central IL-1β expression (Bartolomucci et al., 2003). The differences observed in these studies highlight the challenge of the extrapolation of results across methodologies and models. One explanation of these discrepancies is that specific characteristics of each stress exposure dictate the degree of neuroimmune responsivity, as does the particular brain region in which measurements occur. Additionally, the activation of astrocytes following chronic stress in CRFr2−/− and not WT mice suggest that stress sensitivity and genetic composition of the model are also important factors. Future investigation using other models of stress vulnerability would provide additional insight into which aspects of the neuroimmune response to stress are model-specific, and which may be more generalizable. These common and penetrant factors across many animal models are therefore most likely to translate from the bench to the bedside and offer targets for therapeutics of neuropsychiatric diseases in humans.

Moving forward, there are many exciting areas of research that can be built upon
this recent work. For example, it is known that prenatal stress, a risk factor for stress-related disorders, influences later life response to an inflammatory challenge, but whether it impacts the sensitivity of a neuroimmune response to stress exposure has not been determined. Further, long-term neuroimmune consequences of adolescent stress exposure would shine light on the applicability of these findings to specific human at-risk populations.

**Anti-inflammatory drugs in the treatment of psychiatric disorders**

In Chapter 2, we presented evidence that anti-inflammatory drugs might be beneficial for the treatment of stress-related mood disorders. Indeed, when this work was published, a number of clinical trials had just begun to test this very hypothesis. For example, acetyl-salicylic acid, anti-TNFα agents, and minocycline (an inhibitor of microglia) are all currently under investigation for efficacy as adjunct treatment for bipolar disorder, schizophrenia, and depression [reviewed in (Girgis et al., 2014)]. One such investigation has met with recent success in a subpopulation of treatment-resistant patients with major depressive disorder, those with elevated marks of inflammation (Raison et al., 2013). This double-blind, placebo-controlled clinical trial tested the efficacy of infliximab, a chimeric monoclonal antibody that binds to and inhibits the soluble and transmembrane form of TNFα. This trial revealed that infliximab–mediated inhibition of TNFα was able to reduce depressive symptoms specifically in those patients with an elevated baseline level of C reactive protein (>5 mg/L).

Despite the success with the anti-TNFα treatment, other studies have shown minimal efficacy. This is likely due to the widespread and nonspecific effects on both the
brain and immune system following systemic administration of these anti-inflammatory agents. This points to a clear clinical readout of the known heterogeneity in disease presentation, and importantly, suggests the potential for differences in disease etiology that may explain differences in treatment efficacy amongst individuals and populations. Moving forward, a more targeted approach is clearly necessary for both characterization of patient populations and specific effects of therapeutics.

**Focusing on anatomical loci of vulnerability**

Markers of neuroimmune activation, including changes in astrocyte activation, have been identified in patients with bipolar disorder (Rao et al., 2010) and depression (Shelton et al., 2011). This suggests that a relationship between stress-induced neuroimmune activation and neuropsychiatric disease may serve an important role as a biomarker of disease. Because astrocytes are widely heterogeneous in structure and function, and play distinct roles across different brain areas, astrocyte changes common across regions may be simultaneously causal to or resultant from disease-associated dysregulation. For example, changes within astrocytes found in the DR (e.g. ‘activation’) may be inductive of stress-sensitivity, but those same changes in the prefrontal cortex, while no doubt biologically important, may not be causal of stress-sensitivity due to differences in the functions of the two distinct brain regions.

