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Differential impact of chronic adolescent stress on the glucocorticoid receptor in adult male and female rats

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B.S., Georgia Institute of Technology, 2012

Advisor: Gretchen N. Neigh, Ph.D.

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Abstract

Differential impact of chronic adolescent stress on the glucocorticoid receptor in adult male and female rats

By Sydney A. Rowson

Chronic stress exposure is an important risk factor in the development of disease, and the consequences of exposure to chronic stress may differ in males and females. Furthermore, adolescents undergo extensive neural and neuroendocrine maturation and may be particularly vulnerable to the disruptive effects of chronic stress exposure. A rat model of chronic adolescent stress (CAS) exposure has been useful in studying the sex-specific consequences of CAS. Previously, female, but not male, rats exposed to CAS were found to exhibit enhanced depressive-like behaviors in adolescence. Interestingly, these sex-specific behavioral effects persisted to adulthood. While sex-specific alterations in regulation of the glucocorticoid receptor (GR) exist in adolescence, whether molecular consequences of CAS persist into adulthood is not known. Because the GR is integral in regulation of the stress response and has been implicated in the sex-specific effects of CAS, the studies in this dissertation assessed the extent to which CAS exposure alters regulation and activity of the GR in adult rats in a sex-specific manner. Adult female rats with a history of CAS exposure exhibited reduced nuclear GR localization following exposure to an acute stressor (Chapter 2), consistent with observations in adolescents, indicating that the effects of CAS on GR localization persist in the hippocampus. Adult females exposed to CAS also exhibited increased basal gene expression of *Fkbp5,* a co-chaperone of the GR that reduces its translocation efficiency, and increased interactions with FKBP5 following acute stressor exposure (Chapter 3). Furthermore, CAS altered global transcription in the adult hippocampus differently in males and females, and females had predicted increased activity of the GR following acute stressor exposure (Chapter 4). Together, these data indicate that CAS alters adult regulation and activity of the GR into adulthood, months removed from stressor exposure. Furthermore, the prolonged effects of CAS are sex-specific. These studies establish that there are long-term consequences of exposure to stressors in adolescence on hippocampal regulation of the GR in adulthood.

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List of Abbreviations

BCL associated anathogene 1 (Bag-1)

Chromatin Immunoprecipitation (ChIP)

Chronic adolescent stress (CAS)

Coefficient of variation (CV)

Dentate gyrus (DG)

Differentially expressed genes (DEGs)

Enzyme-linked Immunosorbant Assay (ELISA)

Estrogen receptor (ER)

Fiji is just ImageJ (Fiji)

FK506 binding protein 4 (FKBP4)

FK506 binding protein 5 (FKBP5)

Fold change (FC)

Glucocorticoid receptor (GR)

Ingenuity Pathway Analysis (IPA)

Long-term potentiation (LTP)

Non-stress (NS)

Postnatal day (PND)

Proximity Ligation Assay (PLA)

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Chapter 1

Introduction: The glucocorticoid receptor in chronic stress and disease

Adapted from:
Bekhbat M*, Rowson SA*, Neigh GN. Checks and Balances: The glucocorticoid receptor and NFκB in good times and bad.
*Both authors contributed equally to this work
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Consequences of chronic stress

It has long been recognized that chronic stress exposure is an important risk factor in the development of disease (McEwen and Stellar, 1993). Chronic stress exposure has been associated with the development of varied diseases (McEwen and Stellar, 1993) including cardiovascular (Golbidi et al., 2015), immune (Glaser and Kiecolt-Glaser, 2005; Padgett and Glaser, 2003), and neuropsychiatric diseases (Krishnan and Nestler, 2008), among others. Common stressors such as changing psychological demands at work (Smith and Bielecky, 2012), living environments (Matheson et al., 2006), and peer relationships (Hankin et al., 2015) can contribute to the risk of neuropsychiatric disease, and specifically to depression.

Sex differences in the impact of chronic stress and stress-related diseases

Men and women exhibit different rates of stress-related disorders. For example, men exhibit higher rates of schizophrenia, but women exhibit higher rates of major depressive disorder, generalized anxiety, and post-traumatic stress disorder, among others (Cover et al., 2014). Specifically, women have almost twice the lifetime likelihood of developing major depression than men (Cover et al., 2014). However, the precise mechanisms contributing to the differences in rates of these diseases are not fully clear. Sex hormones can interact with development and activity of the stress response, and estrogen and testosterone have been found to have differing effects on concentrations of stress hormones (Burgess and Handa, 1992; Malendowicz and Mlynarczyk, 1982; Panagiotakopoulos and Neigh, 2014). Furthermore, men and women exhibit differences in the stress response following acute stressor exposure (Kirschbaum et al., 1999). In the brain, men and women with depression exhibit distinct transcriptional profiles in multiple stress-sensitive regions, suggesting that the underlying mechanisms contributing to disease are distinct in males and females (Labonte et al., 2017). Differences in the male and female molecular response to chronic stress are a focus of the studies that will be discussed in the following chapters.

Adolescence as a critical developmental period

Though chronic stress has potentially harmful consequences at any stage of life, exposure to chronic stress during adolescence may be particularly harmful due to its potential to disrupt the developmental processes that occur during adolescence. Adolescents undergo extensive brain changes during adolescence including to dendritic maturation (Markham et al., 2013), changes in brain volume (Giedd, 2004), and extensive maturation of the neuroendocrine system (Foilb et al.,

2011; Romeo et al., 2016). The adolescent period as a period of extensive change is particularly vulnerable to disruption by chronic stress, and exposure to chronic stress may be particularly harmful during this time. Previous studies have shown that adolescents exposed to higher levels of chronic stress related to peers exhibit increased incidence of depression relative to those who exposed lower levels of chronic stress (Hankin et al., 2015). Furthermore, while adult patterns in incidence rates of stress-related disorders are not always apparent during early childhood, sex differences in the rates of major depression become apparent during adolescence (Hankin et al., 2015; Wade et al., 2002). Adolescents are exposed to rises in gonadal hormones which may contribute to the potential for sex-specific disruptions by stressors in adolescence. A brain mapping study that spanned adolescence linked estradiol and testosterone levels with changing brain region volumes through adolescent development (Herting et al., 2014). And consequently, the impact of the chronic stress interaction with gonadal hormones may differ in males and females. Additionally, the ability of severity of stress exposure to predict increased depression outcomes is stronger in females than males (Hankin et al., 2015), suggesting differing disease outcomes in male and female adolescents with chronic stress exposure. As others have discussed, exposure to stressors during adolescence may be more disruptive to behavior than exposure during adulthood (Spear, 2000). These disruptions during adolescence may have lasting consequences into adulthood. In order to better understand the lasting consequences of chronic exposure to stressors in adolescence, we used a rat model of chronic adolescent stress (CAS) in the studies discussed in the following chapters.

A chronic adolescent stress rat model

In order to develop new treatment strategies for stress-related disorders, it is necessary to improve our understanding of the underlying molecular consequences of chronic stress exposure.

Animal models are critical for study of the consequences of chronic stress exposure and to improve understanding of potential mechanisms underlying the neural impact of chronic stress. There are ethical and technical limitations in studying stress-related outcomes in the human brain due to the invasive nature of the methods. The environment and diet of animal model experiments can be well controlled, and in developmental studies, we are able to assess metrics in the brain at specific ages that would not be feasible in humans.

In order to better understand the long-term sex-specific consequences of chronic adolescent stress exposure, in the following studies, we used a mixed-modality chronic adolescent stress paradigm in rats that was found to induce sex-specific behavioral effects in previous studies. In earlier experiments, female, but not male, rats exposed to CAS exhibited increased depressive-like behaviors. Interestingly these sex-specific behavioral effects were apparent in adolescence, when assessed shortly after exposure to the stressors, but these behaviors also persisted into adulthood, removed from exposure to the stressors (Bourke and Neigh, 2011a). However, the mechanisms underlying the sex-specific impact of CAS in adulthood are not fully characterized.

