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Chronic Adolescent Stress Differentially Sensitizes Neuro-Immune Reactivity in Male and Female Rats

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B.A. Vassar College

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An abstract of

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ABSTRACT

Adversity early in life is a reliable predictor of psychiatric disorders such as depression and anxiety which are increasingly recognized to have an immune component. However, the mechanisms by which early life adversity promotes inflammation are not yet fully defined. Using a chronic adolescent stress (CAS) model in rats, I tested the hypothesis that a history of CAS exaggerates induction of the pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway in the adult rat hippocampus without impacting the peripheral immune response. I also assessed potential sex differences because it is unclear whether females, who are twice as likely to suffer from mood and anxiety disorders as males, are disproportionally affected by stress-primed inflammation. Male and female adolescent rats underwent a CAS paradigm or received no stress. Five weeks following the last stressor all rats received a single, systemic injection of either lipopolysaccharide (LPS) or vehicle to unmask possible immune-priming effects of CAS. Through a series of experiments, I demonstrated evidence of central immune sensitization in female CAS rats and both peripheral and central immune sensitization in male CAS rats. These changes were epitomized by an exaggerated NFκB-driven hippocampal transcriptome and altered glucocorticoid receptor signaling in response to LPS. Collectively, these results illustrate one mechanism via which early life adversity promotes neuroinflammation and associated behavioral deficits, and address potential sex-specific mechanisms of CAS-mediated immune priming. I conclude that chronic stress experienced during adolescence leads to enduring changes to immune reactivity in both males and females, and that the mechanism and manifestation of such alterations may be sex-specific.

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Bekhbat M, Neigh GN. Sex Differences In The Neuro-Immune Consequences Of Stress: Focus On Depression And Anxiety. *Brain Behav Immun.* 2017.

Neigh, G., Bekhbat, M., & Rowson, S. (2018-01-24). Neuroimmunology: Behavioral Effects. *Oxford Research Encyclopedia of Neuroscience.* Retrieved 19 Feb. 2018, from http://neuroscience.oxfordre.com/view/10.1093/acrefore/9780190264086.001.0001/acrefore-9780190264086-e-7.

1.1.Mood disorders as imbalance of stress and immune signaling

Throughout the evolution of mammalian physiology, stress-inducing situations reliably required activation of the immune system to help cope with potential injury and to facilitate healing. Conversely, activation of the immune system also consistently called for launching of the stress response to induce alertness and vigilance to aid in survival. As such, our endocrine and immune systems have come to be mutually-regulated (Miller and Raison 2016), generating a physiological concordance that serves vital functions such as regulation of energy allocation, reproduction, and cognition (Maier and Watkins 1998). Beyond these autonomic functions, the stress-immune duo is becoming increasingly recognized to regulate mood and behavior, which leads to a fascinating hypothesis on the origins of psychiatric disorders. The "pathogen host defense" hypothesis of depression (Raison and Miller 2013) posits that in response to a variety of environmental threats and challenges, our stress perception and immune activation have co-evolved to generate a set of "sickness behaviors" following inflammation. Sickness behaviors such as lethargy, social withdrawal, and altered appetite have traditionally helped ancestral humans recover from pathogens. These adaptive behaviors can manifest as depressed mood and behavior in modern humans whose environmental threats have largely shifted away from predators and immediate survival to psychosocial challenges (Raison and Miller 2013, Miller and Raison 2016). In support of this hypothesis, considerable evidence indicates that psychological stress induces immune activation via the same signaling pathways as physiological stress – a process termed "sterile" inflammation. With the resolution of acute threat, both stress and immune axes return to homeostasis in a healthy organism. When an

organism experiences stress chronically, however, the balance between the stress and immune axes is thought to be disrupted, resulting in excessive inflammation in the body and the brain. In contrast to acute inflammatory episodes due to pathogens, chronic stress-related inflammation is chronic and low-grade (Rohleder 2014). Low-grade neuroinflammation in stress-related neural circuitries is increasingly understood to be linked to the pathophysiology of depression (Iwata, Ota et al. 2013).

Few risk factors predict the later development of mood and anxiety disorders more robustly than exposure to significant stressors during development. Childhood adversities are associated with 25-32% of adult-onset psychiatric illnesses (Green, McLaughlin et al. 2010). It is believed that perturbations to an organism during critical developmental periods for its endocrine, neural, and immune systems may sustain lasting impact on later physiology and function. Early life stress in particular is linked to heightened inflammation in adulthood (Danese, Pariante et al. 2007, Miller, Chen et al. 2009). Although adolescence was originally believed to be beyond the window of susceptibility to early life adversity, more recent findings have demonstrated that adversity during adolescence also increases the risk of psychiatric and somatic disorders (Blakemore, Burnett et al. 2010), especially those with an inflammatory component (Mills, Scott et al. 2013).

Neuroimmune response to acute stress

The stress response is the response of an organism to altered homeostasis as a result of real or perceived threats to physiological and psychological well-being (Ulrich-Lai and Herman 2009). Two principal systems mediating the stress response are the sympathetic

nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. Activation of each component mediates aspects of the stress response and modulates the immune response.

The SNS, one of the two divisions of the autonomic nervous system, consists of sensory and motor neurons in the spinal cord, associated autonomic nerves that innervate target organ tissue, and the central circuitries that regulate these neurons (Iversen 2000). The primary goal of SNS activation is to 1) prepare muscles for fighting or fleeing (via norepinephrine released in smooth muscle) and 2) facilitate hormonal regulation of autonomic functions (via epinephrine and norepinephrine secreted from the adrenal medulla into the blood). The resulting catecholamines in circulation bind to adrenergic receptors which are present on nearly all white blood cells (Sanders and Kohm 2002). Adrenergic signaling launches an inflammatory response primarily through enlisting pro-inflammatory transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (Bierhaus, Wolf et al. 2003). Activation of NFκB and its subsequent transcriptional response is a key event in the innate immune response (Hoffmann and Baltimore 2006). NFκB-driven gene expression results in secretion of numerous inflammatory mediators such as cytokines, chemokines, and acute phase proteins into the bloodstream. Following acute psychosocial stressors in healthy humans, NFκB activity in peripheral immune cells peaks within 20 minutes (Bierhaus, Wolf et al. 2003, Wolf, Rohleder et al. 2009, Kuebler, Zuccarella-Hackl et al. 2015). The immune response to acute stressors can be regulated by the endocrine system as detailed below.

Neuroendocrine response to acute stress

During a stress response, the parasymphathetic division of the autonomic nervous system is reflexively triggered to stop SNS activation (Ulrich-Lai and Herman 2009). When an organism experiences prolonged threats, the initial fight/flight response is sustained by the activation of the HPA axis which launches a complex endocrine response. The hypothalamus – endocrine hub of the brain – receives afferents from a variety of regions, including the brainstem which is among the first brain regions to sense physiologic homeostatic perturbations and limbic regions which are involved in the processing and interpretation of psychogenic stimuli (Herman and Cullinan 1997). The paraventricular nucleus (PVN) of the hypothalamus is the primary site of production of corticotropin-releasing hormone (CRH), which acts on the anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH travels in the blood to the adrenal glands where it facilitates production and release of glucocorticoids (corticosterone in rodents, cortisol in primates) from the adrenal glands. Cortisol binds to mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) localized throughout the nervous system and on peripheral tissue. Cortisol displays a higher binding affinity to MR, and as a result, MRs are usually saturated at basal conditions (De Kloet, Vreugdenhil et al. 1998). Therefore GRs are thought to mediate the physiological sequelae of the stress response, including serving as the primary suppressor of inflammatory processes. When inactive, GR resides in the cytoplasm of a cell tethered by a complex of co-chaperone proteins that prevents GR from translocating to the nucleus, a step necessary for its transcriptional activity. Once

glucocorticoids bind to GR, the ensuing conformational changes allow GR to translocate to the nucleus (Wochnik, Ruegg et al. 2005). Subsequently, the activated GR triggers anti-inflammatory gene transcription and represses the actions of pro-inflammatory transcription factors including NFκB (Cronstein, Kimmel et al. 1992, Fantidis 2010, Chinenov, Coppo et al. 2014). Activation of the HPA axis – including the release of adrenocorticotropic hormone (ACTH) and cortisol which peaks by 30-40 minutes following onset of stressor (Bierhaus, Wolf et al. 2003, Kuebler, Zuccarella-Hackl et al. 2015) –serves to suppress NFκB-driven inflammation following acute stress. The crosstalk and balance between NFκB and GR constitute a regulatory checkpoint for inflammatory processes as detailed below.

Chronic stress disrupts endocrine-immune communication

Prolonged exposure to glucocorticoids, such as during chronic stress, has the capacity to lead to glucocorticoid resistance, which broadly refers to decreased glucocorticoid signaling due to impaired activity of GRs. Even the terminology, glucocorticoid resistance, has taken on a dual identity reflecting an impact within both the endocrine and immune systems. Within the chronic stress literature, glucocorticoid resistance primarily indicates *impaired ability of glucocorticoids to terminate HPA axis activity* via negative feedback, which culminates in a prolonged stress response and elevated levels of glucocorticoids available for a longer time. In the context of chronic inflammatory disorders, glucocorticoid resistance indicates *diminished ability of glucocorticoids to suppress inflammatory processes* such as release of cytokines,

proliferation of immune cells and receptors, or, at high concentrations, to trigger apoptosis (Barnes and Adcock 2009).

Chronic stress-related mood disorders represent a multidimensional sum of the two forms of glucocorticoid resistance with increased glucocorticoid levels and a less transcriptionally active GR (Pace, Hu et al. 2007), coupled with greater activation of the innate immune system in the brain and the body that may be further exacerbated by glucocorticoids (Frank, Thompson et al. 2012, Sorrells, Munhoz et al. 2014). Perhaps even more salient than individual alterations in GR or NFκB is the impaired balance and crosstalk between GR and NFκB. In healthy systems, the crosstalk and balance between GR and NFκB activity are precisely coordinated and regulated (Adcock and Barnes 2008). In disease conditions, however, GR is unable to adequately suppress NFκB activity. In turn, inflammatory processes, now disinhibited, further impair GR's anti-inflammatory capacity, thus setting in motion a vicious circle of GR-NFκB imbalance (Pace, Hu et al. 2007). Indeed, a transcriptional signature of overactive NFκB (Miller, Chen et al. 2008), and deficient glucocorticoid signaling and/or glucocorticoid resistance (Miller, Murphy et al. 2014) constitute two of the hallmarks of chronically stressed organisms.

As chronic stress robustly predicts the development of mood and anxiety disorders, the neuroimmune profiles of chronically stressed populations and patients with stress-related psychiatric disorders can be largely similar. A stress-immune balance tipped towards excessive NFκB signaling can recapitulate most of the immune dysregulations seen in these populations including: 1) systemic increases in the pro-inflammatory cytokines interleukin- (IL) 1β, IL-6, tumor necrosis factor (TNF)-α, and C-reactive protein (CRP) (Haug, Mykletun et al. 2004, Dowlati, Herrmann et al. 2010), and 2) altered activity

of both peripheral immune cells (Maes 2011) as well as microglia, the brain's resident immune cells (Setiawan, Wilson et al. 2015). These immune outcomes (see Fig 1.1) have in turn been causally implicated in mood and behavioral deficits as described below.

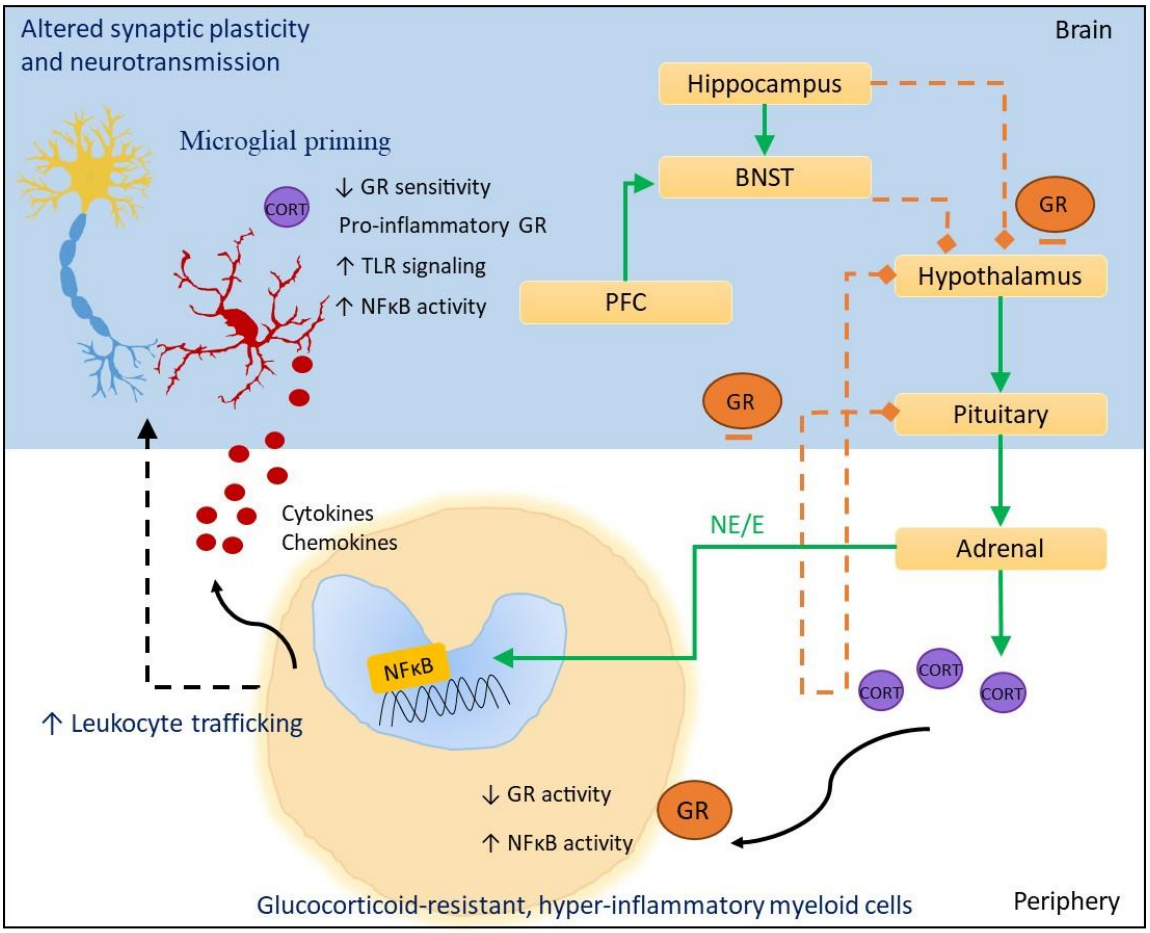

Figure 1.1. GR and NFκB: glucocorticoid resistance in the brain and immune system. Under acute stress conditions, release of catecholamines by the sympathetic nervous system activates NFκB within peripheral immune cells, and leads to the induction of cytokines and chemokines. Activation of the hypothalamic-pituitary adrenal axis results in the release of glucocorticoids, and GR suppresses NFκB in peripheral immune cells. Upon the resolution of the acute stress response, activation of the HPA axis is terminated by a GR-mediated negative feedback mechanism that integrates inhibitory limbic signals to the

hypothalamus. Under chronic stress conditions, impaired GR activity within peripheral myeloid cells can disinhibit NFκB-mediated inflammation. In turn, glucocorticoid-resistant, hyper-inflammatory immune cells can traffic into the brain, eventually becoming recruited to brain regions that regulate the stress response. The disruption in endocrine-immune balance appears to also be due to primed microglia that display decreased GR sensitivity, pro-inflammatory GR, and exaggerated innate immune activation including increased NFκB and Toll-like Receptor signaling. Chronic stress pathology also impairs the GR-mediated negative feedback regulation of HPA axis, thus further perpetuating stress-induced neuroinflammation. The functional significance of the altered GR-NFκB balance may be manifested in the brain as altered synaptic plasticity and neurotransmission. BNST=bed nucleus of the stria terminalis, PFC=prefrontal cortex, TLR= Toll-like Receptor, CORT=cortisol/corticosterone.

Inflammation causes mood and behavioral deficits

Abundant evidence documents causal relationships between inflammation and mood disorders. Some of the most compelling evidence for the behavioral impact of neuroinflammation come from work conducted in patients receiving interferon therapy for the treatment of viral infections and certain types of cancer. Up to half the patients receiving interferon-α develop depressive symptoms including depressive mood, cognitive deficits, fatigue, and abnormal appetite (Capuron, Gumnick et al. 2002, Capuron and Miller 2004, Raison, Capuron et al. 2006). Selective treatment response among patients with major depressive disorder also confirms a causal role for inflammation. For example, depressed

patients who respond to the antidepressant amitriptyline display reduced TNF-α post-treatment, but non-responders do not (Lanquillon, Krieg et al. 2000). Furthermore, the TNF-α antagonist infliximab is effective at reducing depressive symptoms only among depressed patients who present with high serum CRP (Raison, Rutherford et al. 2013). These findings are corroborated by observations that anxiety and depression are strongly correlated with somatic symptoms (Haug, Mykletun et al. 2004).

In addition to clinical manifestations of behavioral deficits, sub-clinical changes in affect and cognition can be induced by immune activation (Schedlowski, Engler et al. 2014). For example, low doses of endotoxin that cause a mild immune response in healthy humans temporarily lead to depressed mood, a feeling of social isolation, anxiety, and anhedonia (Eisenberger, Inagaki et al. 2010, Engler, Benson et al. 2015). Immune effects extend to cognition such that among healthy males administered a typhoid vaccination, greater IL-6 concentrations following the vaccine correlated with slower reaction times in a cognitive task (Brydon, Harrison et al. 2008). Similarly, aging, via a decrease in optimal anti-inflammatory defense in the brain, can lead to sub-clinical deficits in affect and cognition (Deary, Corley et al. 2009, Corona, Fenn et al. 2012).

Inflammation impacts distinct neural circuits to mediate different aspect of mood disorders. For example, acute stress-induced peripheral cytokines are associated with neural activity in brain regions related to stress and emotional arousal including the amygdala, prefrontal cortex, and anterior cingulate cortex (Slavich, Way et al. 2010, Muscatell, Dedovic et al. 2015, Gupta, Labus et al. 2016). Among hepatitis C patients treated with interferon-α (IFN-α), inflammation-induced decline in dopamine synthesis and availability in the basal ganglia – subcortical structures crucial to motivation, reward,

and locomotor activity – is associated with malaise symptoms including fatigue, anhedonia, and psychomotor slowing (Felger and Miller 2012). Furthermore, inflammation-induced anhedonia and psychomotor slowing appear to be mediated by an inverse relationship between CRP concentrations and functional connectivity among cortical regions and the basal ganglia (Felger, Li et al. 2016). Additionally, activation of the anterior cingulate cortex, a region implicated in anxiety and alarm, is associated with cognitive dysfunction in patients receiving IFN-α (Capuron, Pagnoni et al. 2005). The functional consequences of neuroinflammation within distinct neural circuits may be mediated by alterations in synaptic (Pribiag and Stellwagen 2014) and neuronal plasticity (Kubera, Obuchowicz et al. 2011), neurotransmission (Merali, Lacosta et al. 1997, Haroon, Miller et al. 2016), and related behavioral outcomes (Howerton, Roland et al. 2014).

1.2.The neuro-immune axis and its effectors

Once believed to be immune-privileged (Carson, Doose et al. 2006), the brain is now recognized to extensively interact with the immune system via multiple mechanisms that allow extravasation and relay of inflammatory signals from the periphery. The primary mediators of neuroinflammation are cytokines – small immunomodulatory proteins released by immune cells. The pro-inflammatory cytokines IL-1β, interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-a) serve as primary mediators of brain-immune communication, and their role in stress-induced inflammation and sickness behavior has been extensively studied. In addition, inflammatory stimuli can lead to the release of

prostaglandins – hormone-like lipid mediators crucial to various aspects of the sickness behavior – from many different cell types (Pecchi, Dallaporta et al. 2009). In response to injury and infection, the liver also releases acute phase proteins such as C-reactive protein (CRP). All of these peripheral inflammatory molecules can activate receptors localized within the central nervous system (CNS) (Rothwell, Luheshi et al. 1996), thus leading to neuroinflammation and accompanying changes in behavior.

Interestingly, these inflammatory mediators can be released as a result of both pathogens ("classical inflammation") as well as the body's endogenous danger signals in the absence of pathogens ("sterile inflammation"). When the body is infected by micro-organisms such as bacteria and viruses, peripheral immune cells recognize specific components called pathogen-associated molecular patterns (PAMPs) displayed by these invading pathogens (Tang, Kang et al. 2012). PAMPs such as LPS activate toll-like receptors (TLRs) and other pathogen recognition receptors on a variety of immune cell types. In the absence of pathogen-induced inflammation, many environmental and cellular stressors can also lead to pro-inflammatory outcomes (Chen and Nunez 2010). This is accomplished through recognition of danger-associated molecular patterns (DAMPs) present on many endogenous molecules including heat shock proteins, uric acid, RNA, DNA, and S100 proteins. While mechanistically distinct from neuroinflammation that results from invading pathogens, sterile neuroinflammation nonetheless results in the induction of shared pro-inflammatory mediators (Fleshner, Frank et al. 2017). Sterile inflammation may be useful for maintaining neuronal homeostatic functions and synaptic plasticity; yet it can turn maladaptive in disease conditions such as chronic stress.

Brain-immune crosstalk occurs through both a neural route mediated by innervation of peripheral tissue and lymphoid organs as well as a humoral route mediated by peripherally derived inflammatory molecules. The afferent neural route consists primarily of the vagus – the tenth cranial nerve – which innervates visceral organs including the gut and the liver (Pavlov and Tracey 2012). The vagus relays viscerosensory information by projecting primarily to the nucleus tractus solitarius (NTS) and ventrolateral medulla, major relay nuclei in the brainstem that send catecholaminergic projections to forebrain regions involved in autonomic regulation and behavioral responses (Gaykema and Goehler 2011). In particular, the NTS and ventrolateral medulla heavily project to the hypothalamus, thus initiating the well-established neuroendocrine response to immune activation as well as changes to autonomic regulation. In addition to having reciprocal connections to the hypothalamus, the NTS and ventrolateral medulla send projections to limbic structures such as the amygdala and bed nucleus of the stria terminalis (Gaykema, Chen et al. 2007) – areas that are important to orchestrating various aspects of sickness behavior such as fear and pain. Vagotomy experiments lead to attenuated behavioral responses to peripherally administered cytokines (Bluthe, Michaud et al. 1996, Luheshi, Bluthe et al. 2000), suggesting that the vagus nerve mediates parts of sickness behavior.

The humoral route of brain-immune communication occurs through leakage of circulating inflammatory signals (PAMPs and cytokines) into the brain via a permissive blood-brain barrier (BBB), or through cytokines acting on the endothelial cells forming the BBB (Dantzer, O'Connor et al. 2008). Furthermore, cells of the circumventricular organs and the choroid plexus relay peripheral inflammatory signals to adjacent brain parenchyma. These cells can also be induced to produce cytokines and prostaglandins themselves, and

project to brain regions including the NTS and PVN of the hypothalamus that are important to behavioral changes (Quan 2014). Inflammatory signals in the brain parenchyma are amplified and sustained with the enlistment of microglia. As the primary immune effector cells in the CNS, microglia constitute the first-line response to injury and infection, and later on promote repair and resolution of inflammation. When resting, microglia survey the environment, and regulate neuronal excitability, synaptic architecture, neurogenesis, and programmed cell death (Lenz and McCarthy 2015). Parenchymal microglia and neurons express receptors for cytokines and prostaglandins predominantly within the brainstem nuclei, forebrain autonomic nuclei, and limbic structures (Rothwell, Luheshi et al. 1996). Following activation of their immune receptors, these parenchymal cells locally produce their own inflammatory signals and thus are able to propagate this cytokine transmission to yet deeper brain structures.

1.3.NFKB, chronic stress, and mood disorders

NFκB as a central regulator of the immune system

The family of NFκB proteins, when dimerized, serve as a transcription factor that regulates immune and inflammatory responses, cell survival, development, synaptic plasticity, and neurogenesis (Kaltschmidt and Kaltschmidt 2009). NFκB is present in all cell types (Hoffmann and Baltimore 2006) and due to its prominent role in inflammatory signaling, is extensively studied in many diseases including cancer, rheumatoid arthritis,

asthma, cardiovascular and metabolic disease. Five polypeptides in the NFκB family (p65, cRel, Rel, and the precursor proteins p100 and p105 which are proteasomally cleaved into the subunits p52 and p50) form 15 possible dimers that serve as transcriptional activators or repressors, or display no DNA-binding ability depending on dimer composition (Hoffmann and Baltimore 2006). The p65, Rel, and cRel proteins possess a transcriptional activation (TA) domain, which is required for target gene transcription. In contrast, the p50 and p52 subunits do not contain a TA domain, and are unable to regulate gene transcription unless dimerized to another NFκB subunit that possesses a TA domain. Dimers exclusively composed of p50 or p52 are therefore often referred to as transcriptional repressors. Additionally, the p65/Rel, cRel/Rel, and Rel/Rel dimers are unable to bind to DNA.

NFκB signals through two distinct pathways that contrast in function, dimer composition, and molecular regulation (Fig 1.2). The canonical pathway is activated by pro-inflammatory stimuli including the archetypal pro-inflammatory cytokines TNF-α and IL-1β as well as microbial products. The canonical pathways mediates rapid immune activation such as that during acute infection. The non-canonical pathway can be activated by both immune (lymphotoxin-β, CD40 ligand, B-cell activating factor) and non-immune stimuli, and is involved in slow and sustained immune regulation such as that which occurs during development of the immune organs. The canonical and non-canonical pathways also differ in the composition of NFκB. The canonical pathway features the most common form of NFκB, the heterodimer which consists of p65 and p50. In contrast, the non-canonical pathway features heterodimers containing p52 and Rel proteins.