In Chapter 2 we identified this same kind of brain region specificity to the effects of stress exposure on neuroimmune activation. In these studies, the prefrontal cortex was a particularly susceptible brain region to stress, as measured by an increase in the astrocyte-specific protein, glial fibrillary associated protein (GFAP). These results fit
with our understanding of its role in assessing stressor controllability and interpretation of stressor intensity (Amat et al., 2005). However, in subsequent studies presented in Chapter 4, we found that overexpression of IL-1β within this brain region did not impact HPA axis responsivity. This suggests, as stated above, that chronic stress-mediated changes in neuroimmune markers in the PFC may be a secondary consequence to the loci of its impact on behavior. We also found that the ventral hippocampus, but not the dorsal hippocampus, was vulnerable to stress-induced neuroimmune activation. In addition to its role in memory formation and storage, the hippocampus plays a central role in the regulation of the HPA axis, and lesions to the hippocampus influence hormonal stress responsivity. It has been suggested that the dorsal and ventral hippocampus play functionally distinct roles in the regulation of stress and emotion, where the ventral hippocampus play a greater role in regulating HPA stress axis responsivity and negative feedback (Fanselow and Dong, 2010).

To locate the primary area causal to these secondary region-specific effects, we performed a follow-up investigation within the DR, which has serotonergic outputs to both the affected areas, as well as throughout the brain. Previous work in the lab has shown that the DR is vulnerable to chronic stress-induced changes in neuronal survival and in electrophysiological properties of 5-HT neurons (McEuen et al., 2008). Cell death can result in the activation of astrocytes in target brain region, and both 5-HTergic dennervation and lesions to the DR have been shown to directly result in cytokine production and an upregulation of astrocytic expression of GFAP in DR projection sites (Frankfurt et al., 1991). Therefore, we hypothesized that differences in the stress responsivity of DR neurons may be an upstream mediator of the neuroinflammatory
response we observed in the prefrontal cortex and ventral hippocampus.

This hypothesis was examined through cFos immunohistochemistry, a marker of neuronal activation, in the DR following exposure to an acute restraint stress. We predicted that neuronal activation in the DR would cause increased neuroimmune activation in these downstream regions. We surmised that a chronic over-activation of the DR neurons would increase the metabolic demand in the DR and its target areas, eventually leading to increased neuroimmune activation. In line with our prediction, we found that only the chronically stressed CRFr2−/− mice showed enhanced cFos, with levels twice that of any of the other groups. This, along with the neuroimmune activation presented in Chapter 2, was prevented in animals that had been given anti-inflammatory treatment throughout the course of their chronic stress exposure. This finding supports the DR as an upstream mediator of heightened stress responsivity, whose differences in response to a stressor may explain differences in behavior and physiology observed in the CRFr2−/− mice.

Although we were able to determine the region specific to the observed stress-sensitivity, cFos staining alone does not tell us which cell types comprise this increased activation. A major advancement to our understanding of this circuit would be the knowledge of which cell type – the most abundant of which are serotonergic, GABAergic, or glutamatergic – are differentially activated in response to the acute stressor. It is quite possible to explain the observed phenotype with activation of any one (or a combination) of these neuronal sub-types. One such possibility is that this over-activation occurs in GABAergic interneurons, which would likely indicate increased inhibitory tone over the 5-HT neurons, reducing the 5-HT output to target regions,
consistent with the enhanced anxiety-like behavior previously reported in these mice following exposure to chronic stress (Bale et al., 2000; Bale and Vale, 2003; McEuen et al., 2008).

**Sex differences as a model of vulnerability**

To further probe the hypothesis that differences in DR responsivity underlie vulnerabilities in stress coping behavior and physiology, Chapter 3 sought to examine sex differences in the DR, with females representing a different vulnerable population. We hypothesized that some of the sex differences in stress responsivity and responses to drug treatment may also be found in this brain region. In fact, we found this to be the case, where males and females had divergent behavioral, physiological, and electrophysiological responses to DR-infused or bath applied CRF.

The observed sex differences in electrophysiological response to bath applied CRF were particularly intriguing. Specifically, neurons from non-stressed and WT males showed an increase over baseline in IPSC rise time, half-width, and decay time in response to CRF, whereas female neurons showed the opposite responses. This suggests differences in their cellular adaptation to the stress neuropeptide. Interestingly, male mice that had previously undergone social defeat had responses more similar to females, rather than control males (Challis et al., 2013). It should be noted that the 5-HT neurons in which recordings were performed showed reduced average excitability compared to the neurons in the social defeat study. These differences may be attributed to the fact that all of our animals were individually housed, a context which is known to alter neuron excitability (Sanchez et al., 1995).
The findings from this work highlight the importance of identifying sex differences in central stress pathways. Females have an increased predisposition for developing disorders exacerbated by stress exposure; therefore identification of sex-appropriate, and potentially sex-specific, pharmacological treatments is essential.