Mechanisms involved in the stress response: HPA axis

Stress Response: Focus on the HPA axis

Following exposure to a stressor, the sympathetic nervous system and hypothalamic pituitary adrenal (HPA) axis are activated. The sympathetic response is commonly referred to as the "fight or flight" response and involves release of epinephrine and norepinephrine. Epinephrine and norepinephrine influence metabolism, increase heart rate, and shift blood flow through vasodilation and vasoconstriction, among other actions that promote adequate response to a

challenge (Breedlove and Watson, 2013; Nelson, 2000). Following exposure to a stressor, the HPA axis also becomes activated, and in the following chapters, we will focus on the role of the HPA axis in mediating the response to stressors. Following stressor exposure, the hypothalamus releases corticotropin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, and ACTH stimulates the release of glucocorticoids from the adrenal cortex (Breedlove and Watson, 2013) (Figure 1.1). The primary adrenal glucocorticoid is cortisol in humans and corticosterone in rodents. Glucocorticoids have a multitude of effects including action on metabolism and in suppression of non-essential activities such as growth, immunity, digestion, and reproduction (Panagiotakopoulos and Neigh, 2014). The actions of glucocorticoids and ability to suppress some functions while activating others promotes efficient energy usage to redirect energy from non-essential functions to functions that will promote adequate response to a stressor (Panagiotakopoulos and Neigh, 2014). Glucocorticoids act through interaction with the glucocorticoid receptor (GR), a nuclear receptor that will be discussed in greater detail later in this chapter.

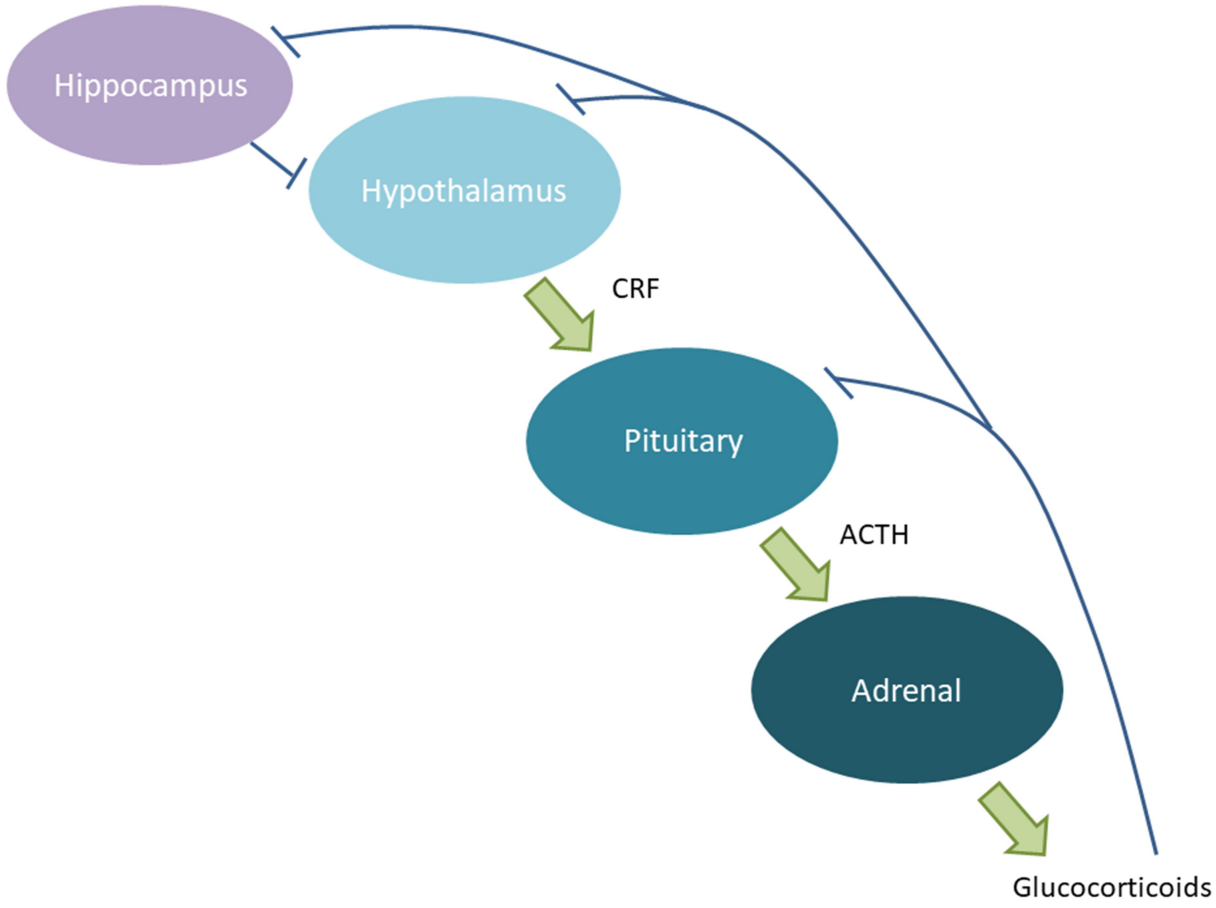

Figure 1.1 HPA Axis. Following exposure to a stressor, the paraventricular nucleus of the hypothalamus releases CRH, stimulating ACTH release from the anterior pituitary. ACTH stimulates the release of glucocorticoids from the adrenal cortex. Glucocorticoids feedback in negative feedback loops in the hypothalamus and pituitary to terminate further HPA activity. Glucocorticoids in the hippocampus also participate in negative feedback of the HPA axis.

In order to prevent prolonged activation of the HPA axis and excessive glucocorticoid exposure, glucocorticoids act in a negative feedback response to terminate further HPA axis activity. Glucocorticoids act on GRs in the hypothalamus and the pituitary in this negative feedback response (Figure 1.1) (Breedlove and Watson, 2013). Though the primary sites of HPA axis negative feedback are the hypothalamus and pituitary, glucocorticoid activity in the hippocampus also contributes to the negative feedback on HPA axis activity (Breedlove and Watson, 2013; Zhu et al., 2014). Lesions of the hippocampus have been found to prolong the corticosterone response following stressor exposure (Feldman and Weidenfeld, 1993), identifying the hippocampus as an additional site of HPA axis feedback. The hippocampus has been implicated in stress-related disorders in humans. Perception of stress exposure was associated with smaller hippocampal volumes in humans (Zimmerman et al., 2016), and depression was also associated with reduced hippocampal volume (Videbech and Ravnkilde, 2004), implicating the hippocampus as a region involved in stress-related disorders. The hippocampus is sensitive to stress as it expresses receptors for stress hormones (Medina et al., 2013; Soares et al., 2015). Furthermore, the hippocampus expresses estrogen (Rivera et al., 2013), progesterone (Bali et al., 2012), and androgen (Qiu et al., 2016) receptors, making the hippocampus an ideal site for assessment of the sex-specific impact of stress. The studies detailed in the following chapters will take place in the hippocampus, as a site of interaction among sex, stress, and stress-related disorders.

Though the HPA axis can be regulated by negative feedback in the hypothalamus, pituitary, and in the hippocampus (Breedlove and Watson, 2013; Sapolsky et al., 1991), chronic stress exposure can dysregulate this feedback system, and negative feedback on the HPA response can be impaired, resulting in prolonged exposure to glucocorticoids. Impaired HPA axis

feedback and excessive exposure to glucocorticoids has been implicated in stress-related disorders such as depression (Pariante and Miller, 2001). The role of HPA axis hyperactivity and impaired feedback has long been investigated in neuropsychiatric disease (Carroll et al., 1968), and specifically, the role of the GR in regulation of the HPA axis is a major focus of much research in studying affective disorders (Pariante and Miller, 2001). Patients with depression exhibit impaired glucocorticoid suppression in the dexamethasone suppression test (Carroll et al., 1968; Nuller and Ostroumova, 1980), a measure of GR-mediated HPA suppression, indicating an impaired role of the glucocorticoid-mediated HPA feedback. Furthermore, the severity of depression has been found to correlate in part with results in the dexamethasone CRH suppression test (Kunugi et al., 2006). The role of effective HPA negative feedback and activity of the GR have been implicated in studies examining mechanisms that may contribute to the prolonged impact of chronic stress.

Furthermore, specifically implicating the role of stress hormones in the consequences of chronic stress exposure, chronic glucocorticoid administration paradigms can induce depressive-like behavior in rodents (Demuyser et al., 2016; Gourley and Taylor, 2009; Kvarta et al., 2015), and humans currently exposed to chronic stress exhibit higher hair cortisol concentrations (Stalder et al., 2017). Specifically in the brain, chronic exposure to glucocorticoids has been found to cause damage in the mouse hippocampus (Zhang et al., 2015).

The glucocorticoid receptor in adolescent stress

The GR has been recognized as a potential mediator of sex-specific consequences of chronic stress (Bourke et al., 2012), and chronic stress paradigms have been found to alter GR expression (Zhang et al., 2017). Other early life stress paradigms have also implicated the GR.