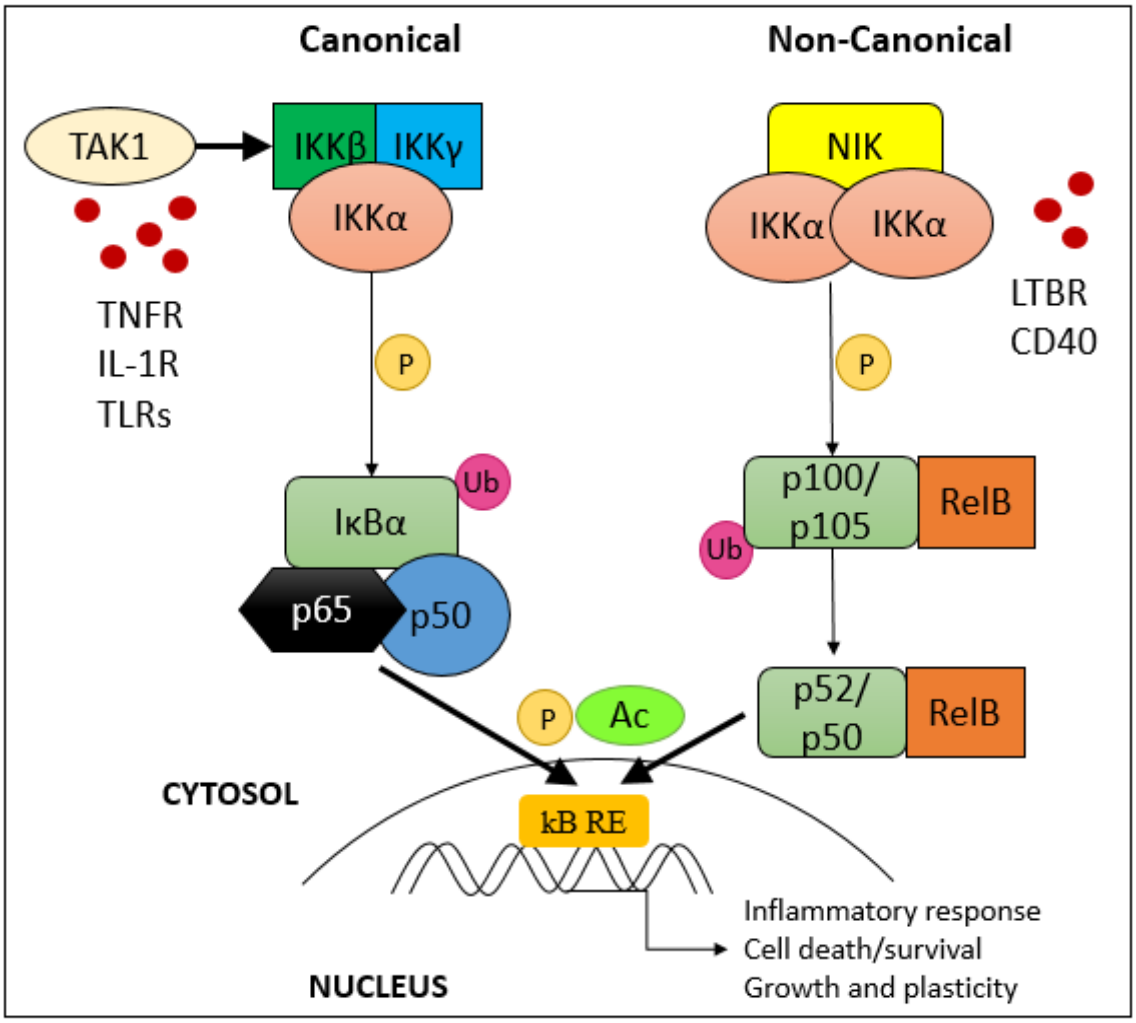

Figure 1.2. Molecular Regulation of NFκB. The canonical and non-canonical NFκB pathways reside in the cytoplasm, tethered by the inhibitor proteins IκBα, p100, or p105. Following TAK1-mediated activation by TNF, IL-1, and Toll-like receptor transduction pathways, the canonical IKK complex phosphorylates IκBα, thus releasing the p65-p50 heterodimer to translocate to the nucleus. In the non-canonical pathway, following activation by lymphotoxin-β receptor (LTBR) or CD40 ligand signaling, IKKα phosphorylates p100 or p105, thus triggering their respective cleavage into p52 or p50. The newly freed p52/p50-RelB complex then translocates into the nucleus. The NFκB complex

may be subject to further post-translational modifications including phosphorylation and acetylation prior to binding to the κREs.

In the absence of relevant stimuli such as cytokines, growth hormones, cellular and oxidative stress, and synaptic transmission, the NFκB dimer resides sequestered in the cytoplasm by inhibitor IκB proteins. While IκB proteins mask the nuclear localization signal on NFκB, IκB does not completely prevent NFκB's nuclear translocation. Rather, IκB is thought to reduce the rate of nuclear translocation of the NFκB-IκB complex that is constantly shuttling between the cytoplasm and the nucleus (Birbach, Gold et al. 2002). The main regulatory event during NFκB activation is the phosphorylation and subsequent degradation of the inhibitor IκB proteins by the appropriately-named IκB kinase (IKK) (Israel 2010), which frees the NFκB complex to translocate to the nucleus. There are two IKK proteins, IKKα and IKKβ, and a third regulatory protein IKKγ (also referred to as NEMO). The canonical IKK complex contains a heterodimer of IKKα and IKKβ, and is regulated by the scaffold protein IKKγ. In the canonical pathway, the IKKβ catalytic subunit phosphorylates IκB, which is then ubiquitinated and proteasomally degraded, thus freeing the NFκB dimer to translocate into the nucleus. In contrast, the non-canonical complex contains only the IKKα homodimer and its upstream kinase NIK (Hoffmann and Baltimore 2006). In the non-canonical pathway, IKKα phosphorylates the p100 or p105 subunit, which are then processed into p50 or p52, respectively. Translocation of the newly released NFκB is mediated via dynein (Mikenberg, Widera et al. 2007) and importin molecules (importin-α) (Fagerlund, Melen et al. 2008).

Once in the nucleus, NFκB binds κB response elements (κRE) located in the promoters of cytokines, chemokines, adhesion molecules, inflammatory enzymes, and receptors. Binding of NFκB to κRE initiates gene transcription through recruiting the basal transcription machinery including RNA Pol II as well as co-factors such as p300 and CREB binding protein (CBP) that are necessary for transcription. All dimers of NFκB can bind to the κB response element; however, the expression and activation of each dimer type is highly cell type-, developmental stage- and stimulus-dependent (Hoffmann and Baltimore 2006, Sen and Smale 2010). In addition, NFκB binding to the promoter has been described to trigger the formation of enhanceosomes, which are nucleoprotein complexes consisting of several cooperating transcription factors, that ensure the spatially- and temporally-specific induction of a gene (Tian and Brasier 2003). Due in part to the extensive redundancy and cross-feedback in inflammatory signaling pathways, different inflammatory transcription factors usually act in concert to yield stimulus-specific activation of each target gene. This is accomplished by varying combinations of transcription factors such as NFκB, SP-1, IRFs, STATs, CREB, and AP-1 sequentially binding to their respective response elements within an enhancer region of the target gene. Another key determinant of the rate of target gene induction by NFκB is elements of the promoter architecture such as number and proximity of κRE present (with greater number of κRE clustered closely together corresponding to stronger induction. Multiple receptor-specific intracellular transduction pathways such as TLRs, TNF-α receptor, IL-1 receptor, T- and B-cell receptor signaling can all converge upon the NFκB pathway. Furthermore, adjacent inflammatory signaling cascades including the p38/MAPK, JNK pathways can interact with, and mutually regulate, the NFκB pathway (Oeckinghaus, Hayden et al. 2011).

Due to its key role in regulating innate immunity, NFκB is positioned to serve critical roles in several mechanisms of stress-induced neuroinflammation. Exaggerated NFκB induction and/or signaling in leukocytes has been evidenced in those with a history of early life stress (Pace, Mletzko et al. 2006, Pace, Wingenfeld et al. 2012) and high perceived chronic stress (Miller, Rohleder et al. 2009), as well as patients with bipolar disorder (Miklowitz, Portnoff et al. 2016), post-traumatic disorder (Pace, Wingenfeld et al. 2012, Guardado, Olivera et al. 2016), and major depressive disorder (Pace, Mletzko et al. 2006, Miklowitz, Portnoff et al. 2016). Many of these studies also report concurrent blunted glucocorticoid sensitivity (Pace, Wingenfeld et al. 2012). Conversely, interventions such as cognitive-behavioral stress management and yogic meditation have been found to reverse upregulation of transcripts with the κB response element (Antoni, Lutgendorf et al. 2012) (Black, Cole et al. 2013).

Furthermore, most known mechanisms of stress-induced neuroinflammation share altered NFκB signaling as either an upstream transcriptional regulator step or a common downstream event in which all other processes culminate. For example, TLR2 and TLR4 signaling within microglia has been demonstrated to be one mechanism underlying the immune-priming effect of stress and glucocorticoids (Frank, Miguel et al. 2010, Weber, Frank et al. 2013). In this model, high mobility group box 1 (HMGB1), one of the best-characterized DAMPs, resides undetected in the nucleus of a cell when unstimulated but is released upon activation by psychological stress (Cheng, Pardo et al. 2016). The released HMGB1 protein can signal through TLRs present primarily on microglia to activate these cells and further promote the spread of inflammation in the brain via NFκB signaling (Weber, Frank et al. 2015, Cheng, Pardo et al. 2016). Activation of NFκB via HMGB1 can

also induce the inflammasome-associated protein nod-like receptor protein 3 (NLRP3), which has been implicated in microglial priming by stress and behavioral deficits due to chronic stress (Weber, Frank et al. 2015).

NFκB in the brain

Constitutive NFκB activity has been demonstrated in several brain regions including the hypothalamus, cortex, hippocampus, amygdala, olfactory lobes, and cerebellum (Schmidt-Ullrich, Memet et al. 1996, Bhakar, Tannis et al. 2002). During systemic inflammation, brain regions most accessible to cerebrospinal fluid (CSF) and blood including the circumventricular organs, neighboring regions, and elements of the blood-brain barrier (BBB) are likely to express the greatest extent of NFκB activation owing to the soluble pro-inflammatory factors present in the CSF and blood (Rivest 2001). NFκB is likely primarily active in the immune-competent cells of the CNS including microglia due to the widespread expression of immune receptors on these cells. However, NFκB is expressed in neurons, and is thought to play roles in synaptic plasticity, brain development (Boersma, Dresselhaus et al. 2011), and synaptic signaling (Meffert, Chang et al. 2003).

Conflicting views exist regarding the origin of NFκB activity in neurons. Whereas some groups have found minimal NFκB activity in primary neuronal cultures (Listwak, Rathore et al. 2013, Mao, Phanavanh et al. 2016) others have found constitutive NFκB activity in neurons (Kaltschmidt, Kaltschmidt et al. 1994, Bhakar, Tannis et al. 2002), and the discrepancy is thought by some to result from varying degrees of purity of neuronal

cultures due to mixed glial populations (Barger & Mao, 2012). It has been suggested that NFκB signaling in glia mediates pathological pro-inflammatory processes whereas signaling within neurons promotes their survival (Camandola and Mattson 2007). Distinct cellular sources of NFκB signaling may therefore account for why NFκB activity seems to lead to varied functional outcomes in chronic stress studies as detailed below.

Chronic stress, NFKB, and hippocampus

The hippocampus has continued to be a primary focus of chronic stress studies given its vulnerability to both stress and inflammation. The hippocampus is part of a circuitry that regulates the HPA axis at baseline and provides negative feedback to suppress its activation following prolonged stress responses (Jacobson and Sapolsky 1991). It is also one of the most plastic regions in the brain, and chronic stress has extensively been demonstrated to alter synaptic architecture and strength in the hippocampus (Christoffel, Golden et al. 2011). The high degree of vascularization in the hippocampus combined with its involvement in the stress-regulatory network render the hippocampus somewhat selectively vulnerable to stress-induced inflammatory changes. For example, hippocampal microglia from stressed rats secrete greater pro-inflammatory cytokines when stimulated *ex vivo* (Frank, Baratta et al. 2007). Additionally, the hippocampus of stress-susceptible rodents display decreased vascular integrity (Menard, Pfau et al. 2017), and increased tissue infiltration of leukocytes (Wohleb, Powell et al. 2013). Among patients with major depressive disorder, a transcriptomic profile characterized by excessive immune signaling has been found in the hippocampus (Mahajan, Vallender et al. 2018) and greater peripheral

IL-6 predicts smaller hippocampal volume (Frodl, Carballedo et al. 2012). Hippocampal NFκB expression and/or signaling has been demonstrated to be altered in several rodent models of chronic stress as detailed below.

Stress-induced alterations to NFκB appear to be dependent on the timing and type of stress, as well as model organism and endpoint tissue. Exposure to 21 days of chronic restraint stress has been shown to increase the expression of NFκB pathway proteins including IκB, p65, p50 both at baseline and also upon a novel acute stressor (Gray, Rubin et al. 2014). Interestingly, the chronic restraint-induced increase in p50 persisted even after a 21-day recovery, potentially suggesting long- lasting impact of chronic stress on NFκB expression. In contrast to the chronicity of this stress paradigm, a single exposure to predator odor in a mouse model of PTSD has also been demonstrated to induce hippocampal NFκB activity which was found to underlie behavioral deficits in extreme behavioral responders (Cohen, Kozlovsky et al. 2011). Chronic stress has also been demonstrated to prime the NFκB pathway in the hippocampus to a subsequent immune challenge. Rats subjected to chronic unpredictable stress did not differ from non-stressed littermates in basal NFκB DNA binding, but upon a systemic LPS probe were found to display exaggerated NFκB DNA binding in the hippocampus (Munhoz, Lepsch et al. 2006). Interestingly, this stress-mediated NFκB priming was attenuated by GR inhibition, suggesting an unexpected facilitative role of GR in these regions on the inflammatory consequences of chronic stress exposure (Munhoz, Lepsch et al. 2006). Furthermore, a higher NFκB-to-GR ratio has been observed in chronic isolation stress (Djordjevic, Adzic et al. 2009), whereas an increase in NFκB and decrease in GR (Hu, Zhang et al. 2016) was found in the hippocampus of mice administered chronic dexamethasone. Collectively,

these data provide support to the idea that chronic stress generally promotes and sensitizes NFκB signaling, which leads to an overall pro-inflammatory outcome.

However, it is important to be mindful of the dramatic variability in chronic stress paradigms which has produced conflicting evidence regarding the impact of chronic stress on NFκB activity and function. In particular, stress paradigms of either considerable severity or long duration may suppress the immune system, thus potentially leading to conflicting findings regarding NFκB activity. By way of example, in a sensory contact model in which subordinate mice bred for long attack latency (LAL) live with highly aggressive, dominant mice bred for short attack latency (SAL), separated by a perforated transparent partition for 25 days, NFκB pathway genes were found to be downregulated in the hippocampus of LAL mice (Feldker, Morsink et al. 2006). Similarly, daily administration of corticosterone to rats for 60 days did not cause hippocampal NFκB activation despite causing behavioral deficits (Plaschke, Feindt et al. 2006). These studies illustrate the complexity of endocrine-immune crosstalk in chronically stressed systems where the default homeostasis may have shifted.

Although ample evidence of changes in NFκB signaling in chronic stress or immune alteration models exists, the implications of such changes for mood and behavior remain incompletely understood. To this end, hippocampal increases in NFκB signaling were found to mediate the pro-depressive actions of four weeks of chronic unpredictable stress (CUS) through suppressing adult neurogenesis and altering the ratio of neural stem-like cells (Koo, Russo et al. 2010). However, in a study employing a six-day CUS paradigm, LaPlant, Chakravarty et al. (2009) found that ovariectomized female mice were protected against the pro-depressive effects of CUS compared to intact females, an effect

that was mediated by NFκB signaling in the nucleus accumbens (LaPlant, Chakravarty et al. 2009). While NFκB is best recognized as a pro-inflammatory signaling pathway that can lead to the release of cytokines and chemokines, apoptosis and cell death, it is also constitutively involved in synaptic plasticity and neural transmission. In fact, contrary to the commonly held belief that neuroinflammation is generally detrimental, a basal degree of inflammatory signaling appears to be necessary for synaptic signaling and long-term potentiation (LTP) in the hippocampus (Rogers, Morganti et al. 2011) and may therefore prove protective in the context of chronic stress.

1.4.Impact of sex and the adolescent period on stress-related inflammation

Sex differences in inflammation and mood disorders

As compelling as the pathogen host defense hypothesis of depression (Raison and Miller 2013) is, it is currently unclear as to what extent stress-immune dysfunction fits with the existing epidemiological facts of mood disorders such as sex differences in their prevalence. Women of reproductive ages display approximately twice the prevalence of depression, anxiety, and post-traumatic stress disorder (PTSD) compared to men (Breslau, Davis et al. 1997, Piccinelli and Wilkinson 2000). Despite well-established sexual dimorphisms in the stress response, immunity, and prevalence of stress-linked psychiatric illnesses, much of current research investigating the neuroimmune impact of stress remains exclusively focused on male subjects.

Converging lines of evidence suggest that the female sex may be associated with increased susceptibility to mood deficits following both long- and short-term inflammation in the body. Although mixed, some evidence for sex differences is provided by studies examining the effects of IFN-α treatment on mood in patients with Hepatitis C. In some cases, IFN-α generated greater depressive symptoms in women compared to men (Koskinas, Merkouraki et al. 2002); however, no sex differences in depressive symptoms following interferon-α treatment have also been reported (Bonaccorso, Marino et al. 2002). In contrast to these smaller individual studies, a recent meta-analysis by Udina, Castellvi et al. (2012) found the female sex to be predictive of major depressive episodes following antiviral treatment. In addition, a series of recent studies in healthy humans have suggested that women are behaviorally more vulnerable to the acute depressogenic effects of endotoxin-induced inflammation (Eisenberger, Inagaki et al. 2009, Moieni, Irwin et al. 2015). While both healthy men and women display increases in pro-inflammatory cytokines when administered a small dose of LPS, cytokine induction was associated with depressed mood and increased feelings of social disconnectedness in women only. Furthermore, the relationship between plasma increases in IL-6 and depressed mood was found to be mediated by an association between IL-6 and increased activity in dorsal anterior cingulate cortex, anterior insula – areas involved in processing social pain – in women who received LPS but not men (Eisenberger, Inagaki et al. 2009). Similarly, an intranasal endotoxin challenge led to depressive-like behavior, and increased expression of TNF-α and IL-6 in the hippocampus and brainstem of female, but not male, rats (Tonelli, Holmes et al. 2008).

Several clinical trials testing non-steroidal anti-inflammatory drugs and other anti-cytokine agents for the treatment of stress-related psychiatric illnesses have demonstrated modest therapeutic benefits (Kohler, Benros et al. 2014), whereas others showed no effect (Eyre, Air et al. 2015). However, conflicting evidence exists regarding the involvement of inflammatory dysregulation in males and females with depression. Although depressed women report a higher prevalence of somatic symptoms (Silverstein 1999) and are more vulnerable to the harmful effects of inflammation (Derry, Padin et al. 2015), the link between stress-related psychiatric illnesses and low-grade inflammation is more consistently found in men than women (Liukkonen, Rasanen et al. 2011, Ramsey, Cooper et al. 2016). In fact, C-reactive protein (CRP), one of the most consistently reported inflammatory biomarkers of mood disorders, was recently found to be associated with anxiety and comorbid anxiety and depression in men, but not women (Liukkonen, Rasanen et al. 2011). Moreover, when large-scale data from the Netherlands Study of Depression and Anxiety (NESDA) were re-analyzed with sex as a variable, several immune markers previously reported to be associated with depression turned out to be male-specific (Ramsey, Cooper et al. 2016). These reports highlight the inherent etiological heterogeneity in mood disorders whereby inflammation occurs in only a subset of affected populations, and potentially demonstrate clinically relevant implications for the generalizability of anti-inflammatory treatments for mood disorders.

Sex differences in stress and immune axes

Studies conducted both in humans and in animal models detail considerable sex differences in the endocrine, behavioral, and neural aspects of the stress response, driven primarily by adrenal and gonadal influences. An important between-species difference emerges with regards to sex differences in HPA axis activity following a stressor. In addition to a more constitutively active HPA axis at baseline conditions, female rodents display a more robust and prolonged activation of the axis following acute stress (Handa, Burgess et al. 1994). In contrast, men display greater induction of ACTH and cortisol compared to women following the Trier Social Stress Test (TSST), a highly standardized laboratory acute stressor (Stephens, Mahon et al. 2016). Sex-dependent disparity in the prevalence of stress disorders is partially attributed to the impact of gonadal hormones on the neuroendocrine system throughout development (Panagiotakopoulos and Neigh 2014). In both rodents and humans, testosterone is negatively correlated with ACTH and corticosterone/cortisol (Stephens, Mahon et al. 2016); whereas, estrogen acts on both the hypothalamus and the adrenal glands to stimulate the output of the HPA axis (Panagiotakopoulos and Neigh 2014). Furthermore, the expression and regulation of GR (Kumsta, Entringer et al. 2007), as well as interaction of sex steroids with GR, are sexually dimorphic (Bourke and Neigh 2011, Bourke, Harrell et al. 2012, Bourke, Raees et al. 2013), and may lead to differential onset, magnitude, and resolution of endocrine responses in males and females during stressor exposure.

Sex differences in neuroinflammation and its consequences

Nuanced effects of sex on different aspects of immunity such as wound healing, immunosuppression, host-defense mechanisms, and chronic, low-grade inflammation have also been reported (Klein, Marriott et al. 2015). Consistently, males and females display distinct vulnerabilities to different types of inflammatory dysregulation which suggests underlying biological differences. Neuroinflammatory diseases such as multiple sclerosis, Alzheimer's disease, and chronic pain are more common among females (Loram, Sholar et al. 2012); and dysregulation of immunocyte function, steroid hormone signaling (Oertelt-Prigione 2012), and gut microbiome-driven changes in autoimmunity (Markle, Frank et al. 2013) have been proposed as potential mediators. However, females are behaviorally and/or immunologically protected in some models of inflammatory diseases such as experimental autoimmune encephalomyelitis (Harpaz, Abutbul et al. 2013), hypoxic-ischemic encephalopathy (Mirza, Ritzel et al. 2015), and microembolic stroke (Nemeth, Reddy et al. 2014), and this protection is thought to be derived, at least partially, from the anti-inflammatory actions of estrogen and progesterone (Czlonkowska, Ciesielska et al. 2006). Both estrogen and progesterone can suppress inflammation in various models of injury and inflammation dependent on the inflammatory milieu (Straub 2007, Deutsch, Espinoza et al. 2013). These underlying sex differences in neuroimmunity are likely to impact stress-evoked inflammation in the brain, and potentially contribute to the differential behavioral outcome of stress in males and females.

Sex differences in peripheral and central inflammatory and immune processes have been studied both *in vivo* and *ex vivo*. Peripheral immune cells exhibit considerable sexual

dimorphism in receptor expression (De Leon-Nava, Nava et al. 2009, Klein 2012). In addition, microglia are known to display a dynamic sexual dimorphism in their number and morphology throughout development (Schwarz, Sholar et al. 2012). As the rat brain develops, the proportion of round or amoeboid-shaped microglia decreases, and microglia with thicker processes or long and ramified morphology become increasingly common in several brain regions regardless of sex. However, the rate of microglial colonization and morphological development differs between males and females such that males have more microglia early in post-natal development, which may confer heightened vulnerability to the negative consequences of immune insult during this time in males. Indeed, exposure to inflammatory insults during early development, especially *in utero* immune stressors, has been suggested to be linked to developmental disorders such as autism and schizophrenia particularly in males (Bale 2015). In contrast, female rats possess more microglia with activated morphology starting around early puberty and in adulthood (Schwarz, Sholar et al. 2012), which interestingly coincides with the onset of a significantly greater prevalence of mood disorders in women but these two temporally congruent events have not yet been mechanistically linked. Glia from males and females have been shown to respond differentially to sex steroids during early development and in adulthood (Loram, Sholar et al. 2012). These dynamic sex differences in both stress response and inflammation may influence stress-induced inflammatory processes in the brain, and thereby, the resulting changes in mood and behavior, as discussed in the next section.

Sex differences in the neuro-immune consequences of stress

Sex differences in stress-induced neuroinflammation may arise from sexual dimorphism at multiple levels including cellular, molecular, and endocrine regulation. The few existing reports on the effects of stress on microglia in the male and female rodent brain have used different stressors and developmental timelines, and examined a variety of brain regions, thus making direct comparisons difficult. Table 1.1 summarizes the available evidence for stress-induced alterations in microglial number and morphology as well as cytokine signaling in microglia and neurons.

These cellular and molecular changes likely reflect modulation of inflammatory processes by adrenal and gonadal hormones. Using an experimental autoimmune encephalomyelitis (EAE) model in which female mice are generally more resistant, Harpaz, Abutbul et al. (2013) found that exposure to chronic variable stress predisposed females to a more pro-inflammatory profile following EAE compared to similarly stressed males. Abolishment of the female-sex protection by chronic variable stress was found to be mediated by corticosterone signaling (Harpaz, Abutbul et al. 2013). This result may be attributable to dysregulated glucocorticoid signaling and sensitivity. In addition to adrenal influences, ovarian hormones have been shown to modulate stress-induced increases in pro-inflammatory cytokines within the female rat brain (Arakawa, Arakawa et al. 2014). Arakawa, Arakawa et al. (2014) used a footshock paradigm in which 80 footshocks were delivered within two hours to naturally cycling female rats. This stressor led to a robust increase in the expression of IL-1β within the paraventricular nucleus at all stages of the estrous cycle

except metestrus. Stress-induced cytokines were further elevated in ovariectomized females compared to sham-operated animals, an effect that was abolished by administration of estradiol and progesterone to ovariectomized females. Although this study by Arakawa, Arakawa et al. (2014) suggests anti-inflammatory functions of ovarian hormones in stressed animals, it is not clear whether a reduction in neuroinflammation necessarily translates to better behavioral outcomes following stress.

Several studies to date have revealed sex differences in the neuroinflammatory priming effect of chronic or repeated stress. Research from our laboratory has demonstrated that chronically stressed male and female rats display distinct neuroinflammatory profiles in the hippocampus (Pyter, Kelly et al. 2013). In this study, male and female rats underwent a chronic adolescent stress (CAS) paradigm, in which experimental rats are exposed to randomized episodes of restraint stress and social defeat by same-sex aggressors. Consistent with the findings of others (Munhoz, Lepsch et al. 2006), when challenged with LPS intraperitoneally in adulthood, male CAS rats displayed exaggerated induction of the pro-inflammatory cytokines IL-1β and TNF-α. Interestingly, CAS females did not display a similar inflammatory sensitization by a history of chronic stress, suggesting sex differences in stress-related neuroimmune mechanisms. Similarly, Hudson, Jacobson-Pick et al. (2014) found that prior exposure to variable stressors led to potentiated IL-1β expression following re-exposure to a brief restraint session in male, but not female, mice. Male mice that underwent both the initial and re-exposure stress displayed exaggerated induction of hippocampal IL-1β compared to males exposed to either stress alone. While female mice displayed elevated IL-1β expression compared to their male counterparts both at baseline and following acute stress, no potentiating effect of re-exposure was evident.

Aside from the studies mentioned above, the currently available literature on sex differences in neuroimmune priming is fairly limited, and mechanistic studies are needed to better understand whether females are particularly vulnerable to stress-induced inflammation.