**Neuroinflammatory regulation of serotonin signaling**

5-HT within the brain acts through a diverse assortment of membrane-bound G-protein coupled receptors and ligand-gated ion channels; to date, 14 subunits of the 5-HT receptor have been identified. Differences in their specific coupling create a diverse array of potential downstream signals. For example, activity through 5-HT$_1$ receptors reduces cAMP, resulting in an inhibitory effect as measured by an increase in frequency and amplitude of IPSCs. 5-HT can also function as an excitatory neurotransmitter through activation of receptors coupled to G$_s$ (5-HT$_4$, 6, 7), Gq (5-HT$_2$), or ligand-gated Na$^+$/K$^+$ cation channels (5-HT$_3$). Therefore, identifying the serotonin receptor subtype in target regions is crucial in determining the mechanism by which it is mediating its effects on behaviors of interest.

In Chapter 4 we presented data that support the hypothesis that the 5-HTergic DR is a locus of susceptibility to neuroinflammation. Specifically, there were marked behavioral changes that clearly link neuroimmune activation, neuropsychiatric disease, and the 5-HT system. In our studies, we described a 5-HT-mediated manic-like phenotype yet 5-HTergic hypofunction is more commonly associated with depressive-like behavior. In fact, selective 5-HT reuptake inhibitors (SSRIs), which increase 5-HT availability by blocking transporter reuptake, treat symptoms in a large percentage of
depressed patients, implicating reduced 5-HT as an aspect of depression’s pathophysiology. Moreover, neuroinflammation has also been linked to depressive aspects of disease. For example, there have been multiple observations of “sickness behavior” following immune activation, or symptoms of depression in patients with rheumatoid arthritis, multiple sclerosis, multiple sclerosis, or following IFNγ treatment.

Despite these associations with depression, substantial evidence also links the 5-HT system to manic behavior. 5-HT is known to regulate hedonic, aggressive, and risk-taking behavior, all aspects of human mania (Ramrakha et al., 2000; Watson et al., 2004; Jones and Fernyhough, 2007; Chandler et al., 2009; Minassian et al., 2011; Haralanova et al., 2012). Furthermore, lesions to the DR have been shown to increase exploration in the open arm of the elevated plus maze (Briley et al., 1990). Interestingly, a recent report has found an association between immune activation and manic symptoms in patients with a depressive disorder (Becking et al., 2013), perhaps helping to link the seemingly conflicting evidence of 5-HT mediating both manic and depressive behavioral phenotypes. Indeed, the prevalence of sub-threshold manic symptoms is ~40% in patients with unipolar depression (Zimmermann et al., 2009; Angst et al., 2010), suggesting that these disorders may be part of a spectrum with potentially overlapping etiology.

IL-1β is known to affect 5-HT synthesis and release. When administered into the hypothalamus, IL-1β augments 5-HT release (Shintani et al., 1993), and direct administration of the cytokine into the DR has been shown to result in reduced spontaneous 5-HTergic neuron firing (Manfridi et al., 2003). However, the link between elevated IL-β and changes to 5-HTergic neurons in the context of behavior remains to be fully explored. We identified numerous changes evoked by our neuroinflammatory
stimulus on the structure and function of the DR that could explain this connection, although further investigation is certainly warranted. In an analysis of gene expression changes, we identified lipocalin-2 (Lcn2) based on its basal sex difference in expression (F>M), and because it presented with one of the greatest fold-change increases following DR inflammation. Lcn2, an iron-binding sideophore, is commonly increased in response to inflammatory insult due to its ability to deprive bacteria of necessary iron. Thus, it serves an important protective role in the case of bacterial infiltration.