Rats that were maternally separated during development exhibit a prolonged HPA response following acute stress exposure and decreased GR mRNA in the hippocampus (Ladd et al., 2004). Furthermore, in humans, polymorphisms of the *NR3C1* gene, the gene encoding the GR, exhibit sex-specific effects in its association with depression. Specifically, a single *NR3C1* polymorphism is associated with depression in females (Sarubin et al., 2016).

Earlier work from our lab has implicated the GR in the consequences of CAS (Bourke et al., 2013). Adolescent female rats exposed to chronic adolescent stress exhibit impaired hippocampal nuclear translocation of the GR and a prolonged corticosterone response following acute stressor exposure, suggesting alterations in HPA output. However, whether sex-specific consequences of CAS persist into adulthood is not yet established.

Because the GR is an important regulator of the HPA axis response and subsequent feedback on its activity that has been implicated in chronic stress-related disorders, we focused on the impact of CAS on regulation of the GR in the following chapters. In these studies, we assessed the extent to which CAS alters function of the hippocampal GR in adulthood in a sex-specific manner. To determine the extent to which CAS regulates the GR in adulthood, months removed from exposure to the adolescent stressors, we assessed cytosolic and nuclear protein expression of the GR in Chapter 2. Furthermore, in order to understand how CAS impacts HPA output, we measured plasma corticosterone. However, localization of the GR is only part of the picture. The GR is regulated through complex mechanisms. The following section will discuss mechanisms of activity and regulation of the GR.

Mechanisms of glucocorticoid receptor regulation

Given the importance of the GR in the stress response, and its potential for sex-specific impact on adult regulation following CAS, it is important to discuss the levels at which the receptor can be regulated. Though there are various mechanisms of GR regulation, we will focus on transcriptional and co-chaperone-mediated regulation of the GR in the following sections and chapters.

Structure, Function, and Regulation of GR

The GR is a 777-amino acid nuclear receptor expressed throughout the body (Hollenberg et al., 1985) including the brain (McEwen, 1973). The GR is encoded by the *Nr3c1* gene and is responsive to its ligand glucocorticoids (Bell and Munck, 1972). As a nuclear receptor, the GR contains a DNA binding domain and a ligand binding domain, both of which are required for nuclear receptor activity (Giguere et al., 1986). The GR resides in the cytosol interacting with a chaperone complex that maintains its activity (Bresnick et al., 1989; Levinson et al., 1972). Following binding of its glucocorticoid ligand, the GR translocates to the nucleus (Davies et al., 2002) where it interacts with specific DNA sequences called glucocorticoid response elements (GREs). Binding of the GR to the GRE binding site results in dimerization of the DNA binding domain of the GR (Luisi et al., 1991) and increases in gene expression of its target genes (Chandler et al., 1983). GRs are also able to bind to negative GRE (nGRE) DNA binding sequences on which the GR reduces transcriptional activity through interaction with transrepression complexes (Surjit et al., 2011). GR binds to the nGRE as two separate GR monomers on opposite sides of the DNA strand in an orientation that prevents GR dimerization, a conformation in contract to GRs at positive GREs where GR binds on the same side of the

DNA strand (Hudson et al., 2013). As a transcription factor, the GR mediates transcription of numerous target genes involved in a range of actions within an organism including effects on metabolism, reproduction, immunity, and regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Baxter et al., 1972; Panagiotakopoulos and Neigh, 2014; Rousseau et al., 1975; Strahle et al., 1987). Given the pervasive effects of GR activation, efficient and effective regulation of receptor activity is essential to organism function and adaptation.

Mechanisms of GR Regulation

Cellular responsivity to glucocorticoids and activity of the GR can be regulated at multiple levels. Though there are multiple levels of GR regulation, we will focus on the transcriptional effects of the GR and co-chaperone regulation of the receptor. An overview of mechanisms of GR regulation that will be discussed is detailed in Figure 1.2.

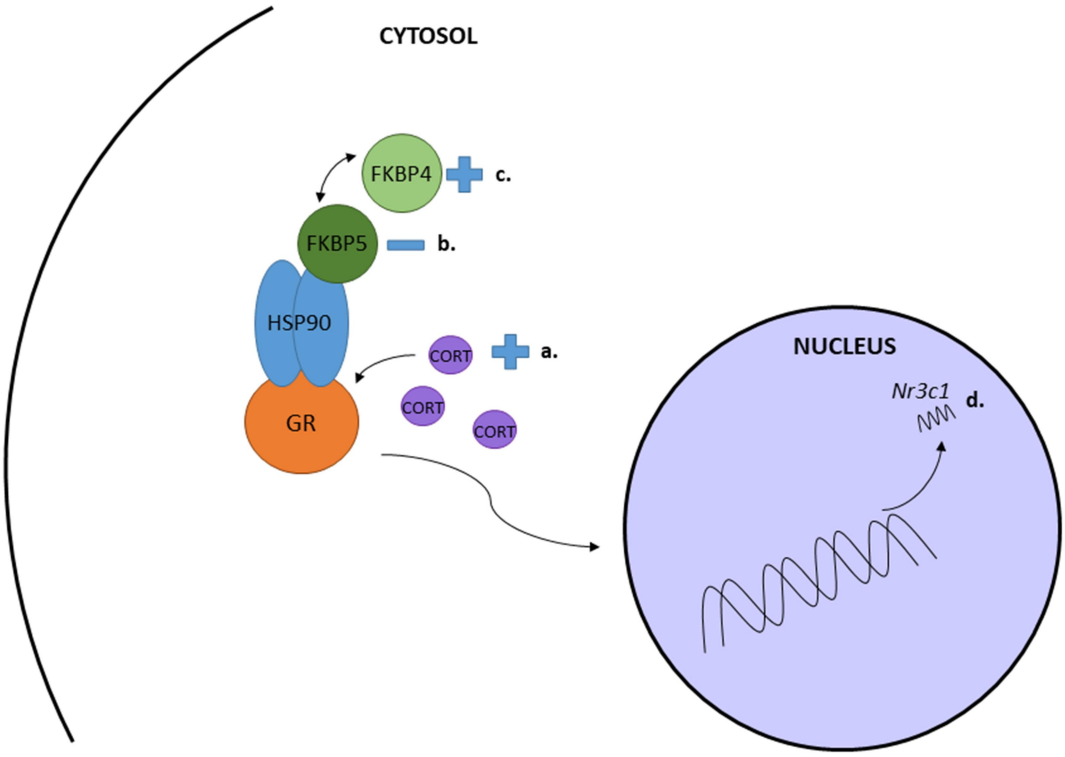

Figure 1.2. Molecular Regulation of GR. Activity of the GR is regulated at multiple levels. Glucocorticoids (CORT), the ligand for the GR, promote nuclear translocation activity of the GR (a.). Co-chaperones of the GR such as FKBP5 (b.) and FKBP4 (c.) impair or promote GR nuclear translocation respectively. Altered transcription of *Nr3c1* (d.) may also impact GR expression and activity.

Glucocorticoid Receptor Expression and Degradation

Regulation of GR protein expression is one level of modulating signaling activity of the GR. Glucocorticoid treatment downregulates GR expression in cell models (Cidlowski and Cidlowski, 1981; Silva et al., 1994), and chronic corticosterone treatment decreases GR protein in specific brain regions (Hu et al., 2016). However, inhibition of proteasomes reverses the effect of glucocorticoid treatment on GR expression, establishing the role of proteosomal degradation in glucocorticoid-mediated regulation of GR protein concentrations. Following treatment with glucocorticoids, the GR can be ubiquitinated, signaling its designation for proteosomal degradation. Changes in GR protein concentrations following glucocorticoid treatment have been found to be functional; reporter gene analysis shows that proteasome inhibition increases dexamethasone-mediated GR transcriptional activity (Wallace and Cidlowski, 2001). Degradation of the GR has been proposed to be a mechanism of cell-type specific glucocorticoid resistance (Mata-Greenwood et al., 2013).