Adolescent stress, inflammation, and mood disorders

In humans, adolescence is typically considered a time of changing social, affective, and reward processing in the brain (Crone and Dahl 2012). Adolescence sees the concurrent maturation of the HPA axis and the brain under the organizational and activational (Romeo 2003) effects of gonadal hormones, and thus the adolescent brain may be particularly vulnerable to stressors (Neigh, Ritschel et al. 2013, Holder and Blaustein 2014). Adolescents repeatedly exposed to stressors during puberty display altered stress responsivity and reduced HPA axis habituation compared to adults exposed to the same stressors (Doremus-Fitzwater, Varlinskaya et al. 2009, Romeo 2010). Furthermore, adolescents display blunted cytokine response to an immune challenge, suggesting that the immune system of adolescents functions differently than that of adults (Doremus-Fitzwater, Gano et al. 2015).

Accumulating evidence suggests that early life stress can lead to long-term programing of adult physiology and behavior via a process called priming. Priming is defined as a sensitized response to a subthreshold or secondary stimulus (Perry and Holmes 2014). While the physiology and behavior of those exposed to early life adversity may appear normal at baseline, upon stimulation with a secondary stressor or challenge, the

priming effect of early life stress may be unmasked (Munhoz, Lepsch et al. 2006, Hudson, Jacobson-Pick et al. 2014). One way to assess priming or sensitization of immune processes in humans is to stimulate leukocyte samples with an antigen *ex vivo* to induce an immune response. For example, leukocytes from people with a history of chronic stress launch an exaggerated immune response when stimulated with the endotoxin lipopolysaccharide (LPS) *ex vivo* (Miller, Murphy et al. 2014). In animal models of stress-induced priming, the secondary challenge can be an injection of LPS or an acute psychosocial stressor. For example, chronic unpredictable stress and inescapable shocks in rats have been demonstrated to prime the cytokine and microglial response in the brain to a peripheral immune challenge (Frank, Baratta et al. 2007). The nature of the secondary stimulus has been reported to impact priming in some instances. Rats with a history of social defeat stress exposure show exaggerated cykokine mRNA expression upon a repeat exposure to social defeat, this potentiation does not take place if the secondary stimulus is LPS (Audet, McQuaid et al. 2014). It is plausible that exposure to stressors during the critical period of adolescence leads to remodeling of neuroimmune processes that persist into adulthood and impact mood and behavior (Pyter, Kelly et al. 2013). However, the mechanisms via which chronic adolescent stress leads to exaggerated innate immune signaling remain incompletely understood.

Rodent models of stress

Human health is influenced by a multitude of genetic, social, and environmental factors that often make it challenging to consider the biology alone. Rodent models offer a

setting where pathophysiology of diseases can be studied without the confounding context. Furthermore, rodents generally make excellent models of human disease due to the benefit of evolutionarily conserved physiological features and relative ease of genetic manipulation (Vandamme 2014). Importantly, the stress circuitry is well conserved between rodents and humans (Hariri and Holmes 2015) and stress-related genes identified from rodent studies have been linked to stress-related psychiatric disorders in humans (Binder, Salyakina et al. 2004, Erhardt, Czibere et al. 2011). Similarly, many aspects of the NFκB signaling pathway including mechanism of activation and protein sequences of pathway members are well conserved across species (Silverman and Maniatis 2001), including rats and mice in which numerous human inflammatory diseases continue to be modeled (Tak and Firestein 2001). Finally, HPA axis function during adolescence is altered in both rats and humans in similar ways (McCormick, Green et al. 2017). Taken together, these studies bolster the translational value of rodent models of chronic adolescent stress and associated inflammatory deficits.

In order to elucidate the mechanisms underlying early life stress and neuroimmune outcomes, researchers commonly use several different models of chronic stress paradigms including social defeat, repeated restraint, chronic unpredictable stress, and footshock stress. Work conducted in these models has generally identified the following neuroimmune consequences of chronic stress: 1) stress-induced cytokine expression and/or signaling in frontal-limbic structures (Audet, Jacobson-Pick et al. 2011), 2) alterations in the number and function of microglia, the brain's resident phagocytes (Bollinger, Collins et al. 2017), 3) inflammatory pathways that mediate the "priming" effect of stress (Frank, Miguel et al. 2010, Weber, Frank et al. 2015), 4) alterations to the neurovascular unit such

as increased BBB permeability (Pearson-Leary, Eacret et al. 2017) and infiltration of peripheral immune cells into brain parenchyma (Wohleb, Powell et al. 2013). In order to study the mechanism via which adolescent stress exerts long-lasting impact on adult neuroimmune reactivity, we focused on the activity and expression of NFκB which may serve as a lynchpin connecting all four above-mentioned mechanisms of neuroinflammation.

Stressors with a psychosocial aspect are argued to be particularly conducive to generating inflammation in the body and the brain and thus are most relevant for the pathophysiology of depression (Slavich and Irwin 2014). To this end, we have used a mixed-modality chronic adolescent stress (CAS) paradigm that incorporates both psychosocial (social defeat, single-housing) and physical (restraint) stressors. For all work included in this thesis research, adolescent stress was modeled in Wistar rats using the following stress paradigm: Male and female offspring of Wistar rats were weaned on post-natal day (PND) 22 into same-sex pairs. On PND 35 rats were randomly assigned to CAS and non-stressed groups. CAS rats were individually housed from this point onward to model social withdrawal and isolation that may be experienced by those undergoing chronic stress. CAS rats then underwent six randomized exposures to each of social defeat and restraint stress over twelve days spanning the mid-adolescent period in the rat (PND 38-49). In the social defeat paradigm, rats were exposed to a larger same-sex adult Long Evans rat that is trained to demonstrate aggressive behavior toward the experimental Wistar rat. Despite a lack of physical injury, repeated exposure to an older defeater rat elicits a sustained stress response in the Wistar rat. On days of restraint stress, for one hour under bright light rats were contained in a plexiglass chamber that restricted movement. Upon

completion of the stress paradigm, CAS rats remained single-housed whereas NS rats remained pair-housed. All rats were allowed to mature into adulthood without further experimental manipulations. On PND 94, all rats received a single intraperitoneal dose of saline or LPS (L3880, Sigma Aldrich) (0.25 mg/kg; 750,000 Endotoxin Units) to induce an immune response and to unmask potential priming effects of CAS. As previously demonstrated (Pyter, Kelly et al. 2013), the CAS paradigm led to priming of neuroinflammatory response in male rats that was detected in adulthood.

1.5.Specific aims of this dissertation

The female bias in the prevalence of depression first arise during puberty (Hankin, Abramson et al. 1998) whereas sex differences in anxiety emerge in mid-childhood and become more pronounced throughout adolescence (Lewinsohn, Gotlib et al. 1998). These trends are thought to stem from the interactions between gonadal hormones and stressful events during adolescence (Brooks-Gunn and Warren 1989, Cyranowski, Frank et al. 2000). In healthy adolescent humans, there are already sex differences in the number and composition of leukocyte subtypes (Bartlett, Schleifer et al. 1998). It is therefore plausible that these existing sex differences in stress response and immunity during puberty are further emphasized by the experience of chronic stress during this critical period (Holder and Blaustein 2014), thus giving rise to the female bias in later development of mood disorders. For example, adverse life events during development were significantly associated with somatic symptoms in females, but not males (Tak, Kingma et al. 2015). In contrast, adverse events during adulthood were associated with somatic symptoms in men.

These results suggest an interaction between developmental timeline, stressor exposure, and sex on later immune outcomes. However, whether females display a more pronounced immune response following stressors, and therefore experience greater maladaptive consequences with regards to mood and behavior, has not been critically evaluated to date. Therefore, modeling adolescent stress in rats via the CAS paradigm offers an ideal setting to study the potential sex-specific impact of early life stress on neuroimmune reactivity. This thesis research aimed to address the mechanisms by which chronic adolescent stress leads to exaggerated neuroinflammation in order to better understand the mechanisms linking stress-induced inflammation and the sex-specific pathophysiology of mood disorders.

	Stressor	Stressor duration	Sex	Outcome	Region / Organ	Age at assessment	Species	Reference
Stress-driven changes in baseline expression of inflammatory mediators	Maternal chronic variable stress	E1-E7	M	↑ inflammation-related genes	Placenta	E 12.5	Mouse	Bronson et al. 2014 *
			F	→ inflammation-related genes				
	Prenatal bright light	45 min * 3 times/day during E12-PND0	M	↑ IL-1β, TNF-α	HC	PND 120	Mouse	Diz-Chaves et al. 2013
	Prenatal restraint		F	↑ IL-6	HC	PND 135	Mouse	Diz-Chaves et al. 2012†
	Maternal deprivation on PND 9	24 hr	M	↑ synaptic expression of IL-1R1	HC	PND 45	Rat	Viviani et al. 2014 *
			F	→ synaptic expression of IL-1R1				
	Maternal deprivation on PND 9	24 hr	M	→ IL-1β, TNF-α, IL-6, CD11b, GFAP	HC, PFC	PND 103	Rat	Burke et al. 2013 *
			F	→ IL-1β, TNF-α, IL-6, CD11b, GFAP				
	Prenatal repeated restraint, prenatal	45 min * 3 times/day during E14-21	M	↓ IL-1β immunoreactivity	Hilus of DG	PND 147 - 161	Rat	Mandyam et al. 2008 *
			F	→ IL-1β immunoreactivity				
	Prolonged restraint stress	6 hr/day for 28 days	F	↓ IL-10 after 1-4 weeks of restraint, ↑ IL-4, IL-1β and ↓TNF-α after 2 weeks of stress, ↑ IFNγ after 2 and 4 weeks of stress	Cortex, HC	Adult (> PND 50)	Mouse	Voorhees et al. 2013
	Footshock		F	↑ mRNA expression of IL-1	HC	> PND 77	Rat	Arakawa et al. 2014 †
Stress-driven changes in acute stress-stimulated immune response	Variable stressor	3 days	M	Stress history potentiated IL-1β following re-exposure to restraint (↑)	HC	In adulthood, 6 weeks after initial stress exposure	Mouse	Hudson et al. 2014 *
			F	Stress history did not potentiate IL-1β following re-exposure to restraint (→)				
Stress-driven changes in antigen-stimulated immune response	Chronic adolescent stress	60 min of restraint or 5 min of social defeat/day during PND 37-48	M	Stress history potentiated LPS-induced IL-1β, TNF-α (↑)	HC	PND 80 (4.5 weeks after the end of stress)	Rat	Pyter et al. 2013 *
			F	Stress history did not potentiate LPS-induced IL-1β, TNF-α (→)				
Stress-induced glial alterations	Prenatal bright light stress	45 min * 3 times/day during E12-PND0	M	↑ percent of Iba-1+ cells with reactive morphology	CA1	PND 120	Mouse	Diz-Chaves et al. 2013
	Prenatal restraint	45 min * 3 times/day	F	↑ percent of Iba-1-positive cells	DG	PND 120	Mouse	Diz-Chaves et al. 2012†
	Maternal separation	3 hr/day during PND 1 - 14	M	↓ number of glia	SNpc, VTA	PND 15	Rat	Chocyk et al. 2011 *
			F	→ number of glia				
	Acute and chronic restraint	3 hr/day for 1 or 10 days	M	→ ratio of primed to ramified microglia	mPFC	Adult (> PND 70)	Rat	Bollinger et al. 2016 *
			F	↓ ratio of primed to ramified microglia				
	Prenatal dexamethasone administration	1 mg/kg on E18 and E19	M	↑ number and length of microglia processes	PFC	PND 90	Rat	Caetano et al. 2016 *
			F	↓ number and length of microglia processes				
Stress-driven trafficking of peripheral immune cells to the brain	Witnessing footshock of cagemate	1 hr/day for 5 days	M	↑trafficking of bone marrow-derived cells into the brain	PVN	Adult	Mouse	Ataka et al. 2013
	Footshock	1 hr/day for 1,2, or 5 days	F	↑trafficking of bone marrow-derived cells into the brain	Ventral HC	Adult (> PND 98)	Mouse	Brevet et al. 2010

Table 1.1. Summary of stress-induced neuroimmune alterations in females. * both males and females assessed in the same study; † female rats were ovariectomized. M = male; F = female. E = embryonic day; PND = postnatal day. HC = hippocampus; DG = dentate gyrus; PFC = prefrontal cortex; mPFC = medial prefrontal cortex, SNpc = substantia nigra pars compacta; VTA = ventral tegmental area; PVN = paraventricular nucleus. ↑ increase; ↓ decrease; → no change.

CHAPTER 2: CHRONIC ADOLESCENT STRESS SEX-SPECIFICALLY ALTERS CENTRAL AND PERIPHERAL NEURO-IMMUNE REACTIVITY IN RATS

2.1.Abstract

Adversity early in life is a reliable predictor of psychiatric disorders such as depression and anxiety which are increasingly recognized to have an immune component. However, the mechanisms by which early life adversity promotes inflammation are not yet defined. Using a chronic adolescent stress (CAS) model in rats, we tested the hypothesis that a history of CAS exaggerates induction of the pro-inflammatory NFκB pathway in adult rat hippocampus without compromising the peripheral immune response. We also assessed potential sex differences because it is unclear whether females, who are twice as likely to suffer from mood disorders as males, are disproportionally affected by stress-primed inflammation. Male and female adolescent rats underwent a CAS paradigm or received no stress. Five weeks following the last stressor all rats received a single systemic injection of either lipopolysaccharide (LPS) or vehicle to unmask possible immune-priming effects of CAS. An NFκB signaling PCR array demonstrated that CAS exaggerated the expression of NFκB-related genes in the hippocampus of both males and females. Interestingly, targeted qCPR demonstrated that CAS potentiated the induction of hippocampal IL-1β mRNA in female rats only, suggesting that some immune effects of CAS are indeed sex-specific. In contrast to the hippocampal findings, indices of peripheral inflammation such as NFκB activity in the spleen, serum IL-1β, IL-6, TNF-α, and corticosterone were not impacted by CAS in female rats. Despite showing no alterations in hippocampal cytokine mRNA, male CAS rats mounted a lower serum corticosterone response to LPS at 2 hours after injection followed by an exaggerated serum IL-1β response at 4 hours. This blunted HPA axis response coupled with excessive innate immune signaling in the periphery is

consistent with possible glucocorticoid resistance in males. In contrast, the innate immune effects of CAS manifested as excessive hippocampal immune reactivity in females. We conclude that while CAS enhances innate immune reactivity in both males and females months later in adulthood, the mechanism and manifestation of such alterations may be sex-specific.

2.2.Introduction

Chronic stress experienced during development is a significant risk factor for developing psychiatric and somatic illnesses later in life (Nusslock and Miller 2016). When experienced during adolescence, chronic stress has been shown to lead to impairments to key endocrine systems including the hypothalamic-pituitary-adrenal (HPA) axis (Bourke, Raees et al. 2013) and its regulation of immune function (Pyter, Kelly et al. 2013) in adulthood. In the latter study, prolonged exposure to stressors during development sensitized the hippocampal immune profile to react more potently when challenged with a bacterial endotoxin months removed from the initial stress experience (Pyter, Kelly et al. 2013). This suggests that while the acute sequelae of a stress response may dissipate soon after the stressor ends, chronic developmental stress is able to generate long-term impact by programming the way immune-related gene expression is regulated. Such broad changes are likely to be mediated by transcription factors; yet the role of transcription factors in stress-primed inflammation remains to be elucidated.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), a pro-inflammatory transcription factor; is ideally suited to mediate stress-induced immune alterations due to its role as a central regulator of innate immunity (Bekhbat, Rowson et al. 2017). NFκB controls the induction of hundreds of immune and inflammatory genes in tissues of multiple organ systems including the central nervous system and immune organs (Hoffmann and Baltimore 2006, Kaltschmidt and Kaltschmidt 2009). The resulting cytokines and other immune mediators following NFκB activation are involved in a number of homeostatic functions under non-pathological conditions (Kaltschmidt, Kaltschmidt et al. 1994), but prolonged or excessive presence of inflammation can cause

mood and behavioral deficits (Raison, Capuron et al. 2006). In healthy humans who endure a brief psychosocial stressor called the Trier Social Stress Test (TSST), NFκB is induced within minutes of stressor onset, and its activation is resolved by an hour thereafter (Bierhaus, Wolf et al. 2003, Wolf, Rohleder et al. 2009, Kuebler, Zuccarella-Hackl et al. 2015). Repeated or chronic exposure to stressors appears to derail this healthy pattern of NFκB induction by increasing its magnitude and delaying its resolution (Pace, Mletzko et al. 2006). Indeed, a transcriptional profile characterized by excessive activity of NFκB appears to be one of the hallmarks of chronic stress (Miller, Chen et al. 2008, Miller, Chen et al. 2009). Exaggerated NFκB signaling has been demonstrated in those with a history of early life stress (Pace, Mletzko et al. 2006, Pace, Wingenfeld et al. 2012), as well as patients with bipolar disorder (Miklowitz, Portnoff et al. 2016), post-traumatic disorder (Pace, Wingenfeld et al. 2012, Guardado, Olivera et al. 2016), and major depressive disorder (Pace, Mletzko et al. 2006, Miklowitz, Portnoff et al. 2016). Furthermore, the degree of chronic stress at a given time can predict the prospective expression of NFκB in white blood cells up to six months later (Miller, Rohleder et al. 2009), suggesting feed-forward loops of inflammatory signaling. Here we investigated altered NFκB signaling as a potential mechanism mediating the priming effects of chronic adolescent stress (CAS).

Identifying the organ tissue from where exaggerated immune responses originate is a first step to understanding the mechanisms underlying stress-primed inflammation. We have previously demonstrated that CAS male rats respond with exaggerated inflammatory gene expression in the hippocampus when assessed 4 hours following a systemic LPS challenge (Pyter, Kelly et al. 2013). Compared to higher doses of LPS that have been demonstrated to induce depressive-like and sickness behavior, a more subtle dose of LPS

(0.25 mg/kg) was chosen in this study to ensure that the LPS challenge uncovers the added impact of CAS on immune processes without over-activating the immune system. It is currently unclear as to whether this exaggerated hippocampal immune reactivity to LPS reflects brain-specific CAS impact or is paralleled and/or sustained by CAS-induced alterations to peripheral immune reactivity. While many rodent stress paradigms such as social defeat impact immune processes in both the brain and peripheral organ tissue (Reader, Jarrett et al. 2015), the extent of immune-priming in the central nervous system and the periphery do not always correlate following stressors (Gibb, Hayley et al. 2011). Therefore, we examined both central and peripheral immune outcomes over a time course following an immune activation by LPS *in vivo*. Furthermore, despite a well-established female bias in stress-related psychiatric illnesses (Breslau, Davis et al. 1997, Piccinelli and Wilkinson 2000), it is not clear whether females are particularly vulnerable to stress-induced immune alterations. Importantly, the onset of increased prevalence in females coincides with the onset of puberty (Bangasser and Valentino 2014). This suggests that developmental stress impacts male and female brains potentially to different extents and/or via distinct mechanisms, leading to sex differences in future psychiatric and somatic dysfunction. All studies to date that examined NFκB signaling following stressors have done so in male subjects only. Thus, an additional goal of the present study was to investigate whether CAS-associated changes in NFκB signaling are sex-specific.

2.3.Methods

Animals

Male and female offspring (*n*=5-7/group, 98 total, see Table 2.1 for design) of Wistar rats (Charles River) were culled to 4 male and 4 female pups per litter. No more than two pups from a litter were assigned to a group to control for litter effects. Pups were weaned into same-sex pairs on post-natal day (PND) 22 on a 14:10 reverse light:dark cycle (on 0000 h, off 1400 h). Standard rat chow and water were provided *ad libitum*. The weight of all animals was recorded at weaning, isolation, Day 1, 5, and 10 of the CAS paradigm, and weekly following the end of CAS until the terminal endpoint. Institutional Animal Care and Use Committees at Emory University and Virginia Commonwealth University approved all animal use procedures. Animal experimentation was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

	Female				Male			
	NS		CAS		NS		CAS	
	Saline	LPS	Saline	LPS	Saline	LPS	Saline	LPS
1 hr		$n = 6$		$n = 6$		$n = 6$		$n = 5$
2 hr	$n = 5$	$n = 6$	$n = 6$	$n = 7$	$n = 7$	$n = 6$	$n = 7$	$n = 7$
4 hr		$n = 5$		$n = 6$		$n = 6$		$n = 7$

Table 2.1 Study design for experiments in Chapter 2.

Chronic adolescent stress

On PND 35 rats were randomly assigned to non-stressed (NS) or chronic adolescent stress (CAS) groups. CAS were individually housed, and shortly after underwent CAS as detailed previously (Pyter, Kelly et al. 2013). Briefly, our mixed-modality chronic stress paradigm consists of 6 random exposures to each of social defeat and restraint stress which

take place across twelve days spanning the mid-adolescent period in the rat (PND 38-49). In the social defeat paradigm, rats are exposed to a larger same-sex adult rat that is trained to demonstrate aggressive behavior toward the experimental rat. Aggressive behavior by male Long Evans rats is typically described in terms of "pinning" behavior whereby the juvenile Wistar rat is pinned to the floor in a supine, immobilized position by the adult Long Evans rat (Weathington, Arnold et al. 2012). Typical aggressive behaviors by female Long Evans rats include kicking, defensive burying, and occasional pinning. Despite a lack of physical injury to the experimental rat, repeated exposure to an older defeater elicits a sustained stress response (Bourke and Neigh 2011). As previously published (Pyter, Kelly et al. 2013), the CAS paradigm leads to increased susceptibility to neuroinflammation in male rats that is present in adulthood. Upon completion of the stress paradigm, rats were allowed to mature into adulthood without further stressor exposure. On PND 94, all rats received a single intraperitoneal injection of either saline or LPS (E. Coli, O:B123, L3880, Sigma Aldrich, 0.25 mg/kg=750,000 Endotoxin units). One, two or four hours following injection, rats were sacrificed via rapid decapitation. All experimental procedures were completed at least two hours before the end of the light cycle to avoid the corticosterone awakening response which takes place at the beginning of the dark cycle. Order of collections was counterbalanced across experimental groups to normalize possible influences of the circadian rhythm.

Quantitative RT-PCR

The hippocampus was dissected under RNAse-free conditions, homogenized, and RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to

the manufacturer's instructions. RNA integrity was assessed by a NanoDrop One spectrophotometer (ThermoScientific, Wilmington, DE, USA) and RNA samples (*n*=5-7/group) were reverse transcribed using the High Capacity RNA to cDNA Kit (Applied Biosystems, Foster City, CA, USA). Primers for rat *IL1B, IL6,* and *TNF* were designed using NCBI's Primer Blast tool (sequences are provided in Table 2.2), and purchased from Invitrogen (Carlsbad, CA). Primer specificity was checked via the melt curve analysis. The universal two-step RT-PCR cycling conditions used on the QuantStudio Flex 6 (Applied Biosystems, Foster City, CA) were: 50°C (2 min), 95°C (2 min), followed by 40 cycles of 95°C (15 s) and 60°C (1 min). Samples were run in triplicate, and the coefficient of variation within the triplicates was no more than 4%. Among the five internal control genes assessed (HPRT1, GAPDH, B2M, ACTB, LDHA), none displayed <15% inter-group variability. Fold changes in gene expression were therefore calculated by the comparative $2^{-\Delta CT}$ quantification method relative to the female, NS, Saline group.

Gene	Forward primer	Reverse primer
IL1B	AGCAGCTTTCGACAGTGAGG	CTCCACGGGCAAGACATAGG
IL6	AGCGATGATGCACTGTCAGA	GGAACTCCAGAAGACCAGAGC
TNF	ATGGGCTCCCTCTCATCAGT	GCTTGGTGGTTTGCTACGAC

Table 2.2. List of primers used.

NFκB PCR signaling array

Rat NFκB signaling array (PARN-025Z) was purchased from Qiagen (Valencia, CA, USA), and performed according to the manufacturer's recommendation. Hippocampal cDNA from n=3/group were pooled, and used for PCR array. Groups compared were: 1) Female/NS/2 hours post-LPS, 2) Female/CAS/2 hours post-LPS, 3) Male/NS/2 hours post-LPS, and 4) Male/CAS/2 hours post-LPS.

p65 DNA-binding assay

Spleens were flash-frozen, and splenic nuclear protein extract was prepared using the Nuclear Extract Kit from ActiveMotif (Carlsbad, CA). p65's DNA-binding activity in spleen was measured using the TransAM Chemi kit (ActiveMotif, Carlsbad, CA). Recombinant p65 protein was purchased from ActiveMotif (Carlsbad, CA) and used to create a standard curve.

Serum cytokine ELISA

Immediately following decapitation, trunk blood was collected into EDTA-treated glass tubes (Vacutainer, BD Biosciences, Franklin Lakes, NJ). Blood was centrifuged at 1800 rcf for 20 min at 4°C, and serum was collected, aliquoted, and stored at -80°C until further use. Serum cytokine concentration was measured via Rat IL-1β, IL-6 and TNF-α ELISA kits that were purchased from R&D Systems (Minneapolis, MN). Assays were performed in duplicate according to the manufacturer's instructions. The coefficient of variance among the duplicates was less than 15%.

Serum corticosterone ELISA

Serum was obtained as described in 2.5. Measurement of serum corticosterone was conducted using an ELISA kit purchased from Enzo Life Sciences (Farmingdale, NY) according to the manufacturer's instructions. The coefficient of variance among the duplicates was less than 15%. The plates were read on a Synergy HTX plate reader (Biotek,

Winooski, VT), and its built-in four-parameter logistic regression software was used for plotting the standard curve and data extrapolation.

Statistical Analysis

Bodyweight data were analyzed via two-way repeated measures ANOVAs (within-subjects: time and between-subjects: stress) within each sex. NFκB PCR array data were analyzed using the web-based software GeneGlobe Data Analysis Center (Qiagen, Valencia, USA). Gene expression, spleen, and cytokine data were analyzed using three-way ANOVAs performed in SPSS 24.0 (sex * stress * stimulus). Because of our *a-priori* hypothesis that males and females of CAS background may display differential neuroimmune reactivity, we also tested for the effect of CAS in males and females separately using a 2x2 ANOVA (stress * stimulus). Bodyweight and peripheral endpoint data are expressed as mean ± SEM. Gene expression was normalized to the female, non-stressed, saline group, and data are presented as mean fold change ± SEM. Significance threshold was set to $\alpha = 0.05$ for all analyses.

2.4.Results

2.4.1. CAS leads to decreased weight

Body weight was assessed in males and females separately using a 2x2 ANOVA with time and stress as main factors. CAS led to a decrease in body weight in females throughout the duration of the paradigm ($F(1,204) = 12.86$, $p<0.001$), and this decrease was significant at the terminal time point ($t(204)=2.674$, $p<0.05$) (Fig 2.1A). CAS did not

significantly impact the body weight of males throughout the paradigm ($F(1,240)=0.321$, $p>0.05$). The total number of pins received by male rats during the CAS paradigm positively correlated with weight gain during the same period (PND 38-47) ($r=0.5245$, $p<0.05$) (Fig 2.1B). The number of pins each animal received did not correlate with adrenal weight normalized to terminal body weight (data not shown).