Despite its normally protective role, Lcn2 upregulation may also have substantial deleterious effects. First, it likely serves to deprive brain cells of iron (Bachman et al., 2009). Importantly, tryptophan hydroxylase (TPH), the rate-limiting enzyme in the synthesis of serotonin, requires ferrous iron (Fe^{2+}) for proper enzymatic activity (Nakata and Fujisawa, 1982a, b). Therefore, the deprivation of this ion could result in an inability to synthesize 5-HT. Second it could reduce the bioavailability of catecholamines, as Lcn2 sequesters iron by binding to available catechol groups (Bao et al., 2010; Miethke and Skerra, 2010). In the brain, maintaining appropriate levels of catecholamines, which include norepinephrine and dopamine, is vitally important to behavioral homeostasis. In the DR noradrenergic input from the locus coeruleus exerts tonic control on 5-HT neurons to increase 5-HTergic tone (Baraban and Aghajanian, 1980, 1981; Vandermaelen and Aghajanian, 1983; Pan et al., 1994). The absence of sufficient norepinephrine results in reduced 5-HTergic neuron firing and less 5-HT release in target brain regions (Pudovkina et al., 2002). Therefore, inflammation-induced 5-HTergic hypofunction could be partly explained by the observed elevation in Lcn2, and consequent reduced TPH functioning and/or norepinephrine in the DR.
To address this theory, it would be important to perform a direct measure of iron in both its oxidized and reduced state. Additionally, this hypothesis would be greatly strengthened by overexpressing Lcn2 directly into the DR, or, alternatively, by performing these experiments in a mouse with a conditional deletion of Lcn2 in the DR. Interestingly, global Lcn2-deficient mice have recently been shown to have increased stress-induced anxiety-like behavior, cognitive impairments in spatial learning tasks, and hyperactivation of the HPA axis, supportive of a role for this protein in stress related behavior and physiology (Ferreira et al., 2013). However, due to the lack of brain region specificity in these experiments, which were performed on whole body knockout mice, further investigation is warranted to better understand the mechanism by which Lcn2 mediates these observed phenotypes. Nonetheless, the evidence that altered Lcn2 can impact stress-related behaviors are of great relevance.

**General implications**

The work presented in this thesis has shown that hyper- or hypo-function of the dorsal raphe nucleus, based on genetic susceptibility, sex, or inflammatory insult, can result in manic- and depressive-like behavior. The novelty of these findings centers on the importance of the loci impacted by these differences. The dorsal raphe nucleus has previously been identified as a vulnerable region to impact stress coping and physiology; here we demonstrate the specific, mechanistic link between risk factors for stress-related disorders, and quantitative changes to this brain region and its functional output.
APPENDIX A: SEX DIFFERENCES IN STRESS BEHAVIOR AND PHYSIOLOGY FOLLOWING INFECTION WITH *TOXOPLASMA GONDII*

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INTRODUCTION

Mood and anxiety disorders are more than twice as common in women than men. As stressful life experiences are known triggers for disease onset, disease prevalence in females may be explained by the well-established sex differences in stress sensitivity (Handa et al., 1994; Goel and Bale, 2009). This sex bias is associated with differences in corticotropin-releasing factor (CRF) neurotransmission, and sex differences have been identified in expression and signaling of both CRF receptor subtypes, CRFr1 and CRFr2. This stress-sensitive signaling cascade is susceptible to perturbations from the environment, including neuroinflammatory stimuli. Interestingly, CRFr2 is located both in the brain and on immune organs and circulating immune cells, where it plays a pivotal role in regulating both stress and immune responsivity. Importantly, there are known sex differences in the immune response of males and females, including a difference in susceptibility to neuroinflammation (Schuurs and Verheul, 1990; Xiao et al., 2012).

Toxoplasma (T.) gondii is an intracellular obligate parasite with the ability to cross the blood-brain-barrier and induce neuroinflammation. In the chronic stage of infection, neuroinflammation is maintained while peripheral infection is cleared. As such, T. gondii infection is a powerful tool to test the hypothesis that neuroinflammation is responsible for sex-specific differences in the neurocircuitry and behaviors associated with stress dysregulation. Furthermore, exposure to T. gondii in utero has been linked with increased risk for the development of sex-biased neuropsychiatric disorder, making T. gondii an etiologically relevant source of neuroinflammation (Henriquez et al., 2009; Arias et al., 2011).