Regulation of GR expression and localization can alter its activity. In order to assess the extent to which CAS impacts adult expression and localization of the GR, protein expression of the GR was assessed in Chapter 2. Because the GR's transcriptional activity first requires translocation of the receptor to the nucleus, the nuclear fraction of the GR protein was used to determine the extent to which CAS alters nuclear content of the GR in male and female rats after acute stressor exposure. Furthermore, we assessed global transcription in adult male and female rats exposed to CAS with RNA sequencing in Chapter 4. Hippocampal transcripts from adult rats exposed to CAS were analyzed to predict activity of the GR in CAS or non-stress (NS) treatment groups using Upstream Analysis software with the RNA sequencing dataset. We used RNA sequencing in Chapter 4 to assess the extent to which CAS effects on the hippocampal

transcriptome are sex-specific and furthermore, the extent to which CAS impacts predicted altered activity of the GR on global transcription in the hippocampus.

Co-Chaperone Regulation of the glucocorticoid receptor

As a nuclear receptor, the GR resides in the cytosol in a complex with a number of chaperone and co-chaperone proteins. The chaperones and co-chaperones impact function of the receptor, and by modulating the receptor's activity have the ability to impact glucocorticoid signaling. Following translation of the GR protein, the GR interacts with the chaperones heat shock protein 90, 70 (Hsp90, Hsp70), and p60 which are required for efficient folding of the GR into a conformation with high-affinity for ligand binding, and interaction with the chaperone p23 further stabilizes the GR-Hsp90 complex (Dittmar and Pratt, 1997; Whitesell and Cook, 1996). The GR resides in the cytosol bound to two molecules of Hsp90, and continued interaction of the GR with the chaperone Hsp90 is required to maintain the GR's high-affinity conformation (Bresnick et al., 1989). In addition to the two molecules of Hsp90, the GR cytosolic complex also contains an immunophilin. The immunophilins FK506 binding protein 51 (FKBP5) and FK506 binding protein 52 (FKBP4) have been widely studied for their regulatory activity of the GR and established links to the expression of mood disorders (Binder, 2009). FKBP5 and FKPB4 bind to Hsp90 through a tetratricopeptide repeat (TPR) domain. This domain, as well as amino acids in the immunophilin's C-terminal domain, impact the immunophilin's binding affinity to Hsp90 (Cheung-Flynn et al., 2003; Silverstein et al., 1999). The GR cytosolic complex consists of two molecules of Hsp90, one molecule of GR, and one immunophilin (FKBP5 or FKBP4). These two proteins, FKBP5 and FKBP4, compete with each other for binding with Hsp90 (Wochnik et al., 2005) and dictate the feasibility of translocation.

Interaction of the GR complex with the immunophilins FKBP5 and FKBP4 have differential action on glucocorticoid signaling. While interaction with FKBP4 facilitates movement of the GR to the nucleus, allowing the GR to elicit its transcriptional activity, interaction of GR-Hsp90 with FKBP5 inhibits GR-mediated transcriptional activity and efficient translocation of the GR to the nucleus. This occurs in part due to reduced interaction of FKBP5 with dynein compared to that of FKBP4 (Tatro et al., 2009; Wochnik et al., 2005). When the GR interacts with its corticosteroid ligand, the GR-Hsp90 complex dissociates from the co-chaperone FKBP5, and FKBP5 is exchanged for FKBP4. FKBP4 then associates with dynein, a motor protein that assists in nuclear translocation of the GR complex to the nucleus (Davies et al., 2002). The peptidyl prolyl isomerase (PPIase) domain of FKBP4 is required for association with dynein and movement of the GR complex to the nucleus (Galigniana et al., 2002; Galigniana et al., 2001; Silverstein et al., 1999). Dynein then interacts with microtubules to assist in the nuclear transport of the GR (Harrell et al., 2004). The balance between FKBP5 and FKBP4 in part dictates the extent to which GR can translocate to the nucleus in order to act as a transcription factor.

BCL associated anathogene (Bag-1) is another co-chaperone that can interact with the GR. Interaction with the GR co-chaperone Bag-1 promotes GR degradation (Demand et al., 2001), and overexpression of Bag-1 decreases protein levels of GR (Mata-Greenwood et al., 2013), having a negative effect on GR activity. Furthermore, PPID, another GR co-chaperone, facilitates GR activity (Duina et al., 1996; Jandova et al., 2013; Renoir et al., 1995).

Evidence for a contribution of FKBP5 in adolescent stress

FKBP5 is one of the most widely studied regulators of GR activity (Binder, 2009), and co-chaperone regulation of GR activity can have pronounced effects on the impact of glucocorticoids. An example of the influential role FKBP5 can play is apparent in multiple species of new world primates that naturally exhibit high levels of circulating glucocorticoids. These primates do not exhibit the characteristic harmful effects of chronic elevated glucocorticoid exposures as they also exhibit high levels of the negative co-chaperone of GR activity FKBP5 which may in part prevent excessive glucocorticoid signaling (Scammell et al., 2001). In humans, a single nucleotide polymorphism (TT at rs1360780) of the *FKBP5* gene that results in increased FKBP5 protein is associated with increased number of depressive episodes and a quicker response to treatment with antidepressants (Binder et al., 2004). Furthermore, rat models of chronic stress have found elevations of FKBP5 protein in the prefrontal cortex and hippocampus (Chen et al., 2016) as well as increased *Fkbp5* gene expression in the prefrontal cortex and hippocampus (Guidotti et al., 2013).

Recently, a specific inhibitor for FKBP5 was developed allowing for mechanistic study of the role of GR that previously had been limited due to the lack of specific inhibitors of FKBP5. Pharmacological inhibition of FKBP5 increases neurite outgrowth, enhances dexamethasone suppression of the HPA axis, and increases active coping and decreases passive coping in the forced swim test (Gaali et al., 2015). Mice treated with the FKBP5 inhibitor also exhibit decreased anxiety behavior (Hartmann et al., 2015) and reduced pain severity (Maiaru et al., 2016).

In the rat model of CAS discussed earlier, in addition to sex-specific impairments in GR nuclear translocation, adolescent female rats exposed to CAS exhibited increased gene expression of the GR co-chaperone *Fkbp5* following an acute forced-swim stressor when assessed at the end of adolescence, shortly after exposure to the adolescent stressors (Bourke et al., 2013). However, these endpoints were assessed in adolescence, shortly after exposure to the stressors, and the sex-specific effects of CAS that are apparent in adulthood have not been fully characterized. In the Chapter 3, we assessed the impact of CAS on expression of co-chaperones and used the gene expression results to direct further investigation of protein interactions.

Summary and Conclusions

A rat model of chronic adolescent stress has shown that female rats exposed to CAS exhibit increased depressive-like behaviors in adolescence that persist into adulthood. Furthermore, adolescent rats exposed to CAS exhibit sex-specific alterations in GR translocation and expression of GR co-chaperones, implicating regulation of the GR in the sex-specific consequences of CAS exposure. However, these effects of CAS on the GR were observed at the end of adolescence, shortly after exposure to the stressors. While the sex-specific behavioral effects of CAS persist into adulthood, the molecular effects underlying the sex-specific adult impact of CAS are incompletely understood.

The experiments in the following chapters assess the extent to which CAS impacts regulation of the GR in adulthood in a sex-specific manner. One mechanism of GR regulation is at the level of nuclear translocation, and in Chapter 2 we discuss the sex-specific effects of CAS on subcellular localization of the GR. The GR associates with co-chaperones that regulate its activity, and Chapter 3 details experiments that assess expression and function of GR co-

chaperones. Finally, because the GR is a transcription factor that regulates expression of numerous target genes, we use RNA sequencing in Chapter 4 to assess the extent to which the CAS alters the hippocampal transcriptome in a sex-specific manner. Together these studies will provide valuable information about the long-term impact of CAS on sex-specific alterations in adult regulation of the GR and its co-chaperones with implications for new interventions for stress-related disorders.

Chapter 2

Chronic adolescent stress alters adult hippocampal localization of the glucocorticoid receptor

Introduction

Chronic stress is an important risk factor in the development of neuropsychiatric disease, and women exhibit increased incidence of stress-related disorders such as depression and anxiety compared to men (Cover et al., 2014; Pratt and Brody, 2014). While chronic stress can have lasting consequences during any stage in life, chronic stress exposure during adolescence may be particularly harmful (Spear, 2000).

Our lab has previously shown that exposure to chronic adolescent stress (CAS) enhances depressive-like behaviors in a sex-specific manner. Interestingly, the behavioral effects of CAS

persist into adulthood (Bourke and Neigh, 2011a); however, molecular effects underlying the adult impact of CAS are not fully characterized.