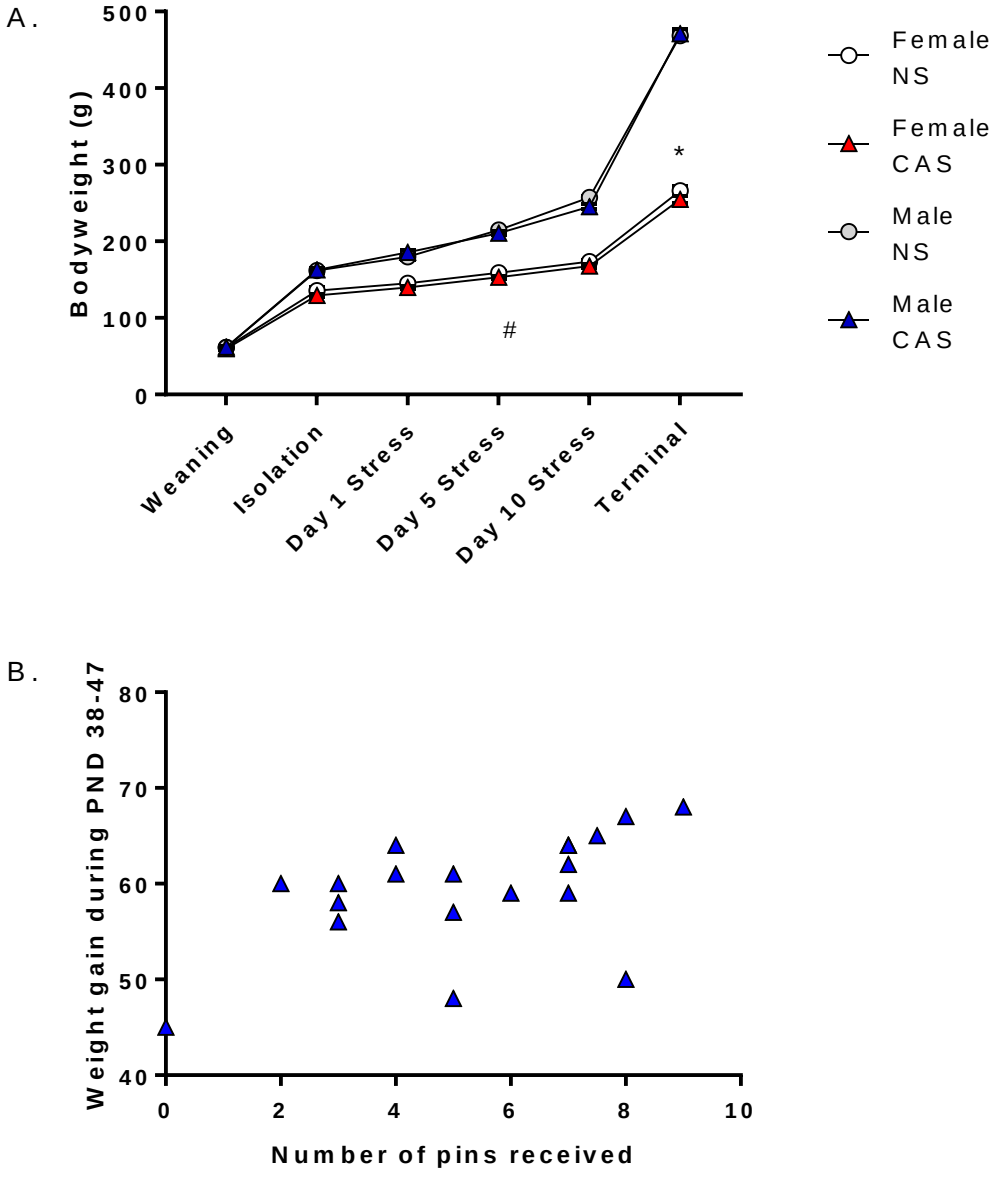

Fig 2.1. Chronic adolescent stress (CAS) leads to a decrease in bodyweight in females. A) Bodyweight was assessed in males and females separately using a 2x2 ANOVA with time and stress as main factors. CAS led to a decrease in bodyweight in CAS females throughout the duration of the paradigm (#, $p<0.001$), and this decrease was significant at the terminal time point ($p<0.05$). B) The number of pins received by male rats during the CAS paradigm positively correlated with weight gain during the same period (PND 38-47) ($p<0.05$). # main effect of CAS, * CAS effect across the indicated comparison.

2.4.2. CAS exaggerates NFκB-related inflammatory gene expression in the hippocampus

NFκB signaling PCR array was performed as an initial assessment to determine whether activation of the overall NFκB pathway was altered by CAS. Within each sex, several genes from the array were found to be upregulated at least 1.5 fold in the CAS animals compared to NS controls 2 hours following an LPS injection (Table 2.3). No gene on the array was found to be downregulated at least 1.5 fold by CAS in either female or male rats. Based on these results and demonstration of these cytokines as the primary mediators of the innate immune response (Dantzer 2004), we chose to evaluate the following panel of pro-inflammatory cytokines by targeted qPCR: *IL1B*, *IL6*, and *TNF*.

Genes Over-Expressed in			
Female CAS vs. NS		***Male CAS vs. NS***	
Gene Symbol	***Fold Regulation***	***Gene Symbol***	***Fold Regulation***
Rel	2.2043	Egr1	1.6948
Raf1	2.0626	Ccl2	1.6916
Casp8	2.0339	Fos	1.6513
Il1r1	1.9968	Irf1	1.6244
Il1b	1.9578	Kat2b	1.5708
Fos	1.945	Jun	1.5548
Tlr2	1.7714	Traf3	1.5396
Ccl2	1.7249	Nfkbia	1.5378
Tnf	1.5279	LOC687813	1.5332
Tnfrsf10b	1.5154		
Ccl5	1.5058		
Bcl2a1	1.5026		
Casp1	1.5007		

Table 2.3. Genes from the NFκB PCR array that are upregulated at least 1.5 fold by CAS. Hippocampal gene expression was assessed in female and male rats of NS and CAS backgrounds at 2 hours following a systemic LPS injection in adulthood. NFκB PCR array data were analyzed using the web-based software GeneGlobe Data Analysis Center (Qiagen, Valencia, USA).

2.4.3. CAS enhances hippocampal IL1B mRNA expression in female rats

To assess CAS-induced alterations in neuroimmune reactivity, we performed qPCR using total hippocampal RNA from male and female rats after either saline or LPS injections. A three-way ANOVA with sex, CAS, and stimulus as main factors revealed that LPS injection significantly upregulated the hippocampal mRNA expression of *IL1B* ($F(3,77)=75.853$, $p<0.001$), *IL6* ($F(3,77)=51.621$, $p<0.001$) and *TNF* ($F(3,77)=74.667$, $p<0.001$) (Fig 2.2). Males displayed a greater abundance of *IL1B* mRNA compared to

females (F(1,77)=6.652, p<0.05). While there was no main effect of CAS on *IL1B* (F(1,77)=1.514, p>0.05), sex and CAS interacted (F(1,77)=4.985, p<0.05) such that female CAS rats displayed greater expression of *IL1B* compared to female NS controls (F(1,38) = 4.956, p<0.05). The expression of *IL6* and *TNF* were not impacted by sex (*IL6*: F(1,77)=0.855, p>0.05; *TNF*: F(1,77)=0.377, p>0.05) or CAS (*IL6*: F(1,77)=1.495, p>0.05; *TNF*: F(1,77)=1.732, p>0.05), and no significant interactions were present (p>0.05).

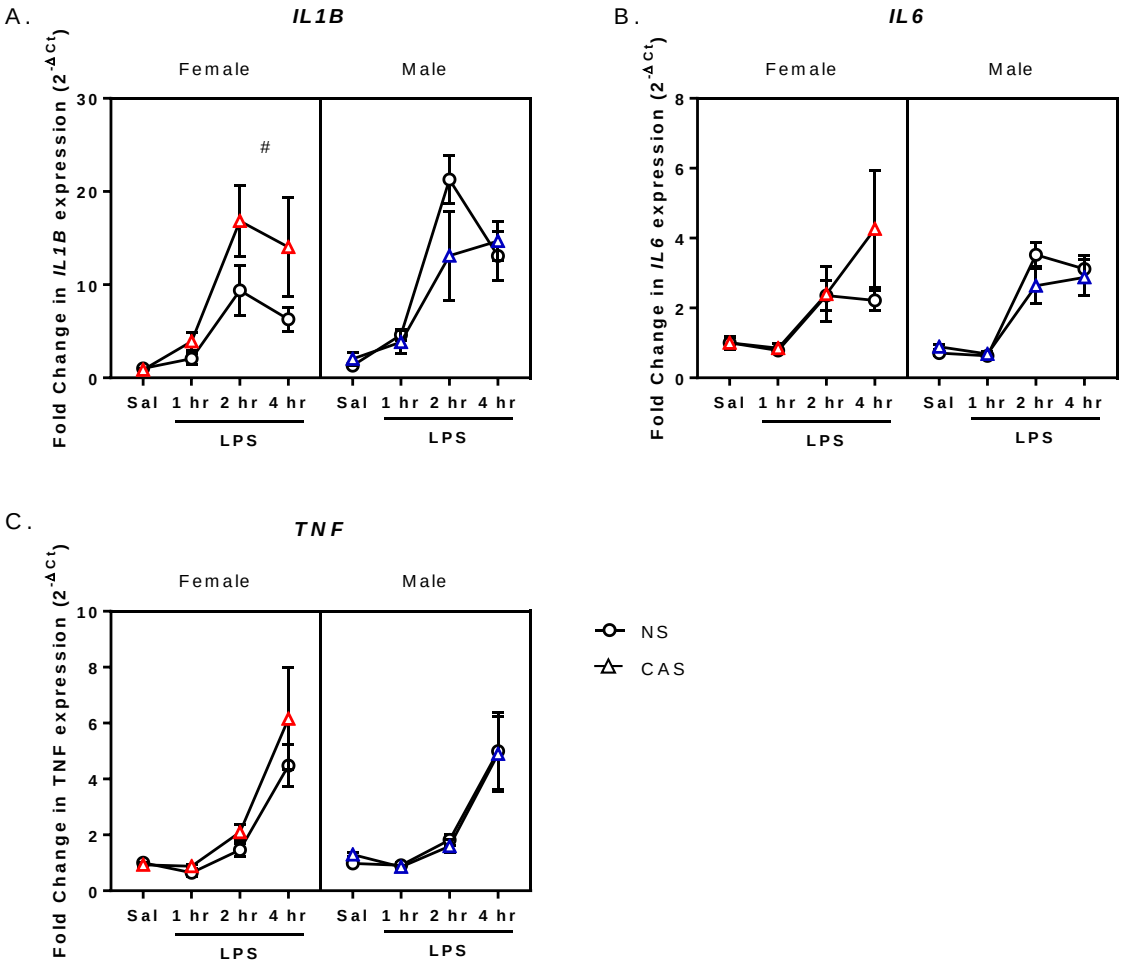

Fig 2.2. CAS leads to priming of hippocampal IL1B gene expression in female rats. Male and female rats of NS or CAS backgrounds received a single systemic injection of

saline or LPS in adulthood to unmask potential immune priming due to stressor exposure (n = 5-7/group). Targeted qPCR experiments confirmed that sex and CAS interacted such that CAS exaggerated the hippocampal mRNA expression of A) IL1B in female, but not male, rats ($p<0.05$). CAS did not impact the mRNA expression of B) IL6 or C) TNF in the hippocampus of either males or females. Data are presented as mean fold change ± SEM. # main effect of CAS.

2.4.4. CAS exaggerates peripheral immune reactivity in males

To determine whether CAS-driven increases in hippocampal NFκB induction was paralleled in peripheral immune tissue, we assessed in the spleen the transcriptional activity of p65, the main subunit of the NFκB complex (Fig 2.3). LPS injection induced an increase in the binding of p65 to the DNA ($F(3,77)=8.703$, $p<0.001$). However, CAS did not impact p65's DNA binding activity in the spleen ($F(1,77)=1.987$, $p>0.05$). There was no main effect of sex ($F(1,77)=1.130$, $p>0.05$).

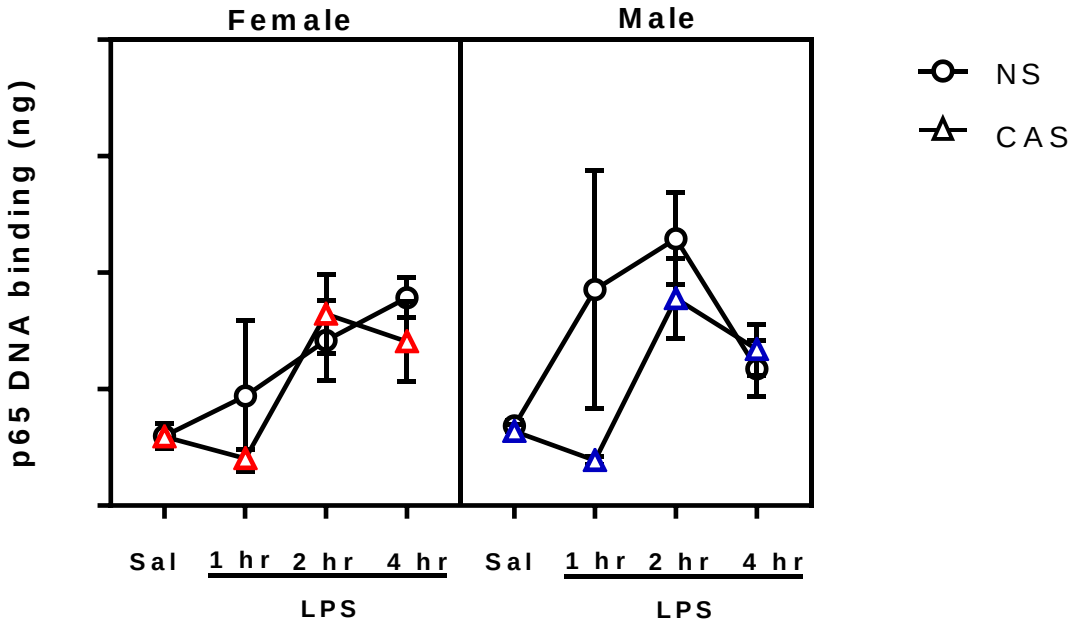

Fig 2.3. CAS does not impact the splenic p65 activity at baseline or following LPS. Male and female rats of NS or CAS backgrounds received a single systemic injection of saline or LPS in adulthood to unmask potential immune priming due to stressor exposure (n = 5-7/group). Splenic p65 activity was analyzed with sex * stress * stimulus as main factors. While stimulus led to a significant induction of p65 (p<0.001), neither sex nor stress impacted p65 activity (p>0.05). Data are presented as mean ± SEM.

IL-1β: As expected, LPS significantly increased serum IL-1β concentrations ($F(3,77)=15.748$, p<0.001) (Fig 2.4 A). Fourteen of twenty five saline-injected rats displayed IL-1β concentrations below the limit of detection (assay sensitivity <5 pg/mL). While there was no main effect of sex ($F(1,77)=0.24$, p>0.05), there was a trend toward a sex * stimulus interaction ($F(3,77)=2.444$, p=0.072). CAS did not impact serum IL-1β ($F(1,77)=0.884$, p>0.05). Because of our a-priori hypothesis that males and females of CAS

background may display differential neuroimmune reactivity and kinetics, we tested for the effect of CAS in males and females separately. There was a trend toward a main effect of CAS in males ($F(1,32)=3.931$, $p=0.056$) which was mostly driven by increased IL-1β concentrations at 4 hours post-LPS compared to NS males ($t(32)= 3.065$, $p<0.05$). There was no main effect of CAS in females ($F(1,30)=0.06283$, $p>0.05$).

IL-6 and TNF-α: LPS significantly increased IL-6 ($F(3,76)=18.68$, $p<0.001$) and TNF-α ($F(3,69)=20.922$, $p<0.001$) concentrations (Fig 2.4 B-C). Eight of twenty five saline-injected rats displayed TNF- α concentrations below the limit of detection (assay sensitivity <5 pg/mL). Sex did not impact serum IL-6 ($F(1,76)=1.788$, $p>0.05$) or TNF-α ($F(1,69)=0.048$, $p>0.05$). However, there was a sex * stimulus interaction on IL-6 ($F(3,76)=0.855$, $p=0.05$) such that females that received LPS displayed the greatest IL-6 concentrations. There was no main effect of CAS on serum IL-6 ($F(1,76)=1.085$, $p>0.05$) and TNF-α ($F(1,69)=0.250$, $p>0.05$) concentrations.

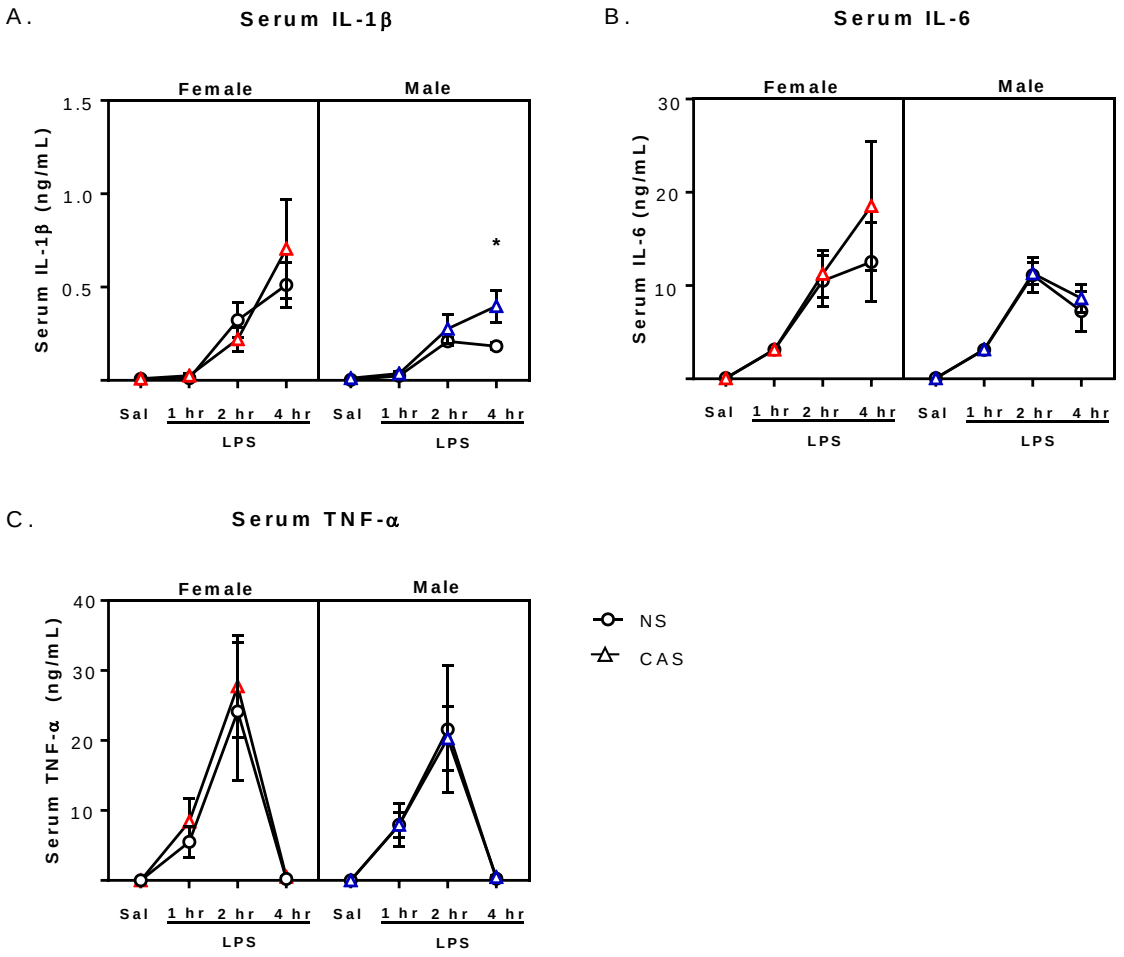

Fig 2.4. CAS leads to enhanced serum IL-1β in males following LPS. Male and female rats of NS or CAS backgrounds received a single systemic injection of saline or LPS in adulthood to unmask potential immune priming due to stressor exposure (n = 5-7/group). Serum cytokine concentrations were analyzed with sex, stress, and stimulus as main factors. While stimulus led to a significant induction of all three cytokines (p<0.001), neither sex nor stress had a main effect on cytokine concentrations (p>0.05). CAS males displayed greater serum IL-1β compared to NS males at 4 hours post-LPS (p<0.05). Data are presented as mean ± SEM. * CAS effect across the indicated comparison.

2.4.5. CAS leads to blunted corticosterone response to LPS in males

We next investigated altered HPA axis response to LPS as a potential mechanism underlying the exaggerated peripheral immune reactivity in CAS males. LPS led to a significant increase in serum corticosterone ($F(3,78)=27.87$, $p<0.001$) (Fig 2.5). As expected, females displayed greater serum corticosterone compared to males ($F(1,78)=13.116$, $p < 0.001$), and there was a significant sex * stimulus interaction ($F(3,78)=4.871$, $p<0.01$). There was no main effect of CAS on serum corticosterone concentrations ($F(1,78)=0.735$, $p>0.05$). However, we assessed the impact of CAS in males and females separately to address our a-priori hypothesis. Within females, CAS did not impact serum corticosterone ($F(1,38) = 0.0003382$, $p>0.05$). Within males, CAS rats showed lower serum concentrations of corticosterone compared to NS males at 2 hours following LPS ($t(40)= 2.793$, $p<0.05$).

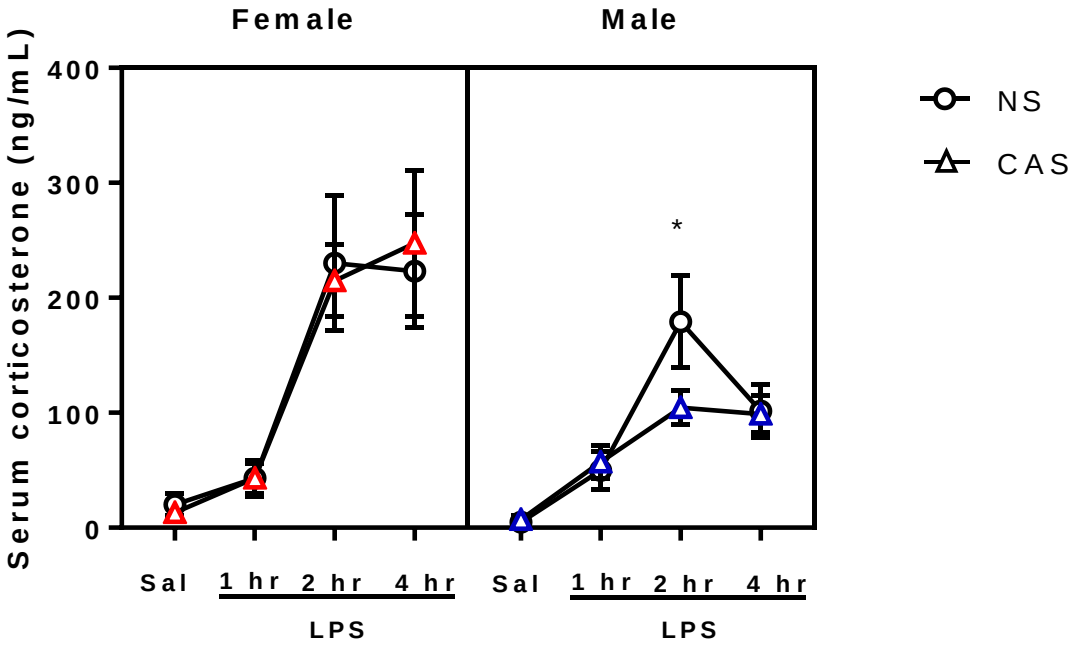

Fig 2.5. CAS male rats display blunted corticosterone release following LPS. Male and female rats of either NS or CAS backgrounds received a single systemic injection of saline or LPS in adulthood to unmask potential immune priming due to stressor exposure (n = 5-7/group). Serum corticosterone concentrations were analyzed with sex * stress * stimulus as main factors. Stimulus led to a significant induction of serum corticosterone (p<0.001). Females displayed greater corticosterone concentrations compared to males (p<0.05). Male CAS rats displayed lower corticosterone concentrations compared to male NS rats at 2 hours post-LPS (p<0.05). Data are presented as mean ± SEM. * CAS effect across the indicated comparison.

2.5.Conclusions

Due to its role as a lynchpin of the innate immune system and its involvement in the stress response, NFκB could be part of a mechanism by which early life adversity sensitizes the neuroimmune profile. Here we demonstrated that experiencing chronic stress during adolescence led to the priming of NFκB-related gene transcription in the hippocampus of both male and female adult rats. In addition, we showed that the induction of IL1B mRNA was potentiated in the hippocampus of female CAS rats following LPS, suggesting that neuroimmune priming due to stress can manifest in a sex-specific manner. Conversely, we delineated a male-specific impact of CAS on the peripheral immune response, and propose sex differences in the manifestation of how CAS alters the innate immune response. Our results therefore complement and add to previous literature in which chronic unpredictable stress was found to exacerbate NFκB's transcriptional activity in male rats (Munhoz, Lepsch et al. 2006).

The functional significance of exaggerated central cytokine signaling is incompletely understood, but may involve altered neurotransmission, synaptic plasticity, and synaptic strength. Under basal conditions, the maintenance of long-term potentiation during hippocampal-dependent learning is associated with expression of a network of cytokines (del Rey, Balschun et al. 2013). Acute or chronic inflammation has been demonstrated to promote glutamatergic signaling (Haroon, Fleischer et al. 2016), and in turn, glutamatergic signaling can activate synaptic NFκB (Scholzke, Potrovita et al. 2003) to further promote a pro-inflammatory-excitatory loop. Moreover, cytokines such as IL-6 can enhance synaptic excitability in via reducing GABAergic inhibition (Garcia-Oscos, Salgado et al. 2012). Inflammation also suppresses dopaminergic and serotonergic systems

via altering the synthesis, release, and reuptake of these neurotransmitters (Miller, Haroon et al. 2013), which is thought to underlie a subset of sickness behaviors (Capuron, Pagnoni et al. 2005, Felger, Mun et al. 2013). The impact of cytokine signaling depends on the cell type as illustrated by the example of IL-1β which perpetuates inflammation when signaling in glia but regulates synaptic properties of hippocampal neurons (Srinivasan, Yen et al. 2004).