The current studies sought to determine the sex-specific impact of T. gondii-
induced neuroinflammation on stress related behavior in male and female mice. As CRFr2 plays an important role in regulating both stress and immune responsivity, we also sought to determine whether *T. gondii*-induced stress dysregulation is mediated through CRFr2. To test this hypothesis, male and female mice were chronically infected with *T. gondii* and assessed for changes in anxiety-like behavior, glucocorticoid production, weight change, and survival. In a parallel study, males and females were sacrificed during acute or chronic infection, and brains were harvested for gene expression analysis and serum collected for cytokine analysis.

METHODS

*Animals*

Mice were generated in-house from CRFr2 heterozygous breeding pairs on a mixed C57Bl/6:129 background to yield WT and CRFr2−/− littermates. Same-sex littermates (14-16 weeks) were housed in groups of 3-5 under a 12h L/D cycle, with food and water available *ad libitum*.

*Assessment of weight and survival*

Body weight was measured weekly, beginning one week prior to infection with *T. gondii*, at approximately 1400 h throughout the study. Mice were monitored daily for health and survival status.

*Toxoplasma gondii infection*

Me49 cysts were harvested from the brains of chronically infected CBA/J mice. Brains
from infected mice were homogenized in sterile DPBS and an aliquot was used to count cysts. The suspension was diluted in DBPS pH7.4 and experimental animals were injected i.p. with 20 cysts. Naïve animals were injected with sterile DPBS alone to control for the stress exposure of injection.

**HPA assessment**

Plasma corticosterone levels were determined following a 15-min restraint stress in male WT and CRF2\(^{-/-}\) mice (N = 7-9 per genotype/treatment group). Testing was administered between 0800–1100 h on the final day of CVS by placing mice in a 50 ml conical tube containing a 50 mm air hole. Tail blood samples (10 \(\mu\)L) were taken at onset and completion of restraint (0 and 15 min, respectively). To examine the stress recovery phase, additional samples were collected at 15 min and 75 min following the culmination of restraint (30 and 90 min, respectively). Samples were collected into 5 \(\mu\)L of 50 mM EDTA, centrifuged, and stored at -80\(^{\circ}\)C until the assay was performed. Plasma corticosterone concentration was measured in duplicate using a \(^{125}\)I-corticosterone radioimmunoassay kit (MP Biomedicals, Orangeburg, NY). The minimum detection limit of the assay was 7.7 ng/ml and intra-assay coefficient of variation was 7.3%.

**Elevated plus maze**

Adult mice (n = 7-10 per group) were placed in the center square of the maze facing the open arm at the beginning of the 5 min test. Light intensity in the open arms was 300 lux. Testing occurred 2-5 h after lights on. Testing was digitally recorded and distance and time spent in each arm were analyzed automatically using ANY-maze v4.75 software.
Brain gene expression

Whole brains were cryosectioned at -20°C. Using a hollow needle (Ted Pella Inc., Redding, CA), brain regions were micropunched according to the Paxinos and Franklin atlas (Paxinos and Franklin, 2001) and the ventromedial hypothalamus was collected. Punches were probe-sonicated for 2.5 min (30 sec on/off cycles) in TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was isolated by chloroform extraction, as previously described (Howerton et al., 2013). cDNA was transcribed, and quantitative gene expression of CRFr2 was determined through TaqMan gene expression assay according to the manufacturer’s instructions (Invitrogen).