The glucocorticoid receptor (GR) as the receptor for the stress hormone corticosterone may be an important underlying factor in the prolonged impact of CAS exposure. The GR mediates negative feedback on the HPA axis, and its dysregulation has been implicated in the development of neuropsychiatric disease (Bourke et al., 2012). Furthermore, adolescent rats exposed to CAS exhibit sex-specific alterations to the GR. Specifically, adolescent female rats exhibit a prolonged corticosterone response following acute stressor exposure, coupled with impaired GR nuclear translocation in the hippocampus, suggesting possible dysregulation of the hippocampal feedback response on HPA axis activity (Bourke et al., 2013). However, whether CAS alters GR function in the hippocampus into adulthood is not known.

In these experiments we used a rat model of CAS developed in our lab that confers sex-specific behavioral and molecular effects in adolescence (Bourke and Neigh, 2011a; Bourke et al., 2013) to assess the extent to which CAS alters hippocampal function of the GR in adulthood in a sex-specific manner. The hippocampus participates in negative feedback on the HPA axis (Sapolsky et al., 1984; Sapolsky et al., 1985) and has been implicated as a site involved in depression (Bremner et al., 2000; Videbech and Ravnkilde, 2004). Furthermore, the hippocampus highly expresses corticosterone (Soares et al., 2015) and sex hormone receptors (Guerra-Araiza et al., 2003; Qiu et al., 2016; Rivera et al., 2013) making it a valuable site for studying the impact of stress and sex in adolescent stress consequences. Specific effects of CAS have been found only when challenged with exposure to a novel acute stressor (Bourke et al., 2013), so in addition to assessment at baseline, we challenged adult male and female rats with a

novel forced swim stressor and evaluated corticosterone and GR activity in separate groups at subsequent time points.

Methods

Animal husbandry

All rats were housed on a 14:10 reverse light:dark cycle in AALAC-approved animal housing at Emory University. Food and water were available *ad libitum.* Pups from Wistar dams (Charles River Laboratories) were culled to litters of eight (4 males and 4 females) on postnatal day (PND) 3. On PND 21, litters were weaned into same-sex pairs. Wistar rats were chosen for these experiments because they are a rat strain that are prone to anxiety-like behavior (Vidal et al., 2011). All experiments were approved by the Institutional Animal Care and Use Committee at Emory University.

Chronic adolescent stress paradigm

On PND 35, male and female rats from the same litter were divided into CAS and non-stress (NS) groups. Male and female rats in the CAS group were individually housed beginning on PND 35 through the end of the study. On PND 38-49, CAS rats were exposed to a mixed-modality CAS paradigm consisting of restraint, social defeat, and isolation as previously described (Bourke and Neigh, 2011a; Bourke et al., 2013). All stress procedures occurred during the light cycle. Briefly, rats were exposed to six days of restraint and six days of social defeat in a pseudorandom order across the twelve days. The restraint paradigm consisted of placing the experimental Wistar rat in a clear plastic rodent restraint (Braintree Scientific) for one hour. Following the one-hour session, Wistar rats were returned to their home cage. The social defeat

paradigm consisted of exposure to a larger, adult Long Evans rat. Male retired breeder Long Evans rats were housed with female adult Long Evans rats. The female Long Evans rats were ovariectomized to prevent estrous cycle changes in aggressive behavior. Prior to the social defeat session, one of the Long Evans rats was removed from the home cage, so the experimental Wistar rat only interacted with a same-sex Long Evans rat. On social defeat days, experimental Wistar rats were placed in the home cage of an adult, more aggressive, same-sex Long Evans Rat. They were separated by a clear plastic barrier that allowed both visual and olfactory cues for two minutes to acclimate. The barrier was then removed for the subsequent five minutes, allowing the rats to physically interact. Interaction often resulted in pinning in males and aggressive behavior from the Long Evans rat. Injury and biting were very uncommon, and rats were excluded from the study if injury occurred. Following the five minutes of physical interaction, the barrier was replaced with the rats on separate sides of the barrier for the following twenty-five minutes. Following the social defeat session, the experimental Wistar rat was returned to its home cage. Rats were weighed weekly, and pair-housed NS controls were undisturbed throughout the study except for weekly weighing. The timeline for experimental procedures are detailed in Figure 2.1.

Acute stress forced swim

In adulthood (at least six weeks following the end of CAS exposure, PND 91-103), rats were exposed to the novel acute stressor of a five-minute forced swim. Acute stressor exposure occurred during the light cycle when circadian corticosterone levels would be low. Rats were acclimated to a room separated from that used for forced swim stressor exposure. Rats were removed for acute stressor exposure and immediately placed in a clear acrylic cylinder (60 cm tall, 22 cm diameter) with 25 °C water for five minutes. Immediately following the forced swim

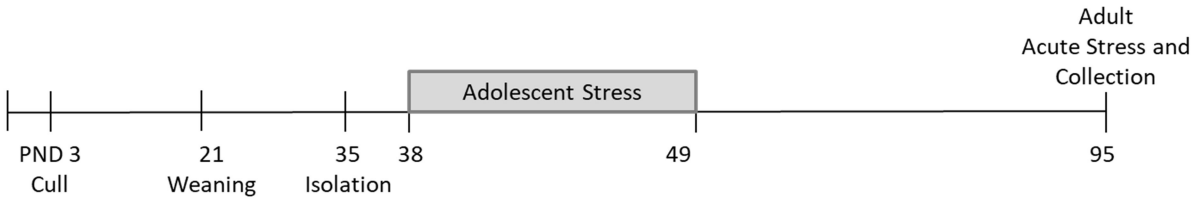

Figure 2.1 Timeline for chronic adolescent stress paradigm.

exposure, rats were replaced in their home cage. 15, 30, or 120 minutes following swim exposure, rats were rapidly decapitated, and brain tissue was collected. Brains were rapidly frozen on dry ice and stored at -80 °C for future processing. Trunk blood was collected in EDTA tubes on ice at the time of collection. Separate groups of CAS and NS rats were collected at baseline, without acute stressor exposure, immediately following removal from the habituation room.

Nuclear and cytosolic protein fraction extraction

Hippocampal hemispheres were dissected on dry ice, and the right hemisphere was used for all protein analyses. Nuclear and cytosolic protein fractions were extracted as previously performed (Bourke et al., 2013). Briefly, tissue was homogenized in 50 mM Tris, 6 mM $MgCl_2$, 1 mM EDTA, 10% sucrose buffer with 1:1000 protease inhibitor. Homogenized protein was spun at 105,000 x g at 4°C for 30 minutes, and the supernatant was collected as the cytosolic fraction. The pellet was homogenized in 0.5 ml buffer consisting of 50 mM Tris, 6 mM $MgCl_2$, 1 mM EDTA, 0.5 M NaCl, 10% sucrose and 1:1000 protease inhibitor. The pellet was centrifuged and washed twice, incubated in an ice bath for one hour and then centrifuged at 8000 x g for 10 minutes at 4°C. The supernatant was collected as the nuclear protein fraction. Protein concentrations were determined using a BCA assay (Pierce, Prod #23227) according to the manufacturer's instructions.

Western blot

A Pierce BCA was used to standardize 10 µg protein in nuclease-free water with 4x Laemmli Sample Buffer (BioRad) and beta-mercaptoethanol and run on a Criterion Precast gel (10-20% Tris HCl, 1.0 mm, BioRad) in a BioRad apparatus for 95 minutes at a constant 150 V. Protein

was transferred to a PVDF membrane (midi size, BioRad). A BioRad Transblot turbo apparatus was used to perform a semi-dry transfer (BioRad, preset Mixed molecular weight setting) in TransBlot turbo buffer according to manufacturer's instructions. The membrane was blocked in 7.5% w/v nonfat dry milk in TBS-T for one hour at room temperature. The membrane was cut to visualize each of three proteins: GR, H3, and GAPDH. The membrane was incubated with anti-GR antibody (1:10000, ab109022) overnight at 4 °C, anti-H3 (1:100000, ab1791) overnight at 4 °C or anti-GAPDH (1:500000, ab181602) for 30 minutes at room temperature. Membranes then were washed three times in TBS-T and incubated with secondary goat anti-rabbit HRP conjugated antibody (1:5000, ab97051). Membranes were washed three times in TBS-T, and chemiluminescence was visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) on a Syngene G:BOX system (Syngene, Frederick, MD). Signal was quantified using Li-Cor Image Studio Lite using the user defined background subtraction method. Arbitrary densitometry units were normalized to a standard protein lane to normalize signal between membranes, and to a housekeeping protein in each lane to normalize between lanes (H3 for nuclear fractions, GAPDH for cytosolic fractions).