We investigated the peripheral immune system as a potential source of this exaggerated hippocampal cytokine response. A history of footshock stress in mice has been shown to accelerate the time course of serum pro-inflammatory factors elicited by re-exposure to the same stressor (Cheng, Jope et al. 2015). Therefore it was important that we differentiate between CAS effects on time course and absolute magnitude of cytokine and NFκB responses. We demonstrated enhanced concentrations of serum IL-1β protein in CAS males at four hours following LPS injection, whereas female CAS rats displayed no alterations in serum IL-1β. The current study cannot rule out the possibility that had the time course following LPS been continued beyond 4 hours, the peripheral immune potentiation in CAS males may have influenced hippocampal outcomes. Furthermore, as we did not assay mRNA expression of IL1B, IL6, and TNF in the serum, the possibility remains that the discrepancy between hippocampal and serum cytokine outcomes at least partially derives from differential mRNA versus protein levels. Stressors of various types have previously been demonstrated to exacerbate LPS-induced production of serum cytokines. Repeated social defeat in mice led to potentiated serum IL-6 response at 4 hours following a systemic LPS injection (Wohleb, Fenn et al. 2012). Similarly, a high but not low dose of LPS in socially defeated rats led to significantly potentiated serum IL-1β

(Carobrez, Gasparotto et al. 2002). Prolonged social isolation followed by re-grouping stress in male mice enhanced the effects of LPS on serum corticosterone and cytokines (Gibb, Hayley et al. 2008). Even a single episode of social defeat stress prior to LPS injection aggravated the ensuing cytokine and corticosterone responses (Gibb, Hayley et al. 2011). CAS was found to not alter serum IL-6 and TNF-α release. Importantly, we did not see an effect of CAS at baseline, 1, 2 or 4 hours following LPS, suggesting that this lack of potentiation is likely not due to altered time course of immune response.

Limited evidence exists regarding stress-induced changes in NFκB activity in peripheral tissue. The spleen acts as a reservoir for immune cells such as monocytes that redistribute into the circulation upon physiological demand such as during acute injury or stress (Swirski, Nahrendorf et al. 2009). We chose to examine the spleen because splenocytes of socially stressed mice have been demonstrated to exhibit potentiated NFκB activity *ex vivo* (Quan 2003). Here we found that CAS in rats did not alter NFκB DNA binding in the spleen, which is consistent with Munhoz, Lepsch et al. (2006)'s report that chronic unpredictable stress did not exacerbate the LPS-induced increase in DNA binding activity of NFκB in the heart of rats. At one hour following LPS injection, three of twelve male and female non-stressed rats displayed splenic NFκB activity that was much greater in magnitude compared to the remaining nine non-stressed rats, suggesting a possible bimodal response among non-stressed rats. In contrast, none of the CAS rats displayed an upregulation of splenic NFκB activity at one hour post-LPS. While the difference in splenic NFκB activity between NS and CAS rats was not statistically significant, it is possible that the lack of splenic activity seen in CAS rats reflects an altered splenic cellular composition due to differential leukocyte trafficking. In fact, there is evidence that suggests that chronic

stress suppresses lymphocyte redeployment required for effective wound healing (Dhabhar and McEwen 1997). Furthermore, repeated stress has also been documented to promote monocytic egress from the spleen (McKim, Patterson et al. 2016). Therefore it is possible that the processing of the whole spleen in the current study as opposed to assessing specific subsets of splenocytes may have diluted the possible immune-priming effects of CAS.

To determine whether exaggerated peripheral immune response could be driven by a deficit in glucocorticoid-mediated anti-inflammatory signaling, we assayed serum corticosterone, the primary output of the HPA axis. Immune activation such as that induced by LPS is known to activate the HPA axis, and therefore serum corticosterone concentrations induced by LPS can be viewed as a proxy of the immune response (Lenczowski, Van Dam et al. 1997, Serrats, Grigoleit et al. 2017). However, corticosterone is a powerful anti-inflammatory mediator, and therefore LPS-induced corticosterone could also indicate an anti-inflammatory compensation. Indeed, high baseline corticosterone is predictive of lower LPS-induced corticosterone (Perez-Nievas, Madrigal et al. 2010). In females, CAS did not impact baseline or LPS-induced serum concentrations of corticosterone, which is in agreement with previously published CAS effects (Bourke, Raees et al. 2013, Pyter, Kelly et al. 2013). However, at two hours following LPS injection, male CAS rats displayed a blunted corticosterone response compared to NS males. While serum corticosterone levels elicited by systemic LPS have been reported to be exaggerated by stress in much the same manner that cytokine concentrations have (Gibb, Hayley et al. 2008, Gibb, Hayley et al. 2011), the serum corticosterone response has also been reported to be diminished in mice that have experienced repeated social defeat (Audet, Jacobson-Pick et al. 2011). Taken together, blunted corticosterone release following LPS at two

hours, followed by exaggerated serum IL-1β at 4 hours, is consistent with a glucocorticoid resistance model in which regulation of the peripheral immune response by the HPA axis has been compromised by exposure to CAS. Surprisingly, CAS did not alter the peripheral inflammatory response to a secondary LPS challenge in female CAS rats, suggesting that the neuroimmune impact of CAS was uninfluenced by changes to peripheral inflammation. Although not assessed in the current study, future investigations should target the impact of CAS on other established metrics of anti-inflammatory defense in the periphery including insulin-like growth factor-1 (IGF-1) (Szczesny, Slusarczyk et al. 2013). Overall, these data suggest that exposure to CAS can impact the neuro-immune reactivity of both males and females. However, this effect was manifested as exaggerated hippocampal neuroimmune signaling in females, whereas it presented as peripheral immune changes in males.

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Declaration of interest

The authors declare no conflict of interest.

CHAPTER 3: CHRONIC ADOLESCENT STRESS CAUSES ENDURING ALTERATIONS TO THE ADULT NEUROIMMUNE TRANSCRIPTOME

3.1.Abstract

Chronic stress during development is a prominent risk factor for mood disorders, and causes neuroimmune alterations that contribute to the etiology of these conditions. We have previously shown that chronic adolescent stress (CAS) primes the hippocampal inflammatory response in adult male rats. However, the mechanism underlying CAS-induced neuroinflammatory priming, and associated sex differences are currently unknown. Here we hypothesized that a history of CAS would exaggerate the hippocampal induction of the pro-inflammatory NFκB signaling pathway in adult male rats only. Male and female adolescent rats underwent the mixed-modality CAS paradigm or received no stress. Five weeks following the last stressor all rats, now adults, received a single, systemic injection of either a low dose of lipopolysaccharide (LPS), a potent immune stimulant, or vehicle to unmask possible priming effects of CAS. Total RNA from the hippocampus was used to perform RNA-Seq, and enriched transcriptional pathways were identified using gene set enrichment analysis (GSEA). Upon LPS stimulation, both male and female rats that underwent CAS displayed an enhanced enrichment of the NFκB pathway in the hippocampus compared to non-stressed, same-sex controls. Targeted qPCR experiments further confirmed that CAS equally primed the expression of the NFκB complex in males and females. Interestingly, CAS also led to an enhanced enrichment of the glucocorticoid receptor (GR) signaling pathway in females. As GR signaling is the primary mechanism of NFκB suppression, our results suggest sex differences in the impact of CAS on NFκB regulation via an altered balance between GR and NFκB signaling. Collectively, our results indicate that chronic stress experienced during adolescence leads to long-lasting changes

to the hippocampal genomic profile, and that CAS alters the balance between the stress and immune pathways in female rats.

3.2.Introduction

Chronic stress experienced during development is a significant risk factor for developing mood disorders including major depressive disorder, anxiety disorders, and post-traumatic stress disorder (PTSD) (McLaughlin, Conron et al. 2010). Women are afflicted with mood disorders twice as often as men (Bangasser and Valentino 2014), and display greater vulnerability to stress as well as more somatic symptoms (Kudielka and Kirschbaum 2005). When experienced during adolescence, chronic stress leads to impairments to key endocrine systems including the hypothalamic-pituitary-adrenal (HPA) axis (Bourke, Raees et al. 2013) and its regulation of immune function (Pyter, Kelly et al. 2013). Early life adversity, including stress during adolescence, is associated with a later pro-inflammatory phenotype that may be linked to psychiatric illnesses (Danese, Pariante et al. 2007, Ehrlich, Chen et al. 2017). Despite well-established sex differences in psychiatric outcomes and the stress response, it is currently unclear whether chronic adolescent stress (CAS) leads to sex-specific neuroimmune consequences.

Mounting evidence suggests that stress exaggerates immune reactivity in multiple brain regions involved in regulating the stress response, including the hippocampus (Pyter, Kelly et al. 2013). Transcriptomic studies reveal excessive immune signaling in the hippocampus of depressed individuals (Mahajan, Vallender et al. 2018) as well as rodents that have experienced chronic stress (Li, Chen et al. 2013, Stankiewicz, Goscik et al. 2015). Such large-scale changes are likely effected by transcription factors which are able to regulate the expression of hundreds of genes at once. Due to their prominent roles as primary mediators of the innate immune system and HPA axis (Bekhbat, Rowson et al. 2017), we examined the pro-inflammatory transcription factor NFκB and the

glucocorticoid receptor (GR), as potential mediators of the impact of CAS on neuroimmune priming. In Chapter 1, we demonstrated that CAS enhanced the mRNA expression of a subset of NFκB downstream targets in the rat hippocampus. These results align with data from human adolescents who display increased mRNA expression of the NFκB subunits p105 (cleaved into p50) and IκBα in leukocytes following targeted social rejection (Murphy, Slavich et al. 2013). Furthermore, we have also demonstrated evidence of peripheral glucocorticoid resistance in CAS males in Chapter 1. However, because the immune system can display considerable compensatory mechanisms and redundant signaling, assessing the expression of select downstream targets of NFκB or GR may not recapitulate the full extent of CAS-induced immune alterations. Therefore, using RNA-Seq we sought to assess the extent of alterations to the overall NFκB and GR signaling pathways in an effort to locate the mechanisms underlying CAS-induced neuroimmune priming (Pyter, Kelly et al. 2013).

Stress has been demonstrated to generate transcriptome-wide changes in gene expression (Li, Chen et al. 2013, Stankiewicz, Goscik et al. 2015). Furthermore, some of these differences are sex-specific (Hodes, Pfau et al. 2015), mirroring the sex-specific transcriptomic profiles seen in humans with depression (Labonte, Engmann et al. 2017). There are also baseline sex differences in immune-related genes in humans (Trabzuni, Ramasamy et al. 2013), thus suggesting a potential basis for sex differences in stress-regulated neuroimmune transcriptome. Here we also sought to elucidate transcriptional pathways differentially regulated by CAS in male and female rats.

3.3.Methods

Animals

Male and female (*n*=117 total, Table 3.1) Wistar rats (Charles River) were weaned and pair-housed on a 14:10 reverse light:dark cycle. Standard rat chow and water were provided *ad libitum*. The Emory University Institutional Animal Care and Use Committee approved all animal use procedures. Animal experimentation was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

RNA-Seq	Female				Male			
	NS		CAS		NS		CAS	
	Saline	LPS	Saline	LPS	Saline	LPS	Saline	LPS
4 hr	*n* = 6	*n* = 6	*n* = 5	*n* = 6	*n* = 6	*n* = 6	*n* = 6	*n* = 6

qPCR	Female				Male			
	NS		CAS		NS		CAS	
	Saline	LPS	Saline	LPS	Saline	LPS	Saline	LPS
2 hr	*n* = 8	*n* = 8	*n* = 6	*n* = 8	*n* = 7	*n* = 8	*n* = 7	*n* = 8
4 hr	*n* = 7	*n* = 7	*n* = 6	*n* = 8	*n* = 6	*n* = 8	*n* = 7	*n* = 8

Table 3.1. Study design for RNA-Seq and qPCR experiments. Female and male rats of either NS or CAS background were challenged with a systemic injection of saline (vehicle) or LPS, and hippocampal tissue were collected two or four hours following injection.

Chronic adolescent stress (CAS)

CAS was performed as detailed in Chapter 2.3. On PND94, all rats received a single intraperitoneal injection of either saline or LPS (L3880, Sigma Aldrich) (0.25 mg/kg; 750,000 Endotoxin Units) and tissue was collected at 2 and 4 hours following injection as described below.

Tissue collection

Following anesthesia with Euthasol overdose (150 mg/kg), rats (n=117) were trans-cardially perfused with ice-cold PBS to remove peripheral immune cells and prevent inflammatory markers present in the blood from confounding gene expression in brain tissue. All experimental procedures were completed at least two hours before the end of the light cycle to avoid the corticosterone peak that occurs prior to beginning of the dark cycle.

RNA-Seq

The hippocampus was dissected under RNAse-free conditions, homogenized, and RNA was extracted using the MiRNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA purity and quality was checked using an Agilent 2100 Bioanalyzer. RNA-Sequencing was performed at the Yerkes Genomics Core at Emory University. Polyadenylated transcripts were purified on oligo-dT magnetic beads, fragmented, reverse transcribed using random hexamers and incorporated into barcoded cDNA libraries based on the Illumina TruSeq platform. Libraries were validated by microelectrophoresis, quantified, pooled and clustered on Illumina TruSeq v3 flowcells. Clustered flowcells were sequenced to achieve target read depth of 15 million reads per sample on an Illumina HiSeq 1000 in 100-base single-end read reactions. Adapter trimming was performed and all files passed. The RNA sequences were aligned to the *Rattus norvegicus* genome rn5 using Subread (Liao, Smyth et al. 2013). The read counts for each gene were then calculated using featureCount (Liao, Smyth et al. 2014). The limma package was used for normalization and differential expression analysis (Ritchie, Phipson

et al. 2015). We filtered out all genes with row sums of 0 and the genes with standard deviations below the 10th percentile. Genes without names or with the prefixes LOC, FAM, RGD, MGG, and MIR were also filtered as they are uninformative for the differential expression analysis. The expression matrix was converted to log2 Transcripts Per Kilobase Million (TPM) and normalized using limma's normalizeQuantiles() function. We performed a principle component analysis (PCA) on the data to check for batch effect as the samples were sequenced over a two day period. PCA revealed batch to be a significant factor in the data. Ward clustering was performed using the hclust package, and two samples were removed based on the clustering. Removal of these samples led to a better clustering based on the batch of the sample. Despite accounting for batch by entering it as a factor in the model, its effect still contaminated the results of the differential expression analysis. Therefore data from batch 2 (n=24) was used for differential expression analysis. Interactions between the factors sex, stress, and LPS were removed from the model as they did not produce any significant results, and their removal provided better results for the main effects of the model. Genes determined to be differentially expressed based on paired contrasts were used to evaluate differentially regulated pathways through KEGG pathway analysis (Ogata, Goto et al. 1999). Additional pathway enrichment analysis was performed using Gene Set Enrichment Analysis (Broad Institute). Refer to Tables 3.2-3.3 for pathways used for performing GSEA.

ABCA1	CCND1	CXCR7	FJX1	IER5	KLF10	NFE2L2	PLEK	SERPINB8	TNC
AREG	CCNL1	CYR61	FOS	IFIH1	KLF2	NFIL3	PLK2	SERPINE1	TNF
ATF3	CCRL2	DDX58	FOSB	IFIT2	KLF4	NFKB1	PMEPA1	SGK1	TNFAIP2
ATP2B1	CD44	DENND5A	FOSL1	IFNGR2	KLF6	NFKB2	PNRC1	SIK1	TNFAIP3
B4GALT1	CD69	DNAJB4	FOSL2	IL12B	KLF9	NFKBIA	PPAP2B	SLC16A6	TNFAIP6
B4GALT5	CD80	DRAM1	FUT4	IL15RA	KYNU	NFKBIE	PPP1R15A	SLC2A3	TNFAIP8
BCL2A1	CD83	DUSP1	G0S2	IL18	LAMB3	NINJ1	PTGER4	SLC2A6	TNFRSF9
BCL3	CDKN1A	DUSP2	GADD45A	IL1A	LDLR	NR4A1	PTGS2	SMAD3	TNFSF9
BCL6	CEBPB	DUSP4	GADD45B	IL1B	LIF	NR4A2	PTPRE	SNN	TNIP1
BHLHE40	CEBPD	DUSP5	GCH1	IL23A	LITAF	NR4A3	PTX3	SOCS3	TNIP2
BIRC2	CFLAR	EDN1	GEM	IL6	MAFF	OLR1	RCAN1	SOD2	TRAF1
BIRC3	CLCF1	EFNA1	GFPT2	IL6ST	MAP2K3	PANX1	REL	SPHK1	TRIB1
BMP2	CSF1	EGR1	GPR183	IL7R	MAP3K8	PDE4B	RELA	SPSB1	TRIP10
BTG1	CSF2	EGR2	HBEGF	INHBA	MARCKS	PDLIM5	RELB	SQSTM1	TSC22D1
BTG2	CXCL1	EGR3	HES1	IRF1	MCL1	PER1	RHOB	STAT5A	TUBB2A
BTG3	CXCL10	EHD1	ICAM1	IRS2	MSC	PFKFB3	RIPK2	TANK	VEGFA
CCL2	CXCL11	EIF1	ICOSLG	JAG1	MXD1	PHLDA1	RNF19B	TAP1	YRDC
CCL20	CXCL2	ETS2	ID2	JUN	MYC	PHLDA2	SAT1	TGIF1	ZBTB10
CCL4	CXCL3	F2RL1	IER2	JUNB	NAMPT	PLAU	SDC4	TIPARP	ZC3H12A
CCL5	CXCL6	F3	IER3	KDM6B	NFAT5	PLAUR	SERPINB2	TLR2	ZFP36

Table 3.2. List of genes in the HALLMARK_TNFA_SIGNALING_VIA_NFKB set. These genes are described as being regulated by NF-kB in response to TNF (Hallmark collection, Molecular Signatures Database, Broad Institute).

ADARB1	BCL6	CYB561	GHRHR	MT1A	PLEKHF1	SLC19A2	VLDLR
AFF1	BMPER	DDIT4	GLUL	MT2A	POU2F1	SLC22A5	XDH
AK2	CALCR	DIRAS2	GOT1	NR3C1	POU2F2	SNTA1	ZFP281
AMPD3	CEBPA	DUSP1	H6PD	PDCD7	RASA3	SPHK1	ZFP36
ANGPTL4	CEBPB	EDN1	HAS2	PDGFRB	RGS2	SPSB1	ZHX3
ANXA4	COL4A2	EHD3	HNRNPLL	PDP1	RHOB	TBL1XR1	
AQP1	CREB1	ERRFI1	KLF13	PER1	RHOJ	TSC22D3	
ARID5B	CREB3	FKBP5	KLF9	PER2	SESN1	USP2	
ASPH	CREB3L4	FOSL2	LOX	PIK3R1	SGK1	USP54	
ATF4	CTGF	GDPD1	MERTK	PLD1	SLC10A6	VDR	

Table 3.3. List of genes in the custom-designed glucocorticoid receptor (GR) signaling pathway. These genes are adapted from the Rat GR Signaling PCR array by Qiagen.

Quantitative PCR

RNA integrity was assessed by a NanoDrop One spectrophotometer (ThermoScientific, Wilmington, DE, USA) and RNA samples were reverse transcribed using the High Capacity RNA to cDNA Kit (Applied Biosystems, Foster City, CA, USA). To ensure uniform amounts of total cDNA across groups, cDNA was quantified via the PicoGreen Assay (Invitrogen, Carlsbad, CA), then standardized so that all samples started quantitative RT-PCR with 2 μg cDNA. The rat gene Hprt1 was determined to be the optimal endogenous control based on an inter-group variance of less than 11% across the experimental groups. Primers were designed and purchased from Invitrogen (Carlsbad, CA), and sequences are provided in Table 3.4. The universal two-step quantitative polymerase chain reaction (qPCR) cycling conditions used on the QuantStudio Flex 6 (Applied Biosystems, Foster City, CA) were: 50°C (2 min), 95°C (10 min), 40 cycles of 95°C (15 s) and 60°C (1 min). Samples were run in triplicate, and the coefficient of variation within the triplicates was no more than 4%. ΔCt values were compared between the Saline 2hr and Saline 4hr groups, and combined into one group if no significant differences were found ($p>0.05$). Significant differences were found for TSC22D3, FKBP5, and SGK1 ($p<0.01$); therefore the Saline 2hr and the Saline 4hr groups were kept separate for statistical analyses. Fold changes in gene expression were calculated by the comparative $2^{-\Delta\Delta CT}$ quantification method relative to the female, NS, Saline group.

Function	Gene	Protein	Forward	Reverse
Internal control	*HPRT1*	HPRT1	TGCTGAAGATTTGGAAAAGG	AATCCAGCAGGTCAGCAAAG
NFκB signaling	*NFKBIA*	IκBα	TGGCCAGTGTAGCAGTCTTG	GACATCAGCACCCAAAGTCA
	NFKB1	p50	AGTCCCGCCCCTTCTAAAAC	CCTGGATCACTTCAATGGCCT
	NFKB2	p52	TCTGGGTGTCCTGCATGTAA	AAGCGTAGCCGTACAATGCT
	RELA	p65	AACACTGCCGAGCTCAAGAT	CATCGGCTTGAGAAAAGGAG
GR signaling	*NR3C1*	GR	GGAAGGTCTGAAGAGCCAAG	GATGATTTCAGCTAACATCTCTGG
	FKBP5	FKBP5	TGGAGGTGAACCCTCAGAAC	TCTTGCTCAATGCTTTGCTG
	DUSP1	DUSP1	CTCCAAGGAGGATATGAAGCG	GTAAGCACTGCCCAGGTACA
	SGK1	SGK1	TGCAGTGACGAGCATCCAGAT	CAATGAAACACCAACGGCTCT
	TSC22D3	GILZ	GGGATGTGGTTTCCGTTAAA	TCAACCAGCTCACGAATCTG
Chemokines	*CCL2*	CCL2	ATGCAGTTAATGCCCCACTC	TTCCTTATTGGGGTCAGCAC
	CXCL11	CXCL11	CCCTGGCTATGATCATCTGG	TTTGTCACAGCCGTTACTCG

Table 3.4. Primers sequences used for experiments in Chapter 3.

Statistical analysis

Statistical analyses were conducted in SPSS 24.0, and visual representations were created using GraphPad Prism 7. qPCR data were analyzed via a three-way ANOVA with sex (male vs. female), stress (NS vs. CAS), and stimulus (Saline vs. LPS 2hr vs. LPS 4hr) as main factors and their interactions. Gene expression data are presented as mean fold change ± SEM. Significance threshold was set to $\alpha = 0.05$ for all analyses.

3.4.Results

3.4.1. RNA-Seq reveals differentially expressed genes and pathways

Differential expression analysis was conducted using limma by modeling the main effects of LPS, sex, and stress (Fig 3.1 A-C). The list of top ten differentially expressed genes (DEGs) from each of the main effects is displayed in Fig 3.2 A-C. Furthermore, the following paired contrasts were created for testing the effects of each factor in the model:

The simple effect of CAS at baseline: 1) M.CAS.Sal vs M.NS.Sal, 2) F.CAS.Sal vs F.NS.Sal; the simple effect of LPS in male and female rats of NS or CAS background: 3) F.NS.LPS vs F.NS.Sal, 4) F.CAS.LPS vs F.CAS.Sal, 5) M.NS.LPS vs M.NS.Sal, and 6) M.CAS.LPS vs M.CAS.Sal. The number of DEGs with an unadjusted *p*-value < 0.05 for each contrast is displayed in Venn diagrams in Fig 3.2 A-C. The genes for each circle and for the intersections formed in the Venn diagrams were then used for a KEGG pathway analysis. The top ten enriched KEGG pathways are displayed below each circle of the Venn diagram (Fig 3.2 A-C).

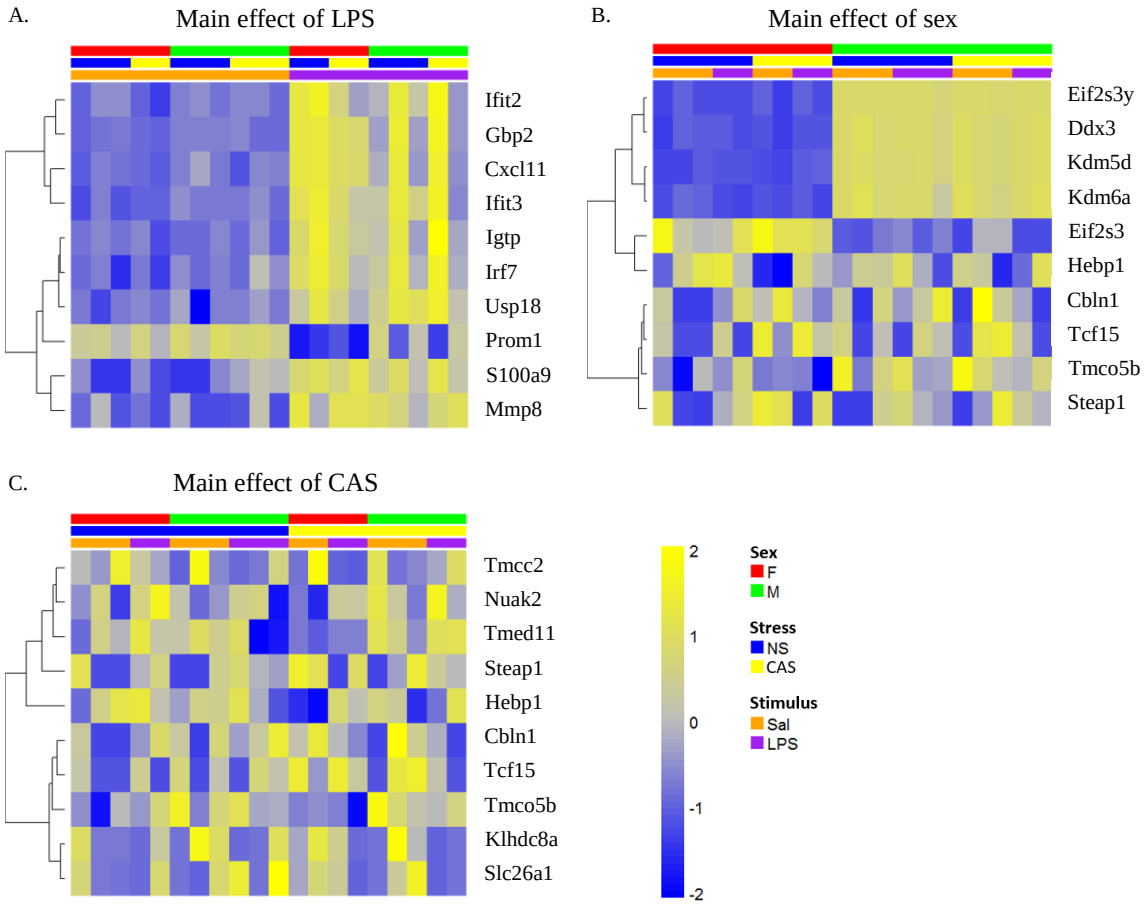
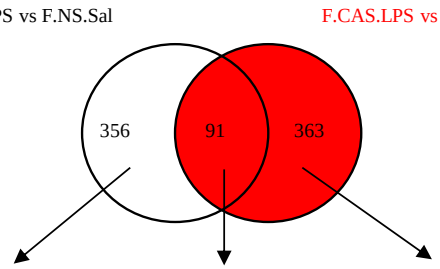


Fig 3.1 RNA-Seq reveals genes differentially expressed according to LPS stimulus, sex, and stress. Male and female rats of NS or CAS backgrounds were challenged with

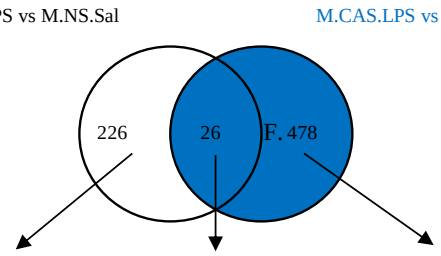
a systemic dose of either saline or LPS, and DEG analysis on the hippocampal transcripts was performed using the package limma in Bioconductor. Top ten DEGs are shown for the main factors of LPS, sex, and stress.