Cytokine Luminex assay

A mouse 20-plex antibody bead kit (BioSource, Cmarillo, CA) was used to measure the level of 20 cytokines and chemokines in 50 µL of serum from naïve, acutely infected (10d post infection) or chronically infected (28d post infection) mice. Analytes included cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF); cytokine receptors: IL-1Rα and IL-2R (CD25); and chemokines: eotaxin (CCL11), IFN-γ-inducible protein (IP-10, CXCL10), Mig (CXCL9), and MCP-1 (CCL2). Data were collected using a Luminex-100 array reader (Luminex Corp., Austin, TX).
Statistics

An investigator blind to animal treatment group conducted all studies and analyses. EPM box measures were analyzed within sex by t-test. Gene expression analysis was analyzed by two-way ANOVA. Weekly measures of weight change were analyzed within-sex using a repeated-measures ANOVA with genotype and infection as the independent variables, and time as a within-subject repeated measure. Plasma corticosterone was analyzed within-sex using a two-way ANOVA for genotype and infection, with time as a within-subject repeated measure. Gene expression was analyzed by t-test comparing naïve and chronically infected male mice. Cytokines were analyzed both by expression level, and as a binomial analysis as to whether the cytokine was detected in a given sample. Significant differences were identified at p ≤ 0.05. All statistics above were performed using JMP8 (SAS) software, and all data are reported as mean ± standard error of the mean (SEM).

RESULTS

Deficiency in CRFr2 rescues female mortality following T. gondii infection

To study sex differences in susceptibility to T. gondii infection, age-matched WT and CRFr2<sup>-/-</sup> male and female littermates, all on a C57:129 mixed background, were infected i.p. with T. gondii (Fig. A.1). Upon infection with 20 cysts of the type II Me49 strain, WT male mice survived at a rate of 91% and females were susceptible with a survival rate of 9%. This susceptibility was rescued in CRFr2<sup>-/-</sup> mice, where CRFr2<sup>-/-</sup> of both sexes showed enhanced survival; 100% of CRFr2<sup>-/-</sup> male mice survived and 76% of female CRFr2<sup>-/-</sup> survived 65 days after IP infection. Fig. A.2 demonstrates time course of susceptibility to infection, which occurred in the chronic stage.
Deficiency in CRFr2 rescues attenuates weight loss following T. gondii infection

Infection with T. gondii resulted in weight loss in all animals during the acute stage of infection (Fig. A.1). This weight loss was most dramatic in WT females, who were unable to regain weight in the chronic stage of infection. By the chronic stage of infection (Fig. A.2), CRFr2\(^{-/-}\) mice regain substantially greater weight.

Infection with T. gondii increases CRFr2 expression in the ventromedial hypothalamus

CRFr2 plays an important role in feeding behavior via its effects within the ventromedial hypothalamus. Since CRFr2\(^{-/-}\) female mice failed to show the same degree of weight loss as WT females, we sought to test the hypothesis that T. gondii infection increases CRFr2 expression in this brain region. Infected animals showed significantly increased expression of CRFr2 in the ventromedial hypothalamus compared to naïve animals (t\(_{1,8}\) = 2.621, p = 0.0306) (Fig. A.3).

Deficiency in CRFr2 protects against T. gondii-induced heightened stress sensitivity

T. gondii infection increased corticosterone production in response to an acute restraint stress (Fig. A.4) in WT male (F\(_{1,36}\) = 9.024, p = 0.0048) and female mice (F\(_{1,20}\) = 24.47, p < 0.0001). Infection had no impact on stress-induced corticosterone production in male (F\(_{1,24}\) = 0.9327, p = 0.344) or female (F\(_{1,20}\) = 0.6717, p = 0.422) CRFr2\(^{-/-}\) mice.

Deficiency in CRFr2 protects against behavioral changes in females infected with T. gondii

The elevated plus maze provokes a stress response, whereby more stress-responsive
animals spend increased time in the closed areas. In both males and females was a significant main effect of genotype ($F_{(3,27)} = 19.66, p < 0.0001$) and infection state ($F_{(1,27)} = 14.64, p = 0.0007$), and an interaction ($F_{(3,27)} = 9.269, p < 0.0002$) to increase time spent in the open arm of the elevated plus maze (Fig. A.6), illustrating that the behavioral effects of infection were specific to WT mice, and time spent in the open arm and distal ends of the open arm was attenuated in CRFr2$^{-/-}$ mice.