Corticosterone ELISA

At the time of collection, trunk blood was collected in EDTA tubes (BD Vacutainer) on ice. Blood was centrifuged at 2500 rpm in a Sorvall RC-5B Refrigerated Superspeed Centrifuge and SM-24 rotor (773 rcf) for 20 minutes at 4 °C. Plasma was collected and stored at -80 °C. An Enzo Life Sciences Enzyme-linked Immunosorbant Assay (ELISA) was used to assess plasma corticosterone according to manufacturer's instructions. Samples were performed in duplicate, and CV% for all duplicates was less than 17%. The limit of detection for this assay is 27 pg/ml.

Corticosterone ELISAs were performed in Dr. Gretchen Neigh's Laboratory at Virginia Commonwealth University.

Weight Analysis

All rats were weighed throughout the experimental paradigm. Weights from animals used in Chapter 2 molecular analyses, Chapter 3 PCR and PLA experiments, and Chapter 4 RNA sequencing experiments were used for analysis of weight through adolescent stress and terminal weight analysis. Animals were weighed on the first day of isolation housing (PND 35) and Day 1, 5, and 10 during the CAS paradigm. NS rats were weighed on the same days as littermate CAS rats. Terminal weight was measured prior to acute stressor exposure on the day of collection. Animals with a missing weight measurement throughout the stress paradigm were excluded from weight gain across stress analysis due to the repeated measures statistical design. One male NS animal was removed from weight gain analysis because its isolation weight was determined to be an outlier with Grubb's test (α=0.05). It was also over four standard deviations from the mean and is physiologically impossible based on previous and subsequent weights and was therefore a recording error.

Statistics

GraphPad Prism 7.02 was used for all analyses with α=0.05. A two-way ANOVA (CAS x acute stress) was used for statistical analysis of GR Western Blot data and corticosterone ELISA separately in males and females because CAS was hypothesized to alter endpoints differently in males and females. The levels of the acute stress factor were the four post- acute stress time points (baseline, 15, 30, and 120 minutes). Sidak's multiple comparisons test was used to assess post-hoc comparisons between NS and CAS groups when a significant interaction was observed.

An unpaired t-test was used for terminal weight analysis. A repeated measures two-way ANOVA was used to assess weight gain across stress (CAS x day). A post-hoc Sidak's multiple comparisons test was used to assess differences between NS and CAS groups within day in males because a significant interaction effect was found.

Results

Chronic adolescent stress blunted nuclear localization of the glucocorticoid receptor in females but not males

Following exposure to an acute novel stressor, females exhibited a significant interaction between time following acute stressor exposure and history of CAS on nuclear GR protein expression ($F_{(3, 70)} = 2.96$, $p = 0.038$, Figure 2.2A). Furthermore, there was reduced nuclear GR protein 30-minutes following acute stressor exposure (Sidak's multiple comparisons test, $p=0.0293$) in adult females exposed to CAS relative to NS controls. There was no effect of time following acute stressor exposure ($F_{(3, 70)} = 1.082$, $p>0.05$) or a history of CAS ($F_{(1, 70)} = 0.57$, $p>0.05$) on nuclear GR content in females. Males also did not exhibit a significant impact of time following acute stressor exposure on nuclear GR ($F_{(3, 63)} = 2.23$, $p =0.093$). Furthermore, a history of CAS ($F_{(1, 63)} = 0.22$, $p>0.05$) or a CAS by acute stress interaction ($F_{(3, 63)} = 0.74$, $p>0.05$, Figure 2.2B) did not impact nuclear GR protein in males.

Cytosolic protein expression was not impacted by acute stress (Female: $F_{(3, 72)} = 1.23$, $p>0.05$; Male: $F_{(3, 64)} = 1.47$) or CAS (Female: $F_{(1, 72)} = 2.11$, $p>0.05$, Male: $F_{(1, 64)} = 2.79$, $p=0.0998$) in females or males (Figure 2.3).

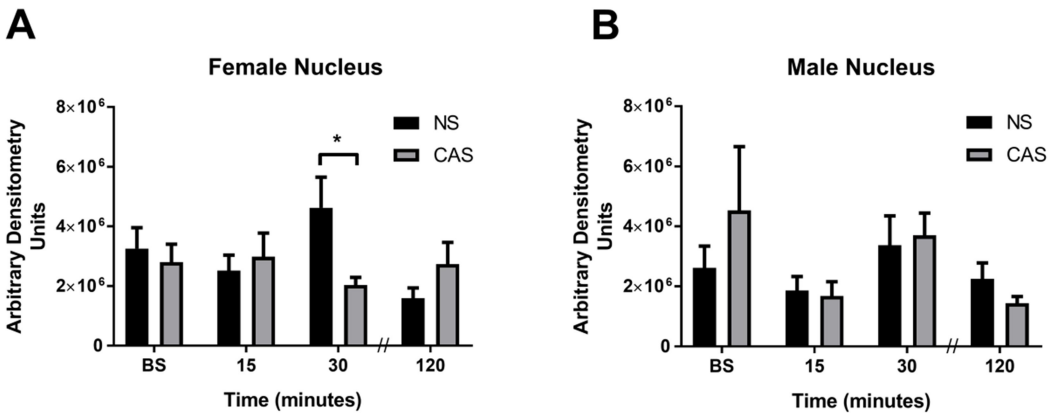

Figure 2.2 Nuclear GR protein in adult male and female rats. Female (A) and male (B) rats were exposed to CAS or NS control conditions. In adulthood, hippocampal tissue was collected at baseline (no acute stressor exposure) and 15, 30, or 120-minutes following exposure to an acute novel stressor. Nuclear GR was assessed with western blot. Arbitrary densitometry units were normalized to H3 nuclear housekeeping protein and a standard protein sample and expressed as mean ± SEM. n=8-10 per group. * indicates significant effect in CAS to NS comparison with Sidak's multiple comparisons test, $p<0.05$.

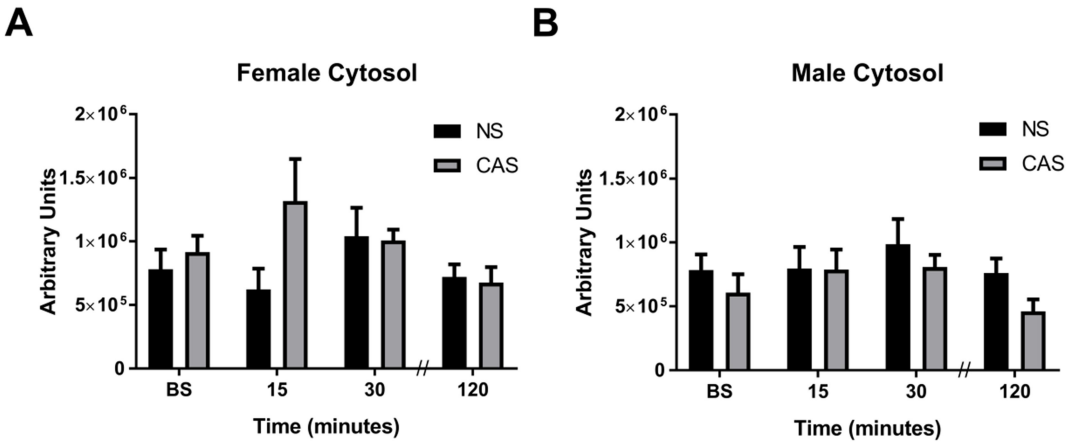

Figure 2.3 Cytosolic GR protein in adult male and female rats. Female (A) and male (B) rats were exposed to CAS or NS control conditions. In adulthood, hippocampal tissue was collected at baseline (no acute stressor exposure) and 15, 30, or 120-minutes following exposure to an acute novel stressor. Cytosolic GR was assessed with western blot. Arbitrary densitometry units were normalized to GAPDH nuclear housekeeping protein and a standard protein sample and expressed as mean ± SEM. n=7-11 per group.

Chronic adolescent stress did not impair the resolution of the corticosterone response

Plasma corticosterone was altered with time following novel acute stressor challenge in both females ($F_{(3, 67)} = 18.87$, $p<0.0001$) and males ($F_{(3, 58)} = 15.97$, $p<0.0001$) (Figure 2.4). However, there was not a significant effect of CAS on the corticosterone response in females ($F_{(1, 67)} = 2.88$, $p=0.0943$) or males ($F_{(1, 58)} = 0.0019$, $p>0.05$).