A.



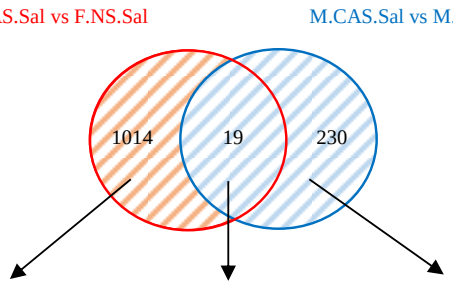
1	Primary immunodeficiency	Cytokine-cytokine receptor interaction	Bladder cancer
2	Antigen processing and presentation	Cytosolic DNA-sensing pathway	Jak-STAT signaling pathway
3	Complement and coagulation cascades	Chemokine signaling pathway	Toll-like receptor signaling pathway
4	Cytosolic DNA-sensing pathway	NOD-like receptor signaling pathway	Pancreatic cancer
5	Taurine and hypotaurine metabolism	Toll-like receptor signaling pathway	Toxoplasmosis
6	Glycine, serine and threonine metabolism	Antigen processing and presentation	Staphylococcus aureus infection
7	Cysteine and methionine metabolism	Cell adhesion molecules (CAMs)	Cytokine-cytokine receptor interaction
8	Pyrimidine metabolism	Malaria	ErbB signaling pathway
9	ECM-receptor interaction	Hepatitis C	Glycosaminoglycan biosynthesis - keratan sulfate
10	Notch signaling pathway	Graft-versus-host disease	

B.



1	Hematopoietic cell lineage	Bladder cancer	NOD-like receptor signaling pathway
2	Vascular smooth muscle contraction	Endometrial cancer	Cytokine-cytokine receptor interaction
3	Complement and coagulation cascades	Non-small cell lung cancer	Chemokine signaling pathway
4	Adipocytokine signaling pathway	Glioma	Apoptosis
5	ECM-receptor interaction	Endocytosis	African trypanosomiasis
6	PPAR signaling pathway	Melanoma	ErbB signaling pathway
7	Pancreatic secretion	ErbB signaling pathway	Hepatitis C
8	Pentose phosphate pathway	Prostate cancer	Toll-like receptor signaling pathway
9	Retinol metabolism	Gap junction	Malaria
10		Toll-like receptor signaling pathway	Jak-STAT signaling pathway

C.



1	Spliceosome	Endometrial cancer	Tight junction
2	Amino sugar and nucleotide sugar metabolism	GnRH signaling pathway	Calcium signaling pathway
3	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	Calcium signaling pathway	Apoptosis
4	Ether lipid metabolism	Bladder cancer	Basal cell carcinoma
5	Vascular smooth muscle contraction	MAPK signaling pathway	Hedgehog signaling pathway
6	RIG-I-like receptor signaling pathway	ErbB signaling pathway	GnRH signaling pathway
7	Pathways in cancer	Neuroactive ligand-receptor interaction	Neuroactive ligand-receptor interaction
8	Glycerophospholipid metabolism	Gap junction	Leukocyte transendothelial migration
9	Ribosome	Tight junction	Hepatitis C
10	Lysosome	Apoptosis	Primary immunodeficiency

Fig 3.2. Paired comparisons reveal KEGG pathways differentially enriched by LPS and chronic adolescent stress (CAS) in males and females. Male and female rats of NS or CAS backgrounds were challenged with a systemic dose of either saline or LPS, and DEG analysis on the hippocampal transcripts was performed using the package limma in Bioconductor. A-C, The number of DEGs with an unadjusted p-value < 0.05 from each of the indicated paired contrasts is displayed along with the top ten KEGG pathways for each of the contrasts and their intersections shown below the Venn diagrams. The simple effect of LPS within female (A) and male (B) rats of either stress background is shown. C) The simple effect of CAS among male and female rats at baseline (saline injection) revealed a greater number of DEGs in female rats. D-F, Heat maps for the top ten DEGs for the main

effects of LPS, sex, and CAS are displayed. F=Female, M=Male, NS=Non-stressed, CAS=Chronic Adolescent Stress, Sal=Saline, LPS=lipopolysaccharide.

3.4.2. NFκB signaling pathway is enriched to a greater extent in the hippocampus of CAS rats following LPS

GSEA assesses whether the expression of a previously defined functional pathway or set of genes is enriched in one biological group (Wang and Cairns 2013). In this case, we used GSEA to identify which inflammatory and endocrine pathways were enriched in the transcriptomes of rats exposed to CAS or NS challenged with either LPS or saline. Normalized counts were used as input data. As the components of a signaling pathway can be defined in many different ways we opted to use the Molecular Signatures Database's Hallmark gene sets which contain 50 pathways described as representing well-defined biological processes (Liberzon, Birger et al. 2015). We assessed the impact of acute LPS challenge (contrast: LPS versus Saline) on the enrichment of these 50 pathways within each of Male Non-stressed (M-NS), Male Stressed (M-CAS), Female Non-stressed (F-NS), Female Stressed (F-CAS) groups. Next, we examined the impact of CAS on neuroimmune signaling by comparing the normalized enrichment scores (NES) of LPS-induced hallmark pathways among same-sex CAS vs. NS rats. Following this analysis, we ranked the differences in NES from largest to smallest, indicating the pathways most impacted by CAS in males and females. Among 50 hallmark pathways, HALLMARK_TNFA_SIGNALING_VIA_NFκB (Table 3.2 for a list of genes contained in this pathway) emerged as the most strongly enriched pathway in CAS rats compared to

NS rats. A visual representation of the LPS-inuduced enrichment of the HALLMARK_TNFA_SIGNALING_VIA_NFκB signaling pathway is shown for all four groups in Fig 3.3 A-B. While this pathway was significantly enriched in all four groups following LPS, the NES appeared greater in magnitude in CAS rats compared to same-sex NS rats, suggesting a greater extent of the enrichment in CAS.

3.4.3. CAS potentiates the induction of canonical and non-canonical NFκB pathway members in the hippocampus of male and female rats

Because several NFκB pathway members including NFKBIA, NFKB1, and NFKB2 were found to be among the leading edge genes driving the enrichment of the HALLMARK_TNFA_SIGNALING_VIA_NFκB pathway (Fig 3.3 A-B), we sought to extend the findings from the RNA-Seq experiment by assessing the mRNA expression of NFκB subunits over a time course following LPS via targeted qPCR. As prior exposure to stressors or stress hormones can exaggerate and prolong the inflammatory response to an immune challenge (Kelly, Michalovicz et al. 2018), we assessed a time course of NFκB response to LPS (See Table 3.4 for qPCR experimental design). NFκB signals through two distinct cascades, canonical and non-canonical, that differ in composition and function (Gilmore 2006). Since it is unclear as to which signaling cascade of NFκB mediates the neuroimmune effects of stress, we chose to examine members of both the canonical (NFKBIA, NFKB1, RELA) and non-canonical (NFKB1, NFKB2) NFκB cascades. LPS stimulus significantly induced the mRNA expression of NFKBIA ($F(2,105)=103.84$, $p<0.001$), RELA ($F(2,105)=15.21$, $p<0.001$), NFKB1 ($F(2,105)=38.66$, $p<0.001$), and NFKB2 ($F(2,105)=120.24$, $p<0.001$) (Fig 3.3 C-F). There was no main effect of sex on

NFKBIA, RELA, NFKB1, and NFKB2 (p>0.05). Interestingly, CAS rats displayed exaggerated hippocampal mRNA expression of NFKBIA (F(1,105)=4.209, p<0.05), RELA (F(1,105)=5.262, p<0.05), and NFKB2 (F(1,105)=8.186, p<0.01) compared to NS rats. CAS did not significantly impact the expression of NFKB1 (F(1,105)=2.718, p=0.102).

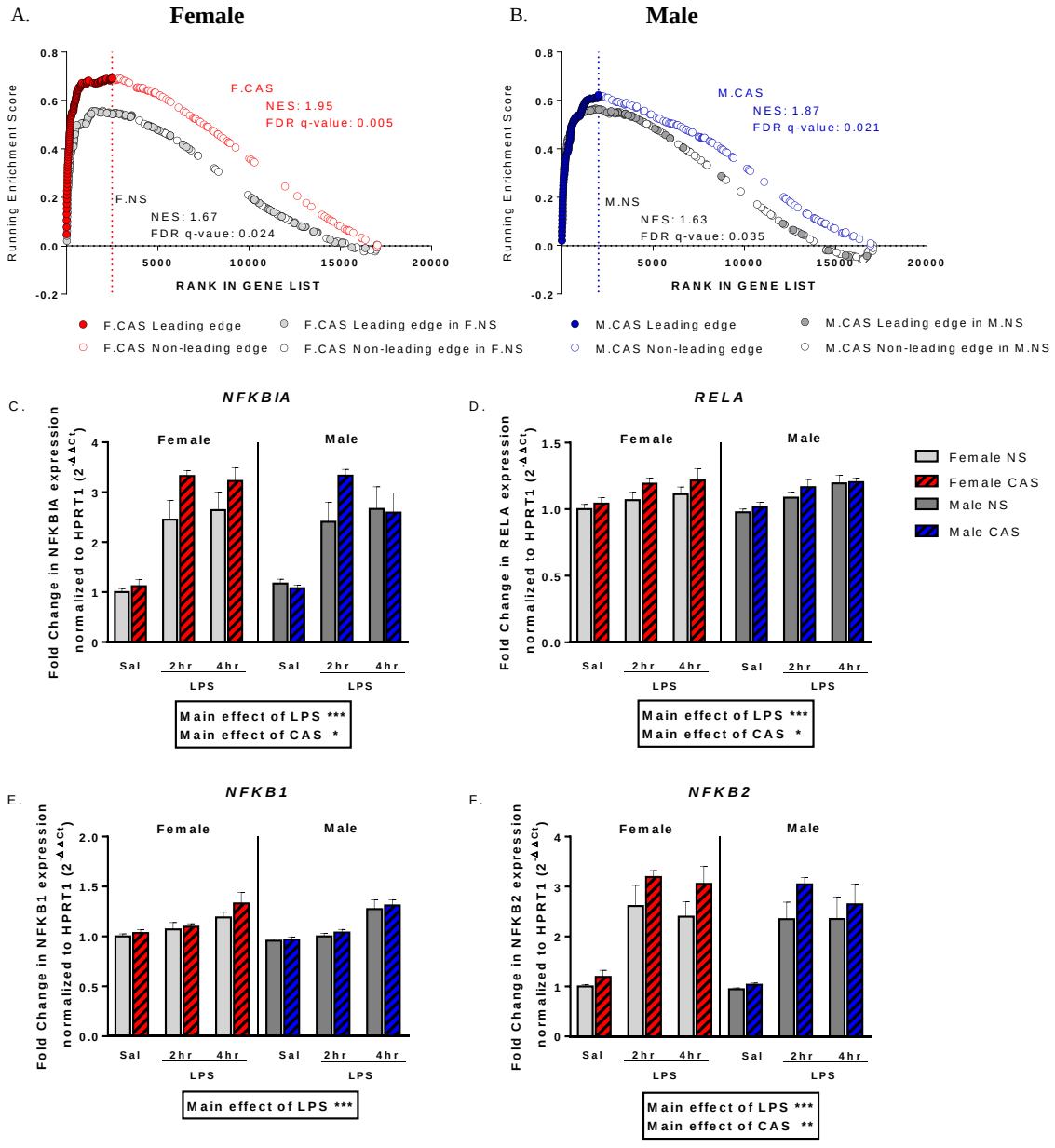


Fig 3.3. CAS enhances the enrichment of the NFκB signaling pathway and exaggerates canonical and non-canonical NFκB pathway genes. Female and male rats of NS or CAS background received a systemic injection of either saline or LPS in adulthood. Among hallmark pathways, HALLMARK_TNFA_SIGNALING_VIA_NFκB emerged as the most strongly enriched pathway in CAS rats compared to NS rats. A-B, Enrichment of the NFκB signaling pathway following LPS was assessed using GSEA, and the normalized enrichment score (NES) for each of the four experimental groups are shown along with the false-discovery rate (FDR) q-values. F.NS=Female, Non-stressed, F.CAS=Female, Chronic Adolescent Stress M.NS=Male, Non-stressed, M.CAS=Male, Chronic Adolescent Stress. Female and male CAS rats exhibited a larger NES compared to same-sex NS rats. C-F, mRNA expression of NFκB pathway members NFKBIA, RELA, NFKB1, and NFKB2 were assessed via quantitative PCR. Stimulus significantly altered the mRNA expression of all four genes ($p<0.001$) whereas sex did not impact the expression of any of the genes ($p>0.05$). CAS rats displayed exaggerated hippocampal mRNA expression of C) NFKBIA ($F(1,105)=4.209$, $p<0.05$), D) RELA ($F(1,105)=5.262$, $p<0.05$), and F) NFKB2 ($F(1,105)=8.186$, $p<0.01$) compared to NS rats. CAS did not significantly impact the expression of E) NFKB1 ($F(1,105)=2.718$, $p=0.102$). Data are mean fold change ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.4.4. CAS potentiates GR signaling in the hippocampus of female rats

LPS-induced enrichment of the GR signaling pathway was compared across the experimental groups to assess immune-induced glucocorticoid response. GSEA was

conducted using a custom-designed GR pathway (see Table 3.4 for a list of genes contained). Results from GSEA demonstrated that the GR signaling pathway was enriched in response to LPS in all four groups (Fig 3.4 A-B). However, the NES appeared greater in magnitude in CAS females compared to NS females, suggesting a greater extent of the enrichment in CAS. NS and CAS males demonstrated similar levels of GR pathway enrichment following LPS.

Next, we examined the impact of CAS on five GR targets from the GR signaling pathway list (Table 3.3). These included two anti-inflammatory genes strongly induced by glucocorticoids: DUSP1, a phosphatase that inhibits the MAPK signaling pathway (Shah, King et al. 2014), and TSC22D3 which inhibits the NFκB pathway (Ayroldi and Riccardi 2009). We also assessed the expression of glucocorticoid-inducible genes FKBP5 and SGK1 which are involved in the stress response and implicated in major depressive disorder (Binder 2009, Frodl, Carballedo et al. 2012). Finally, we assessed the expression of the GR itself (gene: NR3C1). Stimulus significantly altered the mRNA expression of DUSP1 ($F(2,104)=54.771$, $p<0.001$), TSC22D3 ($F(2,101)=19.492$, $p<0.001$), FKBP5 ($F(2,101)=18.085$, $p<0.001$), SGK1 ($F(2,101)=35.534$, $p<0.001$), and NR3C1 ($F(2,105)=4.843$, $p<0.01$) (Fig 3.4 C-G). There was no main effect of sex on DUSP1, TSC22D3, FKBP5, SGK1, or NR3C1 ($p>0.05$). CAS increased the expression of DUSP1 ($F(1,105)=4.174$, $p<0.05$), and displayed a trend toward an interaction with stimulus ($F(2,105)=2.991$, $p=0.055$). Because RNA-Seq suggested potential sex-specific alterations to the GR signaling pathway in CAS rats, we further assessed the simple effect of CAS within males and females each using a Sidak's test. CAS females showed a greater expression of DUSP1 compared to NS females ($F(1,52)=5.914$, $p<0.05$). However, CAS

males did not display potentiated DUSP1 expression (p>0.05). CAS did not impact the expression of TSC22D3, FKBP5, SGK1, or NR3C1 (p>0.05).

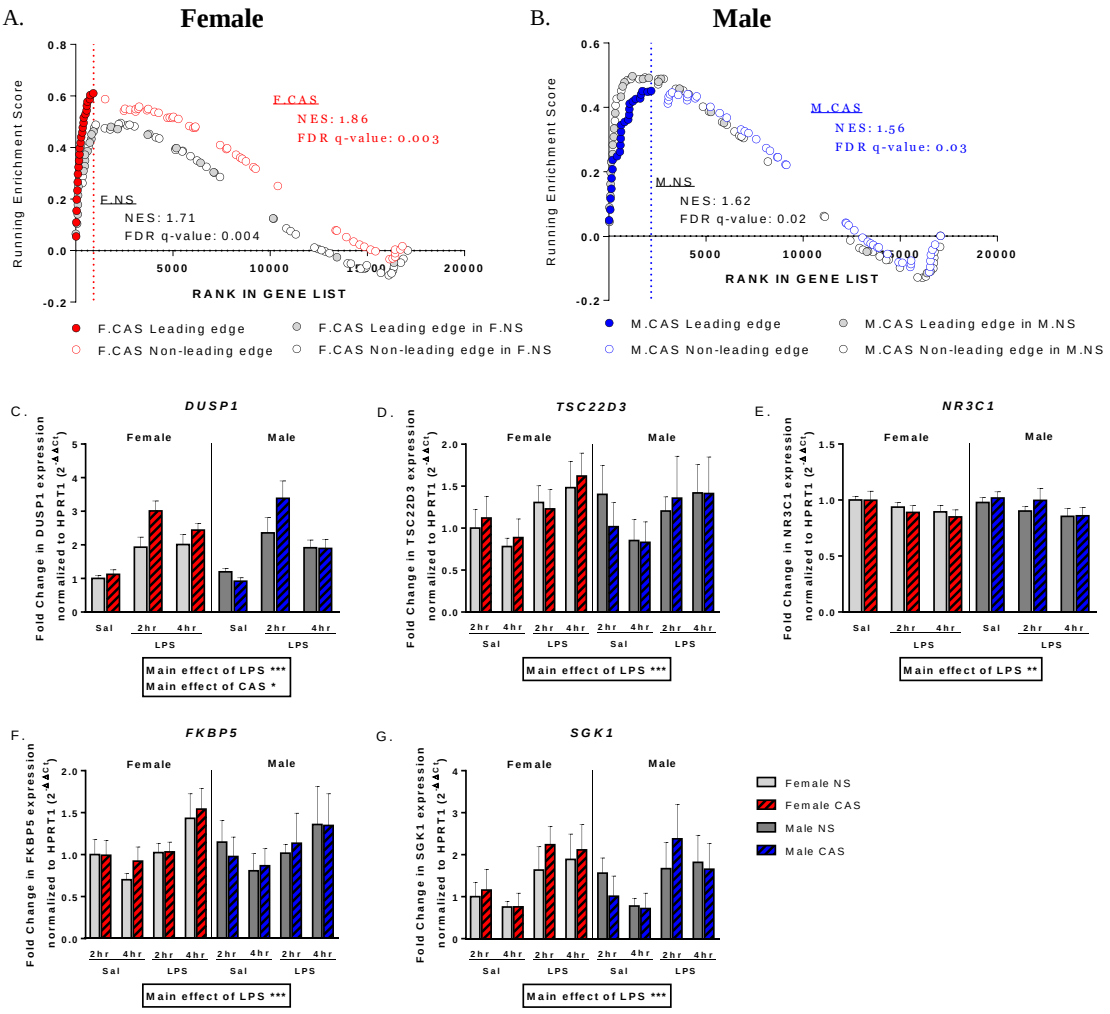


Fig 3.4. CAS exaggerates the enrichment of the GR signaling pathway and leads to potentiation of DUSP1 in the female hippocampus. Female and male rats of NS or CAS background received a systemic injection of either saline or LPS in adulthood. A-B, Enrichment of the GR signaling pathway following LPS was assessed using GSEA, and the normalized enrichment score (NES) for each of the four experimental groups are shown along with the false-discovery rate (FDR) q-values. F.NS=Female, Non-stressed,

F.CAS=Female, Chronic Adolescent Stress M.NS=Male, Non-stressed, M.CAS=Male, Chronic Adolescent Stress. Female CAS rats exhibited a larger NES compared to female NS rats (A) whereas male rats of either stress background displayed similar NES (B). C-G, mRNA expression of GR targets DUSP1, TSC22D3, FKBP5, SGK1, and NR3C1 were assessed via quantitative PCR. Stimulus significantly altered the mRNA expression of all GR targets ($p<0.01$) whereas sex did not significantly impact the expression of any of the five genes ($p>0.05$). CAS rats displayed a potentiated expression of DUSP1 ($F(1,52)=5.914$, $p<0.05$) (A), and there was a simple effect of CAS on DUSP1 expression within females, but not males. CAS did not alter the expression of D) TSC22D3, E) NR3C1, F) FKBP5, or G) SGK1 ($p>0.05$). Data are mean fold change ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.4.5. CAS potentiates chemokine signaling in the hippocampus of male and female rats

RNA-Seq indicated that some of the genes most strongly impacted by CAS are involved in chemokine signaling. Therefore we chose to assess the mRNA expression of the chemokines CCL2 and CXCL11, which were part of the leading edge genes driving the enrichment of the HALLMARK_TNFA_SIGNALING_VIA_NFκB pathway, over a time course post-LPS via targeted qPCR. LPS stimulus significantly induced the mRNA expression of CCL2 ($F(2,105)=83.023$, $p<0.001$), and CXCL11 ($F(2,105)=237.218$, $p<0.001$) (Fig 3.5). There was no main effect of sex on the mRNA expression of either chemokine ($p>0.05$). Interestingly, CAS exaggerated the hippocampal mRNA expression

of both CCL2 ($F(1,105)<0.01$, $p<0.05$) (Fig 3.5 A) and CXCL11 ($F(1,105)=4.201$, $p<0.05$) (Fig 3.5 B).

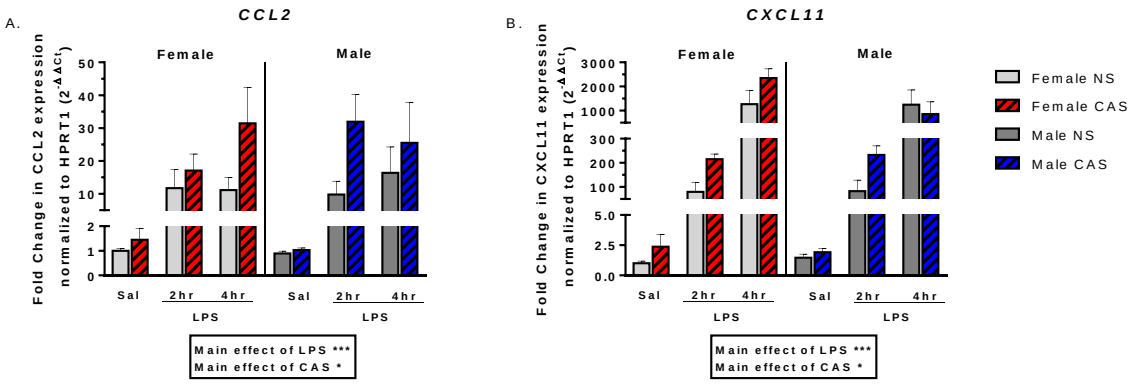


Fig 3.5. CAS exaggerates the mRNA expression of chemokines in the hippocampus. Female and male rats of NS or CAS background received a systemic injection of either saline or LPS in adulthood. LPS stimulus led to a significant increase in CCL2 and CXCL11 mRNA ($p<0.001$). Sex did not impact the expression of either chemokine ($p>0.05$). CAS rats displayed a potentiated expression of A) CCL2 ($F(1,105)<0.01$, $p<0.05$) and B) CXCL11 ($F(1,105)=4.201$, $p<0.05$) compared to NS rats. Data are mean fold change ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.5.Discussion

Here we demonstrated that hippocampal immune transcripts are inducible to a greater extent following LPS in CAS rats compared to NS litter mates. These transcriptomic changes were epitomized by the NFκB signaling pathway several key members of which displayed exaggerated mRNA expression when further examined via targeted qPCR. We demonstrated that CAS led to largely distinct sets of DEGs in the

unstimulated male and female rat hippocampus with only 19 genes overlapping. Consistently, microarray and RNA-Seq studies often reveal little to no overlap in genes differentially regulated by depression between males and females (Labonte, Engmann et al. 2017). In addition to immune-related pathways, CAS enriched several KEGG pathways involved in sugar and lipid metabolism in saline-injected rats, which warrants future investigation into the role of stress in altered metabolism in the brain. When stimulated with LPS, both male and female CAS rats demonstrated exaggerated NFκB-related transcription in the hippocampus compared to same-sex NS controls. Altered NFκB expression and activity have previously been reported in both rodent (Madrigal, Moro et al. 2001, Madrigal, Hurtado et al. 2002, Munhoz, Lepsch et al. 2006, Cohen, Kozlovsky et al. 2011) and human (Nagabhushan, Mathews et al. 2001, Pace, Mletzko et al. 2006) stress literature. While many of these reports have utilized a candidate gene approach, several genome-wide studies confirm our finding of an exaggerated NFκB pathway. In mice subjected to chronic restraint stress, genome-wide changes to gene expression in the hippocampus were detected by a microarray, and similar to our results, the TNFα-NFκB signaling pathway emerged as the top pathway based on the stress-regulated DEGs (Gray, Rubin et al. 2014). Our results are also consistent with the exaggerated expression of transcripts with the response elements for NFκB in chronically stressed humans (Miller, Chen et al. 2008).

While some of the genomic studies in chronically stressed humans (Miller, Chen et al. 2009, Miller, Rohleder et al. 2009, Miller, Murphy et al. 2014) and rodents (Wohleb, Hanke et al. 2011) have found concurrent evidence of diminished expression of transcripts bearing the GR response element, we did not observe this difference in our CAS paradigm.

In contrast, we found that CAS females exhibited enhanced GR signaling which could be interpreted as either a partial anti-inflammatory compensation (De Bosscher, Schmitz et al. 1997) or further demonstration of deficit indicating prolonged HPA axis activation or an overresponse in females (Bourke, Raees et al. 2013). Notably, the degree of immune response to LPS can be regulated through a GR-based mechanism, and environmental factors such as maternal care can modulate this relationship (Parent, Nguyen et al. 2017).