**Sex differences in cytokine expression**

Sex differences in expression levels and probability of expressing a cytokine were compared between WT and CRFr2$^{-/-}$ male and female mice from naïve, acutely infected, and chronically infected cohorts. A 3-way ANOVA was used to compare statistical differences in expression levels by genotype, sex, and treatment group. This analysis revealed differences including effects on genotype and/or sex in IL-1β (genotype x sex interaction, $p = 0.0304$), IL-4 (genotype, $p = 0.0066$, genotype x sex, $p = 0.019$, genotype x treatment, $p = 0.0212$), IL-6 (genotype, $p = 0.0186$), IL-12 (sex, $p = 0.0105$, sex x treatment, $p < 0.001$), MCP1 (sex, $p = 0.0494$, sex x treatment, $p = 0.0108$), MIG (sex x treatment, $p = 0.0059$, sex x genotype x treatment, $p = 0.0112$), TNFa (genotype, $p = 0.0008$, genotype x treatment, $p < 0.0001$, sex x treatment, $p = 0.0003$), and VEGF (sex, $p < 0.0001$, sex x treatment, $p = 0.0197$). When WT mice were looked at separately (Fig. A. 7), KC, IL-2, and VEGF displayed sex differences in probability of expression at all three time points - basally, during the acute stage of infection, and during the chronic stage of infection. IP-10 and MCP1 displayed sex differences in naïve animals, IL-12 displayed sex differences in chronically infected animals, and IL-10, IL-4, and TNFa
displayed sex differences both in naïve and chronically infected animals.

**DISCUSSION**

Sexual dimorphism in the immune system has been widely reported across species, including humans (Schuurs and Verheul, 1990; Nava-Castro et al., 2012; Xiao et al., 2012). With few exceptions, females are more susceptible to inflammatory conditions including asthma, multiple sclerosis, Sjogren’s syndrome, systemic lupus erythrematosus, rheumatoid arthritis, and polymyalgia rheumatica (Meldert et al., 2005; Tiniakou et al., 2013). Inflammation is a known risk factor for the development of neuropsychiatric disorders, including depression, schizophrenia, and bipolar disease, which display sex differences in incidence, presentation, and trajectory of disease. The impact of inflammation on the development of these diseases may occur through its ability to influence an organism’s vulnerability to stress exposure.

CRFr2 is located both in the brain and also on immune organs and circulating immune cells, where it plays a pivotal role in regulating both stress and immune responsivity (Baigent and Lowry, 2000). Therefore, the current studies sought to understand the role of CRFr2 in the susceptibility of females to inflammation-mediated changes in stress related behavior and physiology. *T. gondii*, which is among the natural infections for which females are more vulnerable, was used as a sledgehammer approach to induce neuroinflammation in male and female CRFr2−/− mice.

As predicted, we found females to be more susceptible to the effects of *T. gondii* on survival and weight loss. Females displayed enhanced mortality, as has been reported in other strains of mice exposed to *T. gondii*. Females also lost more weight following
infection, an effect that was absent in CRFr2<sup>−/−</sup> mice. As the susceptibility to <i>T. gondii</i> was observed well into the chronic stage of infection, it is likely that these effects are driven by central expression of CRFr2.

We also observed a substantial sex-specific behavioral effect of <i>T. gondii</i> infection in the elevated plus maze, and on glucocorticoid production in response to an acute stress exposure. Behavioral consequences of <i>T. gondii</i> infection have been previously reported, primarily in the context of disinhibition towards predator odor. Our findings suggest that this may be more applicable to stress-related behaviors in general. CRFr2<sup>−/−</sup> mice, however, displayed attenuated stress-induced glucocorticoid production, and attenuated behavioral changes in the elevated plus maze, suggesting a specific role for this receptor in modulating the interaction between inflammation and stress related behaviors in a sex-specific manner. The brain region specificity of these effects remains to be examined.