Weight gain

Though both male and female adolescent rats gained weight through adolescence during the stress paradigm (Male: $F_{(3, 264)} = 2229$, $p<0.0001$; Female: $F_{(3, 276)} = 461.6$, $p<0.0001$), male rats exposed to CAS gained less weight than NS male controls ($F_{(1, 88)} = 11.43$, $p=0.0011$, interaction: $F_{(3, 264)} = 20.95$, $p<0.0001$, Figure 2.5). On day 5 and 10, CAS males exhibited reduced body weight compared to NS male controls ($p<0.0001$, Sidak post hoc multiple comparisons test). The impact of CAS in males normalized by adulthood ($t_{99}=0.538$, $p>0.05$, Figure 2.6). CAS did not significantly impact weight gain across adolescence in females ($F_{(1, 92)} = 1.57$, $p>0.05$), and CAS did not impact terminal weight in females ($t_{109}=1.25$, $p>0.05$, Figure 2.6).

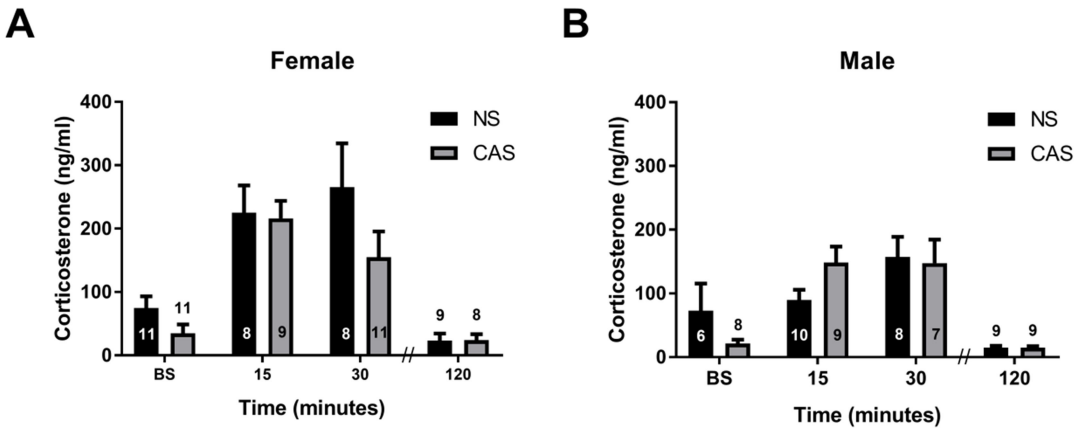

Figure 2.4 Plasma corticosterone following acute stressor exposure in male and female rats exposed to CAS. Trunk blood was collected immediately following rapid decapitation in female (A) and male (B) adult rats at baseline (no acute stressor exposure) or 15, 30, or 120-minutes following a five-minute novel acute forced swim stressor challenge. Both males and females exhibited a main effect of time following acute stressor ($p<0.05$) but CAS did not elicit a significant impact on plasma corticosterone. Numbers denote sample size per group. Data are presented as mean ± SEM.

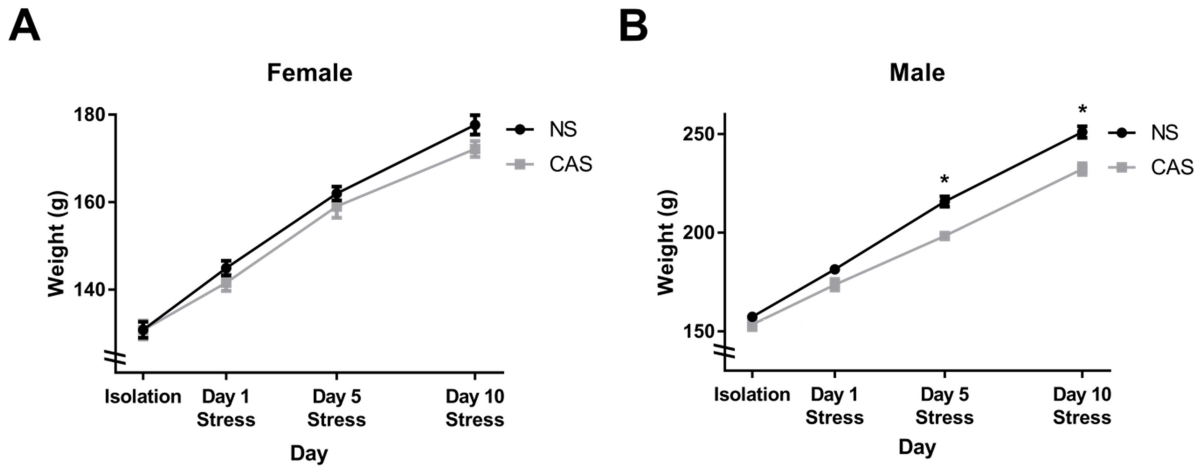

Figure 2.5 Weight gain through the chronic adolescent stress paradigm. Female (A) and male (B) rats were weighed on isolation day (PND 35), the first (PND 38), fifth (PND 42), and tenth (PND 47) day of the stress paradigm. Both males and females gained weight through adolescence, but males exposed to CAS gained less weight than NS male controls ($p<0.05$). Female NS n=35, CAS n=35. Male NS n=33, CAS n=33.* indicates significance in Sidak's multiple comparisons test. Data are presented as mean ± SEM. $\alpha=0.05$.

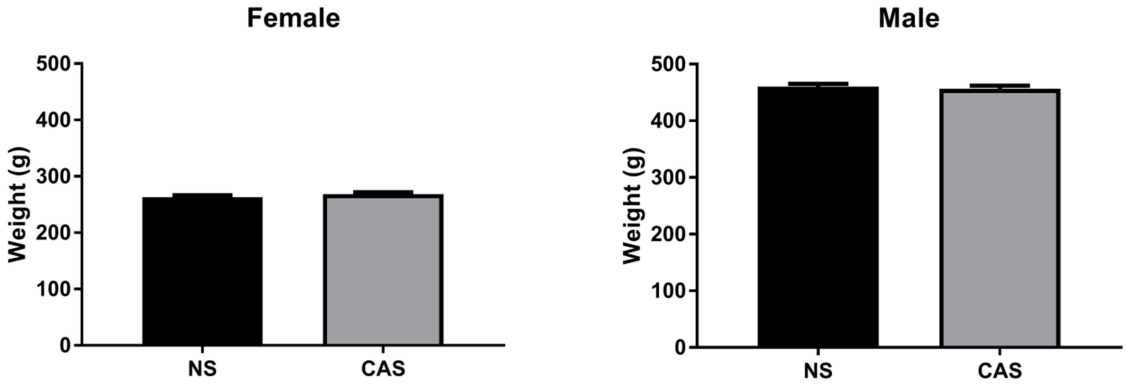

Figure 2.6 CAS does not impact terminal weight. CAS does not alter terminal weight in female (A) or male (B) adult rats exposed to CAS. Weight was measured for all animals the morning prior to rapid decapitation. ($p>0.05$). Female NS n=56, CAS n=55. Male NS n=50, CAS n=51. Data are presented as mean ± SEM.

Discussion

The GR is an important regulator of the HPA axis response, and regulation of the GR has been implicated in the consequences of CAS exposure, when assessed in adolescence, shortly after exposure to the stressors. These studies were designed to determine whether CAS altered localization of the hippocampal GR and impacts the HPA response following acute stressor exposure in adulthood, months removed from stressor exposure. We assessed all endpoints in both males and females to assess the extent to which the CAS impact on GR localization is sex-specific.

CAS altered GR localization in the hippocampus following an acute stressor exposure in females, but not males, such that GR protein content in the nucleus was reduced 30-minutes following exposure to an acute stressor in females with a history of chronic adolescent stress. This blunting of nuclear GR suggests that there may be reduced translocation responsivity of the receptor following acute stressor exposure in females with a history of CAS exposure. Impairment of nuclear GR translocation is consistent with GR activity patterns observed at the end of adolescence (Bourke et al., 2013), indicating that the impact of CAS on the GR is lasting into adulthood. When assessed at the end of adolescence, female but not male rats exhibited blunted GR nuclear translocation following an acute forced swim stressor exposure (Bourke et al., 2013), consistent with patterns observed in adulthood. These data show that the effects of CAS on GR localization in the hippocampus in adulthood following acute stressor exposure are sex-specific and apparent long removed from exposure to the stressors.