The functional significance of exaggerated NFκB in CAS animals remains to be investigated. Conflicting reports exist in the literature with regards to NFκB activity and functional outcomes following psychosocial stressors. While NFκB has been demonstrated to be necessary for alterations in synaptic strength (Christoffel, Golden et al. 2011) and neurogenesis (Koo, Russo et al. 2010) that underlie behavioral susceptibility in stressed male rodents, NFκB also appears to protect against ovarian hormone-related depressive-like behavior following chronic unpredictable stress in females (LaPlant, Chakravarty et al. 2009). The signaling pathway through which NFκB activity is induced also helps determine potential anxiolytic outcomes following NFκB activation (Zimmerman, Shaltiel et al. 2012). Chronic stress has been associated with dendritic atrophy and retraction (Conrad 2008), region-specific alterations in dendritic spine density (see Christoffel, Golden et al. (2011) for review) and reduced long term potentiation (LTP) that were reversible by anti-glucocorticoid treatment (Krugers, Goltstein et al. 2006). Altered NFκB activity has been implicated in chronic stress-induced synaptic alterations in the nucleus accumbens (NAc), a region that is involved in anhedonia, one of the core symptoms of depression. However, the functional implications of NFκB induction by stress appears to vary according to stressor type and duration, as well as the sex of the animal. On one hand,

NFκB signaling in the NAc has been shown to protect against the pro-depressive effects of six days of chronic unpredictable stress (CUS) in ovariectomized female mice compared to intact females (LaPlant, Chakravarty et al. 2009). Conversely, following social defeat stress in male mice, IKK-mediated NFκB signaling promotes social avoidance behavior (Christoffel, Golden et al. 2012) and led to an increase in the number of stubby dendritic spines on medium spiny neurons in the NAc (Christoffel, Golden et al. 2011). In these studies, activation of IKK signaling pathway was found to be necessary and sufficient to mediate both behavioral and structural alterations. Environmental stressors, including exposure to several drugs of abuse such as cocaine (Ang, Chen et al. 2001, Russo, Wilkinson et al. 2009) and alcohol (Crews, Nixon et al. 2006), also upregulate NFκB signaling in the brain. NFκB-mediated alterations in synaptic plasticity also appears to be important for encoding and retaining fear memory particularly in the amygdala (Yeh, Lin et al. 2004, Garabadu, Reddy et al. 2015). Because the cell type via which NFκB signals likely impacts the functional outcome, future investigations should tease apart the impact of CAS on neuronal and microglial NFκB activity in order to better understand the functional consequences of a primed NFκB pathway.

A recently proposed neuroimmune network hypothesis proposes that early life adversity strengthens immune-to-brain traffic (Hostinar, Nusslock et al. 2018). In fact, there is evidence of exaggerated NFκB signaling both in the periphery as well as in the brain of stressed individuals (Munhoz, Lepsch et al. 2006, Miller, Chen et al. 2008). To assess whether there is a basis for CAS to alter the crosstalk between the brain and the peripheral immune system, we assessed the expression of chemokines that have been demonstrated to be primed by stress (Girotti, Donegan et al. 2011, Pyter, Kelly et al. 2013)

and upregulated in humans with depression (Eyre, Air et al. 2016). Chemokine expression in the brain can be inducible up to several thousand fold following acute systemic inflammation (Fil, Borysiewicz et al. 2011). Here we've shown that the mRNA expression of the chemokines CCL2 and CXCL11 were upregulated following LPS injection several hundred to thousands fold, and that the extent of this upregulation was greater in CAS rats compared to NS rats. CCL2, also known as monocyte chemoattractant protein-1, is chemotactic for monocytes whereas CXCL11 is the primary ligand for CXCR3, and is chemotactic for interferon-activated T-cells. Here we performed transcardial perfusion to prevent blood from confounding the hippocampal immune results (Stankiewicz, Goscik et al. 2015). However, it is possible for chemokine-recruited leukocytes to infiltrate into the brain tissue and contribute to exaggerated neuroimmune signaling (Wohleb, Fenn et al. 2012). Therefore, in Chapter 4 we assessed the degree to which CAS altered the infiltration of leukocytes (Miller 2010).

Collectively, our results demonstrate evidence of hippocampal immune priming in CAS rats of both sexes, and suggest potential sex differences in LPS-induced glucocorticoid signaling that may be indicative of anti-inflammatory compensation in females. When considered in light of the GR signaling data presented in Chapter 2, these findings paint a picture of sexually dimorphic effects of CAS on the HPA axis-inflammation relationship whereby CAS possibly promotes an overactive HPA response in females but diminishes it in males.

Acknowledgements

We would like to thank the Yerkes Genomic Core for performing the RNA-Seq experiments, and Gregory Tharp, Dr. Mikhail Dozmorov, John Stansfield, and Dr. Zhaohui Qin for assistance with statistical analysis of the RNA-Seq data. The authors would also like to thank Sydney A. Rowson and Sean D. Kelly for helping with the chronic adolescence stress paradigm.

Declaration of interest

The authors declare no conflict of interest.

CHAPTER 4: CHRONIC ADOLESCENT STRESS LEADS TO AN ANXIETY-LIKE PHENOTYPE THAT IS ASSOCIATED WITH ALTERED IMMUNE CELL PROPERTIES

4.1.Abstract

The neuroimmune network hypothesis proposes that early life adversity strengthens immune-to-brain crosstalk via sensitizing immune cells of the brain as well as myeloid cells in the periphery. Importantly, the hyper-inflammatory activity of microglia and leukocytes from stressed rodents have been implicated in the development of anxiety-like behavior. We have previously demonstrated that chronic adolescent stress (CAS) in rats leads to enduring changes to neuro-immune reactivity both in the hippocampus and the periphery. Furthermore, CAS has also been reported to lead to sex-specific depressive-like behavior. One possibility thus far not addressed in the previous chapters is the impact of CAS on promoting immune-to-brain traffic, and the influence of exaggerated neuroimmune processes on affective-like behavior. Here we report that CAS leads to immediate and long-lasting anxiety-like behavior that may be associated with microglial number and activation following an LPS challenge in adulthood.

4.2.Introduction

In Chapter 2, we demonstrated the potential of chronic adolescent stress (CAS) to exaggerate the peripheral immune response to a systemic challenge received in adulthood. In parallel, Chapter 3 elucidated one potential mechanism via which CAS can prime inflammatory gene expression in the hippocampus. One possibility thus far not addressed in the previous chapters is the influence of CAS on promoting immune-to-brain traffic. The neuroimmune network hypothesis proposes that early life adversity strengthens immune-to-brain crosstalk via sensitizing immune cells of the brain as well as myeloid cells in the periphery (Nusslock and Miller 2016). Both the brain's resident immune cells, microglia (Wohleb, Fenn et al. 2012, Frank, Hershman et al. 2014), as well as monocytes and macrophages of the spleen (Stark, Avitsur et al. 2001, McKim, Patterson et al. 2016) and bone marrow (Wohleb, Powell et al. 2013) have been reported to assume a hyper-inflammatory and primed phenotype following various forms of stressors. Importantly, the inflammatory activity of microglia and leukocytes have been implicated in anxiety-like behavior in these stress models (Wohleb, McKim et al. 2014). Because CAS is known to lead to long-lasting deficits in affective-like behavior (Bourke and Neigh 2011) as well as exaggerated chemokine signaling in the hippocampus (Chapter 3), it is feasible that at least some of the exaggerated neuroimmune reactivity and behavioral deficits seen in CAS rats is due to hyper-activated microglia and increased trafficking of leukocytes to the hippocampus. Here we sought to extend the previous findings of the behavioral impact of CAS to include anxiety-like behavior in the open field test and sociability in the social interaction test. Importantly, plasma levels of IL-6 prior to stressor exposure has been found to be inversely correlated with performance in the social interaction test in mice

(Hodes, Pfau et al. 2014) thus supporting a causal role for inflammation to impact affective-like behavior.

Although stress-induced infiltration of leukocytes to the brain has been reported to occur in female mice (Brevet, Kojima et al. 2010, Ataka, Asakawa et al. 2013), and microglia from stressed adult male and female rats display distinct morphological profiles (Bollinger, Bergeon Burns et al. 2016), currently it is not clear whether there are sex differences in the extent to which early life stress dysregulates immune cells in the brain. Therefore we also aimed to determine whether CAS impacts the number, composition, and activation of immune cells of males and females differentially.

4.3.Methods

Animals

Male and female (*n*=49, see Table 4.1 for design) Wistar rats (Charles River) were weaned on PND 22 and pair-housed on a 14:10 reverse light:dark cycle. Standard rat chow and water were provided *ad libitum*. The Emory University Institutional Animal Care and Use Committee approved all animal use procedures. Animal experimentation was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

	Female				Male			
	NS		CAS		NS		CAS	
	Saline	LPS	Saline	LPS	Saline	LPS	Saline	LPS
2 hr	*n* = 6	*n* = 6	*n* = 6	*n* = 6	*n* = 6	*n* = 6	*n* = 7	*n* = 6

Table 4.1. Design for experiments in Chapter 4.

Chronic adolescent stress (CAS)

CAS was performed as previously described in Chapters 2 and 3. On PND 94, all rats received a single intraperitoneal injection of LPS (L3880, Sigma Aldrich) (0.25 mg/kg; 750,000 Endotoxin Units), and endpoints were collected at 2 hours post-injection.

Behavioral assessments

Rats underwent the open field test and social interaction test immediately following the end of the CAS paradigm (PND 50-52), and again in adulthood (PND 91-93) to test for the immediate and enduring effects of CAS on anxiety-like and social behavior (See Fig 4.1). On day 1 of behavioral testing, rats were placed in a rat open field apparatus (90x90x30 cm) and allowed to freely explore for 15 minutes. Cumulative time spent in the center and the corners of the open field were analyzed using Ethovision XT 12.0 (Noldus Information Technology Inc., Leesburg, VA). The number of fecal boli was recorded as a potential metric of anxiety-like behavior. The apparatus was cleaned with 70% ethanol in between trials. During days 2 and 3 of behavioral testing, the social interaction test took place in the same open field apparatus where now two metal perforated cups (12cm (d) x 33cm (h)) have been placed upside down along opposite walls of the open field. On day 2 both cups were empty, and the experimental rat was placed into the middle of the open field and allowed to explore the arena for 3 minutes. Time spent investigating each of the cups as well as time spent in the corners of the arena were recorded via Ethovision XT 12.0. On Day 3, one of the mesh cups contained a same-sex, unfamiliar Long-Evans rat. The experimental rat was introduced to the arena as previously and the

total time spent in the cup-interaction zones, and the corners of the arena was recorded. Following each trial, the experimental and the stimulus animals were each returned to their home cage, and the arena cleaned with 70% ethanol.

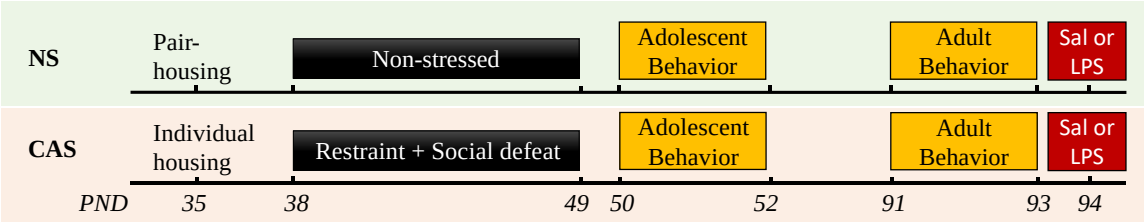


Figure 4.1. Timeline for experiments in Chapter 4.

Tissue collection

Following anesthesia with Euthasol overdose (150 mg/kg) on PND 94, rats were trans-cardially perfused with ice-cold PBS to remove peripheral immune cells and prevent inflammatory markers present in the blood from confounding gene expression in brain tissue. All experimental procedures were completed at least two hours before the end of the light cycle to avoid the corticosterone peak that occurs prior to beginning of the dark cycle.

Isolation of hippocampal cells for flow cytometry

Following brief saline perfusion, brains were extracted and one half hippocampus was dissected under sterile conditions. Neural Tissue Dissociation Kit was purchased from Miltenyi Biotec Inc. (#(P) 130-092-628, Auburn, CA) and enzymatic tissue dissociation was performed according to manufacturer's recommendations. Myelin and debris were removed via a 30% isotonic Percoll gradient (P4937 Sigma-Aldrich, St. Louis, MO).

Pellets were washed with PBS, cells were counted, and processed for staining as described below.

Flow cytometry

Cells were incubated with a Live/Dead fixable stain (#L23101 Thermofisher) for 30 minutes on ice followed by a wash with PBS. Next, non-specific binding was blocked for 5 minutes at 4°C with Purified Mouse Anti-Rat CD32 (#550270 BD Pharmingen, San Diego, CA). Cells were subsequently stained with anti-rat APC-CD11b (#562102 BD Pharmingen, San Diego, CA), anti-rat PE-Cy7-CD45 (#202214 Biolegend, San Diego, CA), and anti-rat PE-RT1B (#554929 BD Pharmingen, San Diego, CA) for 20 minutes at 4°C. Products used for isotype controls were as follows: APC Mouse IgA, κ Isotype Control (#562140 BD Pharmingen, San Diego, CA), PE/Cy7 Mouse IgG1, κ Isotype Control Antibody (#400125 Biolegend, San Diego, CA), and PE Mouse IgG1, κ Isotype Control (#550617 BD Pharmingen, San Diego, CA). Following two washes in FACS buffer, cells were immediately analyzed on a BD LSRII Fortessa. Ten thousand $CD11b^+$ events were recorded.

Immunohistochemistry

Following saline perfusion, one half hemisphere of the rat brain was post-fixed in 4% PFA for 48 hours, then transferred into PBS at 4°C until sectioning. Coronal sections of 40 uM thickness spanning Bregma -1.80 mm to -6.60 mm (Paxinos and Watson 2009) were cut on a Leica VT1000 S vibratome (Buffalo Grove, IL). Six consecutive coronal sections per animal including three sections containing anterior hippocampus (anterior of

-4.36 mm) and three sections containing posterior hippocampus (posterior to -4.36 mm) were stained with a primary antibody for Iba-1 (Wako Chemicals USA Inc., Richmond, VA).

Microglial count

To estimate the number of microglia in the hippocampus, unbiased stereology was performed using StereoInvestigator (MBF Bioscience, Williston, VT). In the Optical Fractionator probe, the grid size used was 675x1000 and the counting frame size was 150x150.

Statistical Analysis

Behavioral data were analyzed via three-way ANOVAs in SPSS by modeling age (within-subjects: adolescent vs. adult), sex (between-subjects: male vs. female), and stress (between-subjects: NS vs. CAS) as main factors, and their interactions. Flow cytometry and stereology data were analyzed via three-way ANOVAs (sex * stress * stimulus) performed in SPSS 24.0. Correlation analyses between behavioral and molecular endpoints were performed in GraphPad Prism 7.0 using data from LPS-injected rats without further stratifying by sex or stress. Data are expressed as mean ± SEM. Significance threshold was set to $\alpha = 0.05$ for all analyses.

4.4.Results

4.4.1. CAS leads to anxiety-like behavior in the open field in adolescence

We assessed the impact of CAS on anxiety- and depressive-like behavior in adolescence and again in adulthood. Female rats overall spent more time in the center ($F(1,48)=18.209$, $p<0.001$) and less time in the corners ($F(1,48)=21.388$, $p<0.001$) of the open field compared to males (Fig 4.2). Females also traveled a greater distance in the open field ($F(1,48)=6.992$, $p=0.011$) with higher velocity ($F(1,48)=6.959$, $p=0.011$) compared to males. Adolescent rats spent more time in the center ($F(1,48)=15.998$, $p<0.001$) and less time in the corners ($F(1,48)=27.459$, $p<0.001$) of the open field compared to themselves as adults. There was a trend toward a age*stress interaction on time spent in the center ($F(1,48)=3.622$, $p=0.063$) such that the effects of CAS were more prominent in adolescence immediately following the CAS paradigm. This finding was accompanied by a significant age*stress interaction on distance traveled ($F(1,48)=5.180$, $p=0.027$) and velocity ($F(1,48)=21.388$, $p<0.027$). We therefore assessed the impact of CAS in adolescence and adulthood on time spent in the center separately using a 2x2 ANOVA with sex and stress as main factors and their interaction. In adolescence, CAS rats displayed a trend toward decreased time in the center ($F(1,47)=2.802$, $p=0.1008$) (Fig 4.2 A) and a significant increase in time spent in the corners ($F(1,48)=4.729$, $p=0.0346$) (Fig 4.2 B). These effects of CAS were no longer observed in adulthood (center: $p=0.3626$; corner: $p=0.6792$).

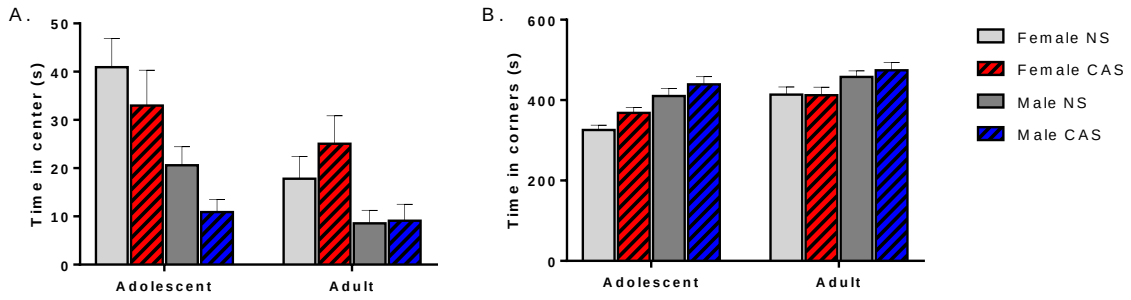


Fig 4.2. Chronic adolescent stress (CAS) led to an anxiety-like phenotype in the open field in adolescence that was no longer present in adulthood. Male and female rats of NS or CAS background were tested in the open field at baseline once in adolescence on postnatal day (PND) 50, and again in adulthood on PND 91. Adolescent rats spent more time in the center (p<0.001) and less time in the corners (p<0.001) of the open field compared to themselves as adults. Female rats spent more time in the center (p<0.001) and less time in the corners (p<0.001) compared to males. A) Adolescent CAS rats displayed a trend toward decreased time spent in the center compared to NS controls (p=0.1008). B) Adolescent CAS rats also spent more time in the corners of the open field (p<0.001). Data are presented as mean±SEM.

4.4.2. CAS males display anxiety-like behavior during social interaction in adulthood

Adolescent rats spent significantly more time in the interaction zones surrounding the Target-containing cup ($F(1,45)=45.794$, p<0.001) compared to themselves as adults (Fig 4.3 A). While neither sex ($F(1,45)=1.310$, p=0.259) nor stress ($F(1,45)=0.242$, p=0.625) impacted target interaction, sex and stress significantly interacted

(F(1,45)=5.013, p=0.030). We further probed this interaction with a 2x2 ANOVA (sex and stress as main factors, followed by a Sidak's posthoc testing for the simple effect of CAS within each sex) in adolescence and adulthood. The simple effect of CAS was not significant within females (p=0.2150) or males (p=0.1790) in adolescence or adulthood (female: p=0.5710; male: p=0.9999). Next, we assessed how much time experimental rats spent in the corners of the arena in the presence of a target rat as a metric of social anxiety (Henriques-Alves and Queiroz 2015). Three-way ANOVA revealed a main effect of age (F(1,44)=36.287, p<0.001), and trends toward age*sex (F(1,44)=3.547, p=0.066) and age*stress (F(1,44)=3.210, p=0.080) interactions (Fig 4.3 B). There was a trend toward a main effect of sex (F(1,44)=3.226, p=0.079) and a significant sex*stress interaction (F(1,44)=5.420, p=0.025). Upon assessing the simple effect of CAS within males and females at each age, we found that CAS led to a significant increase in time spent in corners in adult males (t(46)=2.787, p=0.0154), but not adult females (p=0.9825). Furthermore, CAS males did not display a similar increase in time spent in corners when the target rat was not present on the first day of the social interaction test (data not shown). CAS did not impact time spent in the corners in adolescence (female: p=0.1434; male: p=0.3030).

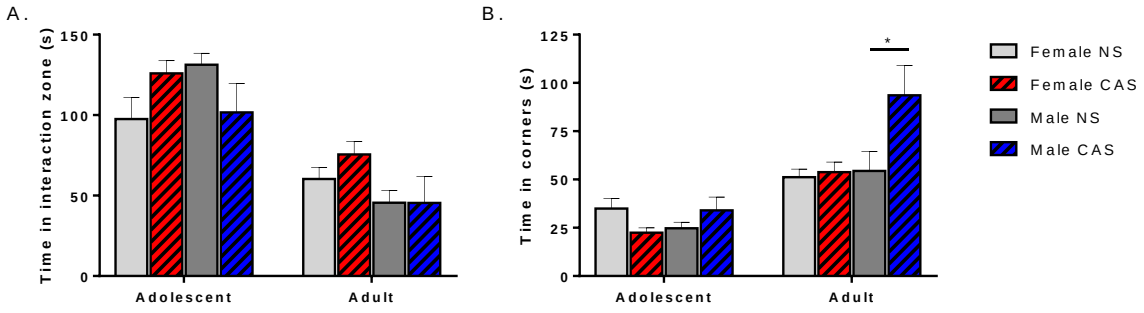


Fig 4.3. CAS did not impact social interaction in either adolescence or adulthood, but increased social anxiety in adult males. Male and female rats of NS or CAS background

were assessed in the social interaction test at baseline once in adolescence on PND 51-52, and again in adulthood on PNDs 92-93. Adolescents spent significantly more time in the interaction zones surrounding the Target-containing cup (p=0.001) compared to themselves as adults. Sex did not impact the duration of social interaction (p=0.625). A) CAS did not impact time spent interacting with a same-sex rat in adolescence (p=0.9633) or adulthood (p=0.4924). B) CAS male rats displayed increased time spent in the corner of the open field during social interaction (*, p=0.0154). Data are presented as mean±SEM.

4.4.3. CAS does not alter the number of total microglia in the hippocampus

The total number of hippocampal microglia was estimated in Optical Fractionator using mean section thickness. While LPS stimulus did not significantly impact the estimated number of microglia ($F(1,40)=2.156$, p=0.150), there was a trend toward a sex * stimulus interaction ($F(1,40)=3.028$, p=0.090) such that male LPS-injected rats displayed the greatest estimated number of microglia (Fig 4.4). There was also a trend toward a greater number of microglia in males compared to females ($F(1,40)=2.778$, p=0.103).

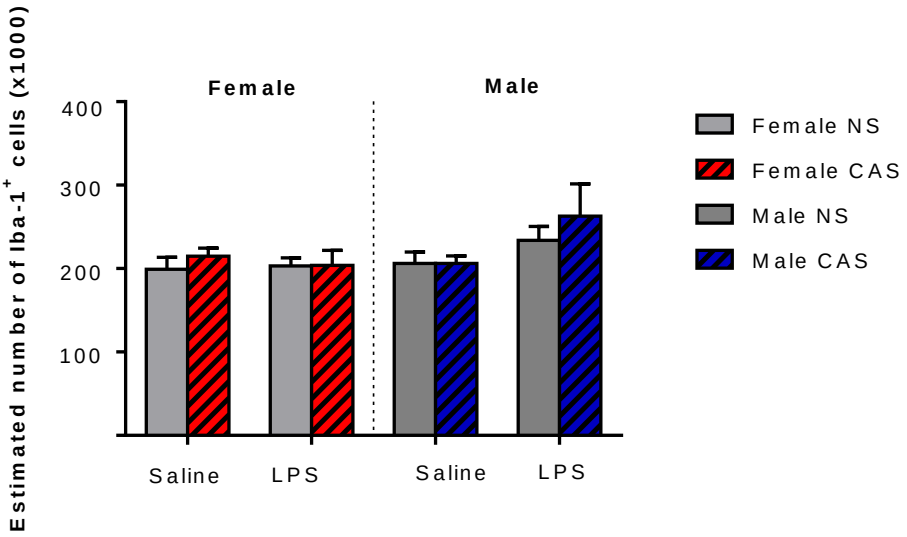


Fig 4.4. CAS did not impact the total number of hippocampal microglia. The number of total hippocampal microglia was assessed at two hours following either saline or lipopolysaccharide (LPS) *i.p.* in adult male and female rats of NS or CAS background on PND 94. Males displayed a trend toward a greater number of microglia compared to females (p=0.103). LPS stimulus did not significantly impact the estimated number of microglia (p=0.150). Data are presented as mean±SEM.

4.4.4. CAS does not impact leukocyte infiltration or MHC-II presentation by microglia

We performed flow cytometry on cells isolated from hippocampal tissue to examine whether the proportion of infiltrating leukocytes in the hippocampus was impacted by CAS. CD45 and CD11b expression was used to identify immune cells and separate them into microglia: $CD11b^{+}/CD45^{int}$ (CD11b-positive, CD45-intermediate) and myeloid cells, which include monocytes and neutrophils: $CD11b^{+}/CD45^{high}$ (CD11b-positive, CD45-high). The extent of leukocyte infiltration was expressed as percent of $CD11b^{+}/CD45^{high}$

population from overall $CD11b^+$ cells (Fig 4.5 A-E). The proportion of infiltrating leukocytes was not impacted by sex ($F(1,40)=1.783$, $p=0.189$), CAS ($F(1,40)=0.222$, $p=0.640$), or LPS ($F(1,40)=0.086$, $p=0.771$) (Fig 4.5 F). We also assessed whether the percentage of microglia with surface expression of MHC-II (RT1B) was different among the groups (Fig 4.5 G). There was a trend toward increased microglial $MHC\text{-}II^+$ presentation in LPS-injected rats ($F(1,40)=3.225$, $p=0.080$) (Fig 4.5 H). However, the proportion of infiltrating leukocytes was not impacted by sex ($F(1,40)=0.134$, $p=0.716$) or CAS ($F(1,40)=0.097$, $p=0.757$).