Centrally, CRFr2 densely populate the ventromedial hypothalamus (VMH), where their activation results in enhanced signaling of excitatory projects to POMC neurons of the arcuate nucleus to inhibit feeding behavior (Poulin et al., 2012). Deficiency of CRFr2 in this brain region leads to a disinhibition of this circuit, resulting in enhanced food intake (Chao et al., 2012). Therefore, it is possible that an inflammation-mediated increase in activation of CRFr2 in the VMH leads to enhanced weight loss observed in WT mice following infection with <i>T. gondii</i>. In CRFr2<sup>−/−</sup> mice, this inflammation-induced weight loss is abrogated by a lack of VMH activation of this circuit. To test this hypothesis directly, we would need to perform a site-directed knockdown of CRFr2 in this brain region prior to <i>T. gondii</i> infection, however, our findings that CRFr2 expression
is increased following infection is supportive of this hypothesis.

To investigate the underlying cause of this susceptibility, male and female mice of both genotypes were euthanized either naively, or 10 days (acute) or 28 days (chronic) following infection. Serum was collected for the analysis of cytokines and chemokines that may be differentially regulated during these different stages in males and females. We detected a subset of these molecules that displayed sex differences across different time points, suggesting an additional peripheral immune response difference between males and females that may underlie their difference in susceptibility.

In summary, CRFr2 appears to function as an important modulator of the interaction of the sex-specific effects of inflammation on stress responsivity. Our data indicate that CRFr2 may play a previously unappreciated role in signaling immune-brain interactions that mediate female stress sensitivity in neuropsychiatric disease.

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Figure A.1 Deficiency in CRF2 resuces female susceptibility to infection with *T. gondii*. Females are more susceptible to infection with *T. gondii*. Deficiency in CRF receptor-2 rescues fatality of *T. gondii* infection, resulting in no deaths amongst males, and significantly fewer deaths amongst females.
Figure A.2 Survival curve of male and female mice following infection with *T. gondii*. Mortality following infection with *T. gondii* occurs in the chronic stage of infection (>28 dpi), and is specific to WT mice. WT female mice are the most susceptible group.
Figure A.3 CRFr2 deficiency attenuates weight loss following *T. gondii* infection. Both males and females lost weight following infection with *T. gondii*. Females are the most susceptible, losing up to 20% of their body weight and failing to re-gain weight. Both male and female CRFr2−/− mice are less susceptible to the weight affects of *T. gondii* infection.
Figure A.4 Infection with *T. gondii* increases expression of CRFr2 in the ventromedial hypothalamus of WT mice. Gene expression of CRFr2 within the ventromedial hypothalamus was compared from naïve or chronically infected male mice. Data are presented as mean values ± SEM (n=4-6). *, $P < 0.05$ following t-test.
Figure A.5 CRFr2 deficiency rescues *T. gondii*-mediated heightened corticosterone response to an acute restraint stress. Both male and female WT mice display enhanced corticosterone production following an acute restraint stress (shaded bar) when infected with *T. gondii*. CRFr2−/− mice show no difference in stress responsivity following infection. Data are presented as mean values ± SEM (n = 4-7).
Figure A.6 CRFr2<sup>−/−</sup> mice are rescued from female-specific behavioral effects of infection with *T. gondii*. WT females spend more time in the open arms of the elevated plus maze.
Figure A.7 Sex differences in basal and *T. gonii*-induced cytokine and chemokine production. Venn diagram of sexually dimorphic cytokine and chemokines in naïve, acutely infected, and chronically infected males and females. Proteins listed are those with significantly different probabilities of expression between the sexes at each time point. Notably, VEGF, IL-2, and KC (keratinocyte chemoattractant) display sex differences in probability of expression at all three time points.


Dahlström A, Fuxe K (1964) Evidence for the existence of monoamine-containing neurons in the central nervous system. Uppsala.


Melgert BN, Postma DS, Kuipers I, Geerlings M, Luinge MA, van der Strate BW, Kerstjens HA, Timens W, Hylkema MN (2005) Female mice are more susceptible to the