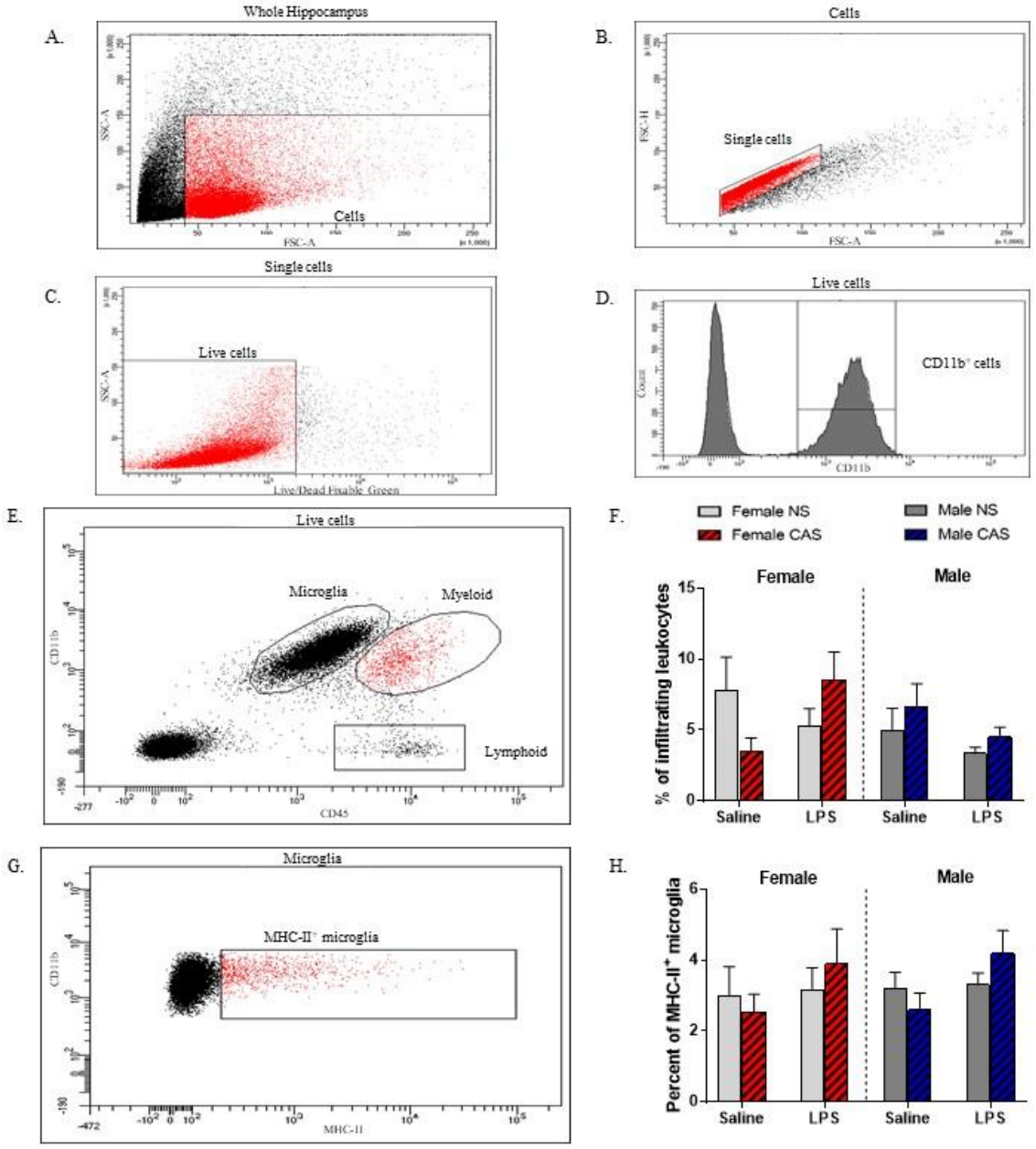


Fig 4.5. CAS did not impact the percentage of CD11b^{+}/CD45^{high} leukocytes or proportion of MHC-II^{+} microglia. Flow cytometry was performed on hippocampal cells from male or female, NS or CAS rats that received either saline or LPS and collected at 2 hours following injection (*n*=5-7/group). Whole hippocampus cell suspensions were gated for cells (A), which in turn were gated for single cells (B). Live/Dead fixable green dye-negative, live cells (C) were selected and gated according to CD11b and CD45 status as

shown in D, E, and G. E) Populations of $CD11b^{+}$, $CD45^{int}$ microglia, $CD11b^{+}$, $CD45^{high}$ myeloid cells, and $CD11b^{low}$, $CD45^{high}$ lymphoid cells were discernible. F) The percent of $CD11b^{+}/CD45^{high}$ myeloid cells was similar across sex (p=0.189), stress (p=0.640), and LPS stimulus (p=0.771). G) MHC-II presentation by microglia was gated as shown. H) The percent of $MHC\text{-}II^{+}/CD11b^{+}/CD45^{int}$ myeloid cells was similar across sex (p=0.716) and stress (p=0.757). LPS stimulus led to a trend toward increased MHC-II presentation by microglia (p=0.080). Data are shown as mean± SEM.

Upon finding no increase in leukocyte infiltration by LPS, we conducted a pilot experiment to assess whether leukocyte infiltration was dependent on LPS dose and time point of assessment. In this experiment, one male Long Evans rat was systemically injected with saline whereas another male Long Evans rat received 1 mg/kg LPS *i.p.*, and hippocampal leukocyte presence was assayed by flow cytometry at 12 hours following injection. Although preliminary, the results shown in Fig 4.6 suggest that the cell isolation and flow cytometry protocols utilized in the CAS study are sufficient to detect leukocyte infiltration that was induced by a higher dose of LPS and at a later time.

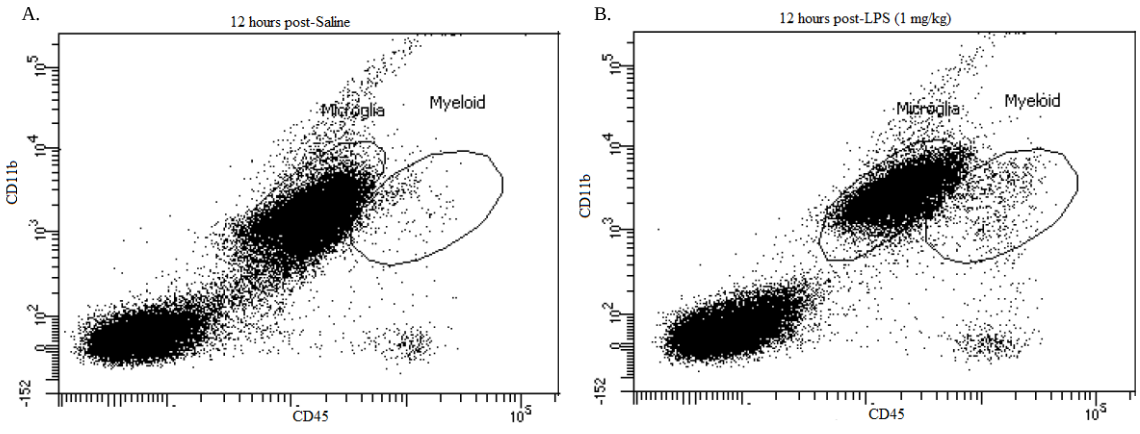


Figure 4.6. Increased leukocyte infiltration may occur following a higher dose of LPS and at a later time. A Long Evans male rat was systemically injected with saline (A) or 1

mg/kg LPS (B), and hippocampal presence of leukocytes was assessed 12 hours following injection. More myeloid cells were observed in the LPS-injected rat, suggesting that an LPS dose higher than the 0.25 mg/kg used in Figure 4.4 may lead to increased leukocyte infiltration if assessed at a later time point than 2 hours.

4.4.5. Adolescent behavior is associated with microglial number in adulthood

We examined whether behavioral performance in adolescence and adulthood was associated with activity or number of immune cells in adulthood were using correlation analyses. Microglial data from adult LPS-injected rats were used without stratifying by sex or stress because these factors did not significantly impact microglial outcomes. There was a trend toward an inverse association between time spent in the center of the open field in adolescence and the number of total microglia following the LPS challenge in adulthood (R^2=0.2549, p=0.0549) (Fig 4.7 A). A similar association between adult open field behavior and microglial number post-LPS in adulthood was not present (R^2=0.1103, p=0.2266) (Fig 4.7 B). There was also a trend toward an inverse association between time spent interacting with a same-sex Long Evans rat in adolescence and the number of total microglia following the LPS challenge in adulthood (R^2=0.2177, p=0.0926) (Fig 4.7 C). Similarly, this association was no longer present with regards to adult social interaction behavior (R^2=0.0001, p=0.9720) (Fig 4.7 D).

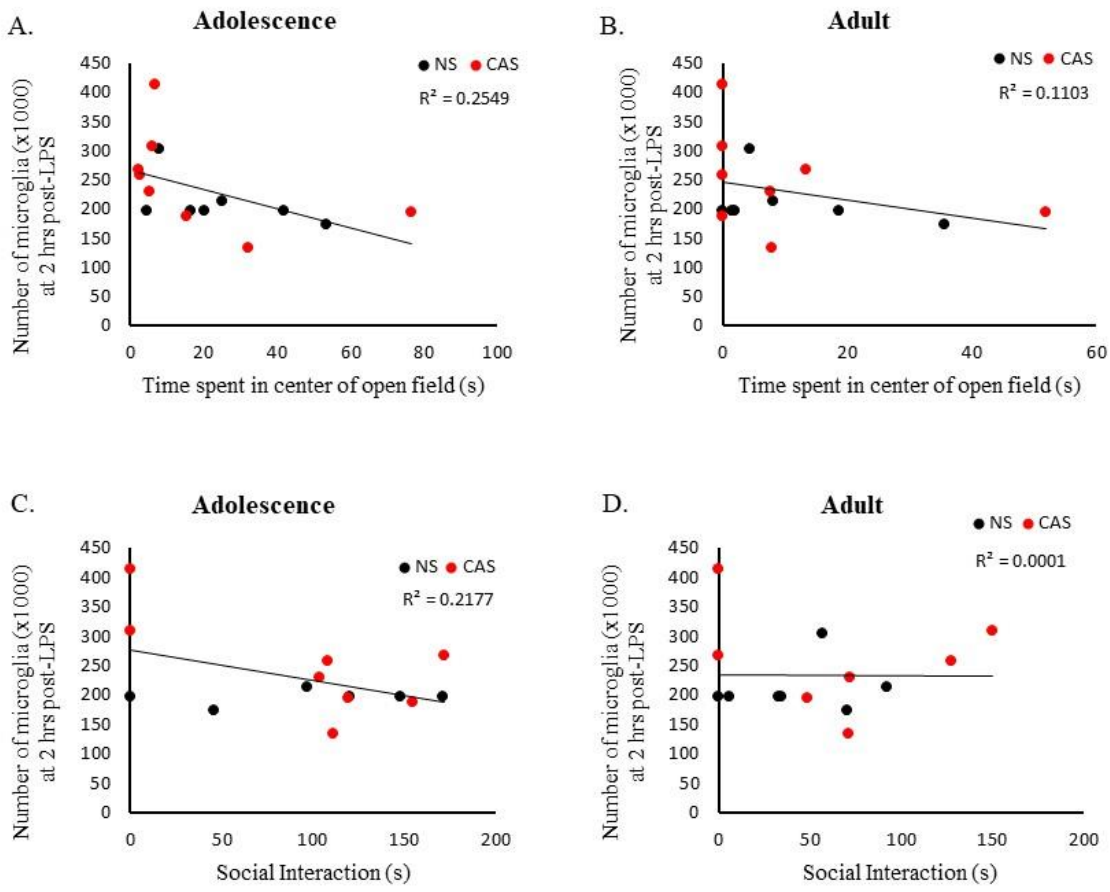


Fig 4.7. Adolescent, but not adult, behavior may be associated with microglial number following LPS in adulthood. Individual data points from LPS-injected NS and CAS groups are shown; but correlation was performed without stratifying by sex or stress. A) There was a trend toward an inverse association between time spent in the center of the open field in adolescence and the number of total microglia following the LPS challenge in adulthood (p=0.0549). B) This association was no longer present in adulthood (p=0.2266). C) There was also a trend toward an inverse association between time spent interacting with a same-sex Long Evans rat in adolescence and the number of total microglia following the LPS challenge in adulthood (p=0.0926). D) Similarly, this association was no longer present in adulthood (p=0.9720).

4.5.Discussion

Here we demonstrated that CAS leads to immediate anxiety-like behavior as indicated by increased time spent in the corners of the open field in male and female adolescent rats. Furthermore, CAS also leads to a delayed-onset social anxiety during interaction with a same-sex conspecific in CAS adult male rats. Interestingly, performance on these behavioral tests may be associated with microglial number and activation following an LPS challenge in adulthood. Previously, CAS has been shown to lead to sex-specific depressive-like behavior whereby CAS females, but not males, displayed reduced sucrose consumption and decreased latency to float along with decreased struggling in the forced swim test (Bourke and Neigh 2011). These behavioral effects of CAS were present when rats were tested in adolescence immediately following CAS or later in adulthood. The current study demonstrated a complementary anxiety-like phenotype in adolescence as indicated by more time spent in the corners of the open field and a trend toward decreased centrality. We also found that in either adolescence or adulthood, CAS did not alter time spent interacting with a con-specific in a two-cup social interaction test where decreased interaction time is traditionally thought to indicate anhedonia. However, CAS led to a "social anxiety" phenotype in adult males as indicated by increased time spent in the corners during social interaction. Spending more time in the corners in the presence of a con-specific that typically-developing rats would find stimulating (Hodes, Pfau et al. 2014) may suggest social anxiety in CAS males (Henriques-Alves and Queiroz 2015). Interestingly, CAS males were not different from NS males regarding time spent in the corners while there was no stimulus animal present, which indicates that this anxiety-like behavior is socially motivated. Furthermore, this social anxiety did not emerge in CAS

males until adulthood, therefore suggesting a potential role for the "incubation" period between stressor exposure and the emergence of behavioral deficits in adulthood.

Next, we assessed whether the number and activation state of immune cells present in the hippocampus was changed due to CAS using unbiased stereology and flow cytometry. The number of microglia in the hippocampus displayed a trend toward increasing following a systemic LPS challenge. Consistently, other studies have demonstrated that either a single injection of a higher dose of LPS (Fukushima, Furube et al. 2015) or repeated administration of LPS (Chen, Jalabi et al. 2012) led to an increase in proliferation of microglia in various brain regions including the hippocampus. We found no sex differences in the total number of microglia in the hippocampus, consistent with previous reports from adult male and female rats (Nemeth, Reddy et al. 2014). In the literature, microglial density in the hippocampus has been shown to be increased in female, but not male, rats with a background of chronic restraint stress compared to same-sex, non-stressed controls (Bollinger, Collins et al. 2017). In contrast to this study in which the animals were collected immediately following the last stressor exposure, we found that CAS did not impact the estimated total number of microglia in the hippocampus in either males or females, suggesting potential washout of CAS effects in the months between stress and terminal endpoint. Similarly, rats injected with LPS displayed a trend toward increased surface presentation of MHC-II by microglia, potentially indicating a subtle increase in activation. As with the number of microglia, neither sex nor stress impacted the percent of microglia displaying MHC-II. Contrary to reports that show increased leukocyte infiltration in repeatedly defeated mice (Wohleb, Powell et al. 2013), we did not find evidence of increased infiltration in rats due to sex, stress, or LPS. It is worth noting that

other social defeat studies in mice have not confirmed increased monocyte trafficking to the brain (Lehmann, Cooper et al. 2016). A systemic injection of LPS has been demonstrated to increase leukocyte infiltration to the brain of mice (Wohleb, Fenn et al. 2012), but contrary evidence also exists (Chen, Jalabi et al. 2012). No similar reports are available for rats to date. The phenomemon of leukocyte infiltration is possible in rats with more severe inflammatory insults such as ischemia (Campanella, Sciorati et al. 2002), experimental allergic encephalomyelitis (Mensah-Brown, Shahin et al. 2011), traumatic brain injury (Truettner, Bramlett et al. 2017), and glioma (Badie, Schartner et al. 2000). Although many studies have successfully used the CD11b and CD45 markers to differentiate microglia from myeloid cells, it has been widely documented that microglia increase their surface expression of CD45 during activation (Li, Tan et al. 2013). It is therefore possible that some of the more recent developments in microglial surface markers such as transmembrane protein 119 (Tmem119) (Bennett, Bennett et al. 2016) may help better differentiate the populations in question. Furthermore, although the current study investigated only cells of the innate immune system, it is plausible that cells of the adaptive immune system play a role in mediating the impact of chronic stress (Brachman, Lehmann et al. 2015).

Nonetheless, the extent to which microglia responded to an adult LPS challenge in terms of cell number was found to be inversely correlated with behavioral performance in adolescence. Although it is difficult to establish whether behavioral deficits cause the changes underlying altered microglial reactivity or vice versa, these results offer several directional possibilities. First, it is possible that the behavioral phenotype of adolescent rats over time influences their immune sensitivity into adulthood. In fact, there is some

supportive evidence from longitudinal studies in humans whereby affective response to daily stressors predicted mortality risk over 20 years in individuals with one or more chronic illnesses (Chiang, Turiano et al. 2018). Secondly, the association between adolescent behavior and adult microglial reactivity may indicate that the inflammatory processes which ultimately lead to exaggerated neuroimmune reactivity (see Chapter 3) and microglial activation and proliferation may have already begun at the time of behavioral assessment in adolescence. Consistently, inflammatory signaling assessed at a given time can predict future immune sensitivity (Miller, Rohleder et al. 2009, Hodes, Pfau et al. 2014). Third, individual differences in some other variable such as the HPA axis could be regulating both adolescent behavioral and adult microglial endpoints. Future studies utilizing inhibitors of inflammation during or prior to stressor exposure can help distinguish between these possibilities. Furthermore, because stress-induced sensitization of immune response can be detected up to several days following the immune challenge (Johnson, O'Connor et al. 2002), investigating the behavioral consequences of CAS in the days following LPS can help clarify the functional significance of stress-induced inflammation in future studies.

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Declaration of interest

The authors declare no conflict of interest.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1.CAS-induced changes to NFκB-GR dynamics

We have performed a series of experiments demonstrating that CAS led to the priming of peripheral (Chapter 2) and central (Chapter 3) immune reactivity that may be mediated by increased activity or expression of NFκB. Although the studies presented in this thesis utilized LPS to induce immune activation, it would be reasonable to expect that other, non-pathogenic sources of inflammation, including psychological stressors, would similarly engage the NFκB pathway (Bierhaus, Wolf et al. 2003, Wohleb, McKim et al. 2014) in the periphery and possibly in the brain. Therefore, individuals exposed to chronic stress during adolescent may be expected to display exaggerated neuroimmune processes both during systemic inflammation such as that induced by somatic illnesses (Capuron and Miller 2011) and also in response to psychological and physiological stressors if the latter are severe enough to cause detectable increases in inflammation. Concurrently, we have presented evidence of LPS-induced GR signaling in CAS females that was similar to the NS female response in the periphery (Chapter 2) but exceeded it in the hippocampus (Chapter 3). In contrast, male CAS rats displayed a hippocampal induction of GR signaling to a similar extent as NS males, but displayed blunted corticosterone concentrations in the periphery compared to NS males (see Table 5.1). Consistent with previously demonstrated sexually dimorphic HPA axis responses to LPS in mice (Frederic, Oliver et al. 1993) our data indicate greater LPS-induced serum corticosterone output in female rats compared to male rats. It is possible that the CAS background exaggerates the sexual dimorphism that normally exists among NS rats.

	CAS female	CAS male
Hippocampus	↑ NFκB pathway	↑ NFκB pathway
	↑ IL1B mRNA	→ IL1B mRNA
Serum	→ Serum IL-1β	↑ Serum IL-1β
	→ Serum Cort	↓ Serum Cort

Table 5.1. CAS sex-specifically impacts immune and endocrine outcomes in the brain and periphery.

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The functional implications of enhanced hippocampal GR pathway enrichment in CAS females remain to be studied. One likely explanation is that this enhancement is part of a compensatory mechanism aimed at neutralizing the exaggerated immune processes otherwise seen in CAS females. It should be noted however, that although the effects of GR are commonly conceptualized as anti-inflammatory, there are several contexts in which GR has been documented to enhance pro-inflammatory processes. In particular, timing of glucocorticoid release has been proposed as a key determinant of this shift (Dhabhar 2009, Horowitz and Zunszain 2015) in which glucocorticoid presence preceding the onset of immune activation has a priming or stimulatory effect, whereas glucocorticoid release that follows immune activation exerts a suppressing effect on inflammation (Frank, Miguel et al. 2010). In fact, glucocorticoids released by both acute inescapable shock stress (Frank, Thompson et al. 2012) as well as acute and chronic exogenous administration of glucocorticoids (Frank, Miguel et al. 2010, Frank, Hershman et al. 2014) sensitize the central and peripheral inflammatory response, and can sensitize hippocampal microglia to hyper-respond (Barrientos, Thompson et al. 2015). Furthermore, in mice experiencing seizures or ischemia, glucocorticoid signaling can promote inflammatory processes including NFκB activation, and perpetuate CNS tissue damage (Sorrells, Caso et al. 2013,

Sorrells, Munhoz et al. 2014). Similarly, a prior history of stress exacerbates LPS-induced neuronal damage (Espinosa-Oliva, de Pablos et al. 2011). In contrast to CAS females, CAS males did not display enhanced GR pathway enrichment in the hippocampus, and mounted a hypo-response of the HPA axis in the periphery. In particular, the blunted serum corticosterone response coupled with exaggerated serum IL-1β concentrations following LPS is consistent with a model of glucocorticoid resistance in which glucocorticoids such as corticosterone do not effectively suppress ongoing inflammation (Horowitz and Zunszain 2015). Our results therefore indicate potential sex differences in the ability of the HPA axis to regulate inflammation following CAS.

5.2.Cellular origin of exaggerated neuro-immune reactivity in CAS rats

NFκB is thought to act in a pro-inflammatory fashion when signaling via microglia whereas its signaling within neurons promotes survival and homeostatic functions (Camandola and Mattson 2007). Because the current studies were conducted on whole hippocampal tissue, it is not clear whether the exaggerated NFκB signaling observed in CAS rats is derived from neuronal or microglial activity. However, as neurons and microglia both express receptors for cytokines (Rothwell, Luheshi et al. 1996) inflammatory signaling within either cell type could impact functional outcomes.

While CAS did not impact the number of hippocampal microglia or increase MHC-II presentation, it is possible that other indices of microglial activation, such as microglial morphology, would provide more complete information about the reactivity of microglia.

Furthermore, microglial phenotypes can be studied in more detail using newer flow cytometry markers and RNA-Seq techniques (Hirbec, Marmai et al. 2018). Our finding of altered NFκB and GR signaling is especially relevant in the context of previous research demonstrating microglial sensitization in stressed rats is mediated via glucocorticoid signaling (Frank, Thompson et al. 2012). Consistently, microglia have been demonstrated to be critical in neuroinflammatory and behavioral profiles seen in other models of chronic stress. For example, microglial depletion using a CSF1R antagonist prior to social defeat stress has been demonstrated to prevent some but not all of stress-induced neuroimmune alterations (McKim, Weber et al. 2017). To address whether NFκB signals through microglia, future experiments should assess NFκB expression and/or activity within microglia that have been magnetically isolated or sorted from the hippocampus. A number of NFκB inhibitors and modulators with varying degrees of specificity of action are also available (Gyrd-Hansen and Meier 2010). Furthermore, modulators of glucocorticoid signaling, including FKBP5 inhibitors, may provide an alternative opportunity to modulate NFκB (Rein 2016).

Beyond primed microglia, increased immune-to-brain traffic has been demonstrated as another mechanism via which stress sensitizes the neuroimmune reactivity in mice (Wohleb, Fenn et al. 2012). In our rat model, although CAS exaggerated chemokine signaling in the hippocampus, flow cytometry data indicate that CAS did not impact leukocyte infiltration. While it is possible that a longer time course or a higher LPS dose would have revealed the impact of CAS on leukocyte trafficking, the experiments presented here demonstrate that altered leukocyte trafficking could not account for CAS-induced neuroimmune priming.

5.3.Sex differences in stress-induced neuro-inflammation

The existing literature on sex differences in stress-induced neuroinflammation is sparse. Data from human studies suggest that women respond to acute stressors in a more pro-inflammatory fashion with increased mobilization of various immune cells and decreased glucocorticoid sensitivity. Furthermore, some evidence suggests that chronic stress may lead to exaggerated immunosuppression in women compared to men. These data are consistent with the behavioral susceptibility of women to inflammatory challenges, yet they do not explain the mechanisms by which inflammatory biomarkers are more consistently linked to depression in men compared to women. However, much of the evidence generated from rodent studies suggest that males may be more likely to display stress-induced inflammatory changes. Our results hereby confirm in female rodents the occurrence of stress-induced immune priming, which has previously been demonstrated in male rodents only (Munhoz, Lepsch et al. 2006, Pyter, Kelly et al. 2013, Hudson, Jacobson-Pick et al. 2014). We furthermore demonstrated that the LPS-induced levels of a subset of pro-inflammatory cytokines were exaggerated in CAS females only. However, these data do not readily support the hypothesis that female susceptibility to mood disorders is fueled by sex-specific neuroimmune responses following stressor exposure as male CAS rats also display a sensitized neuroimmune transcriptome.

It should be noted that important methodological issues such as developmental timeline of stressor exposure, usage of behavioral assays validated in males only, and technical aspects of accurately measuring inflammatory outcomes in naturally cycling females may be introducing a potential bias toward increased immune changes in stressed

males and continued efforts towards maximizing experimental control in preclinical models will be essential. In order to further understand sex differences in the neuro-immune consequences of psychosocial, physical, and/or emotional stress, many of the existing stress paradigms and immune measures need to be replicated in females. In particular there is a dearth of studies utilizing stress paradigms that take place in adulthood and during puberty. Part of the challenge in assessing sex differences in existing paradigms of stress-related inflammation is related to the lack of stress models in which male and female animals are subjected to equivalent stressors, fluctuations in ovarian hormone-mediated regulation of inflammatory processes, and differential kinetics displayed by males and females to antigen stimulation. Because non-lactating female rats and mice do not spontaneously display a similar range of aggressive behavior as their male counterparts, most of social defeat or social confrontation-based stress paradigms are exclusively performed in males (Solomon 2017). However, the lower incidence of physical injuries sustained in group-housed or socially-interacting females has been cited as a reason to use females in studies assessing inflammatory endpoints (Voorhees, Tarr et al. 2013). Importantly, sex-dependent behavioral phenotypes influence experimental designs and thereby the questions that can be addressed, but paradigms exist in which males and females can both be exposed to social stressors (Barnum, Pace et al. 2012, Bourke and Neigh 2012, Burgado, Harrell et al. 2014). Furthermore, in cases such as social defeat in mice where a direct male-to-female comparison metrics have not been established within the stress paradigm, measuring "witness stress" as an alternative that could be equally experienced by animals of both sexes may help bridge the sex gap. For example, Ataka et al (2013) utilized a chronic psychological stress paradigm in which the experimental

animal witnessed another, non-experimental animal undergoing footshock stress. Importantly, witnessing the social defeat of another animal has been demonstrated to generate endocrine and gene expression changes in mice comparable to a first-hand social defeat experience (Warren, Vialou et al. 2013). It is imperative that researchers seek out and employ designs that facilitate assessment of sex differences in stress-induced inflammatory processes given that these preclinical models must inform translational research and ultimately clinical practice. Collectively, increased attention to comparison between the sexes in both rodent and human investigation and awareness regarding the differential function of the rodent and human immune systems will provide for advances in our understanding of stress-induced neuro-immune alterations and their relevance to clinical manifestation of affective disorders and potential novel therapeutic paths.

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