In contrast from what was observed at the end of adolescence, adult female rats in the current studies did not exhibit a prolonged elevation of circulating corticosterone following acute

stressor challenge. CAS did not significantly impact the corticosterone response in adult females (Figure 2.4). Adolescent female rats exposed to CAS exhibited a prolonged corticosterone response after exposure to a novel stressor, suggesting potentially reduced feedback on the HPA axis response (Bourke et al., 2013). The absence of a prolonged corticosterone response in adult females despite blunted GR nuclear content suggests a more, rather than less, active GR once in the nucleus, suggesting potentially enhanced feedback on the corticosterone response that may be adaptive in adult females to the repeated stressor exposures in adolescence. Chronic stress does not always induce a prolonged corticosterone response, and interestingly, other labs have observed that chronic stress paradigms can produce a blunting of the corticosterone response rather than prolonging the corticosterone response following novel stressor exposure (Smith et al., 2017). These preclinical data are in line with blunted HPA response observed in the clinic in adults with a history of childhood maltreatment (Carpenter et al., 2007). Alternatively, feedback on the HPA axis also occurs in the hypothalamus and the pituitary. It is possible that these other sites of HPA feedback compensate for reduced nuclear availability of the GR in the hippocampus and enhance their contribution to feedback on the corticosterone response.

We did not observe changes to cytosolic GR expression in males or females with a history of CAS. Other groups have reported elevated GR cytosolic protein following an adult chronic stress paradigm in the ventral but not dorsal hippocampus (Guidotti et al., 2013) though we did not observe an effect of CAS on GR localization at baseline. Another study showed that rats exposed to repeated restraint stress in adulthood exhibit elevated nuclear GR at baseline and 30-minutes following a restraint session relative to non-stressed controls (Noguchi et al., 2010). However, these studies were adult male rats, suggesting that age of stress exposure could impact the sex-specific effects of chronic stress on GR localization following acute stressor exposure. It

is therefore possible that the sex-specific impact on nuclear GR shown is unique to adolescent exposure to chronic stressors. In order to test that the sex-specific impact is unique to adolescent exposure to chronic stressors, animals would need to be exposed to the same stressors used in these experiments at multiple ages because it is possible that differences in the stressor used or the rat strain could also impact these outcomes. Though there was reduced nuclear GR following acute stressor exposure in adult females exposed to CAS, interestingly, there was not an accumulation of GR in the cytosolic compartment. One mechanism of GR regulation is through degradation of GR protein (Demand et al., 2001), and it is possible that females exposed to CAS exhibit higher rates of GR protein degradation following acute stressor exposure resulting in reduced nuclear protein without cytosolic protein accumulation.

There were also sex-specific effects of CAS on body weight. Adolescent male rats gained less weight during adolescence compared to NS male rats (Figure 2.6), suggesting an initial impact of the stressor exposure that normalizes by adulthood (Figure 2.7) because CAS did not impact male terminal weight. The impact of CAS on weight was specific to males; females did not exhibit differences in weight through adolescence or in adulthood.

Together, these data show that CAS alters the GR response to acute stressor exposure in a sex-specific manner. Specifically, nuclear localization of the GR following acute stressor exposure is impacted in lasting manner such that the effects of CAS are apparent months removed from exposure to the CAS paradigm into adulthood.

However, factors contributing to reduced nuclear GR protein in adult female rats are still unclear. Translocation of the GR is regulated by a complex chaperone and co-chaperone complex in the cytosol, and participation of different co-chaperones in the GR cytosolic complex

can alter GR translocation activity (Davies et al., 2002; Wochnik et al., 2005). Previous studies in adolescents that assessed the impact of CAS on co-chaperones of the GR shortly after exposure to the stressors, found that CAS altered expression of GR co-chaperones in a sex and CAS-dependent manner (Bourke et al., 2013). However, the impact of CAS and sex on transcriptional regulation of these co-chaperones has not yet been assessed in adulthood, many weeks removed from exposure to the adolescent stressors. In Chapter 3, we will assess the extent to which CAS alters gene expression of GR co-chaperones to identify co-chaperones that may participate in sex-specific effects of CAS on GR nuclear localization.

Chapter 3

Chronic adolescent stress alters FKBP5 interaction with the glucocorticoid receptor

Introduction

In Chapter 2, we showed that adult female rats exposed to chronic adolescent stress (CAS) exhibit alterations in localization of the glucocorticoid receptor (GR) following exposure to an acute stressor, but mechanisms underlying the functional impact of CAS on GR localization are unclear. Though localization of the GR can be regulated through numerous mechanisms, the influence of GR's co-chaperones is a likely candidate for mediating the impact of chronic stress in a sex-specific manner. The GR is a nuclear receptor that resides in the cytosol in a complex that interacts with multiple chaperones and co-chaperones that impact its function.

Interaction with the immunophilin FK506 binding proteins 4 and 5 (FKBP4 and FKBP5) promote or inhibit translocation of the receptor respectively (Wochnik et al., 2005). In addition to the widely studied FKBP co-chaperones, the GR can also interact with Bag-1, a protein that reduces GR transcriptional activity (Schmidt et al., 2003) and PPID (also known as cyclophilin 40 or cyclophilin D), that facilitates GR activity (Duina et al., 1996; Jandova et al., 2013; Renoir et al., 1995).

Previous studies that occurred at the end of adolescence, shortly after exposure to the adolescent stressors, found sex-specific alterations in gene expression of the GR co-chaperones *Fkbp5, Ppid, and Bag1.* More specifically, adolescent female rats with a history of CAS exposure exhibited elevated gene expression of *Fkbp5* following exposure to a novel acute stressor (Bourke et al., 2013). However, these experiments were performed at the end of adolescence, shortly after exposure to the stressors. Whether alterations in co-chaperone gene expression are a lasting consequence of CAS has not yet been fully studied. In this chapter we assessed gene expression of *Fkbp5, Fkbp4, Ppid,* and *Bag1* in order to determine the extent to which CAS alters gene expression of GR co-chaperones in a sex-specific manner. The most pronounced effects of CAS on gene expression were observed in *Fkbp5,* so we further assessed the functional role of FKBP5 on interactions with the GR.

Many studies have focused on the role of FKBP5 in affective disorders (Binder, 2009). In humans, polymorphisms of the *FKBP5* gene have been associated with increased number of depressive episodes and altered response to antidepressant treatment (Binder et al., 2004). However, the impact of CAS in adulthood on FKBP5 interaction with the GR has not yet been assessed. While the impact of FKBP5 on the GR complex has previously been investigated in cell lines (Davies et al., 2002; Wochnik et al., 2005) and in neuronal cultures (Tatro et al., 2009),

interactions between the GR and its co-chaperone FKBP5 are shown here *in situ* in the rodent brain. And furthermore, these studies investigate the interaction of chronic adolescent stress and acute stress on interactions between these two proteins. In order to understand the extent to which CAS alters adult expression of the GR co-chaperone *Fkbp5* and interactions between the GR and FKBP5, we used a CAS paradigm that confers sex-specific behavioral and molecular effects on the GR and its co-chaperones (Bourke and Neigh, 2011a; Bourke et al., 2013). A proximity ligation assay (PLA) was used to assess interactions between GR and FKBP5 proteins *in situ*, allowing region-specific impact of CAS and acute stress impact on interactions of these two proteins in the adult hippocampus.

Methods

Animal husbandry

All rats were housed on a 14:10 reverse light:dark cycle with standard rat chow and water available *ad libitum* in AAALAC-approved facilities at Emory University. All procedures were approved by the Institutional Animal Care and Use Committee at Emory University. Pups from Wistar dams (Charles River Laboratories) were culled to litters of eight (4 males and 4 females) on postnatal day (PND) 3. On PND 21, litters were weaned into same-sex pairs. Separate cohorts of rats were used for PCR and proximity ligation assays due to tissue requirements for the assays.

Chronic adolescent stress paradigm

On PND 35, male and female rats were divided into CAS and non-stress (NS) groups. Male and female rats in the CAS group were individually housed beginning on PND 35 through the end of

the study. On PND 38-49, CAS rats were exposed to a mixed-modality CAS paradigm consisting of restraint, social defeat, and isolation as previously described (Bourke and Neigh, 2011a; Bourke et al., 2013). All stress procedures occurred during the light cycle. Rats were exposed to six days of restraint and six days of social defeat in a pseudorandom order across the twelve days. The restraint paradigm consisted of placing the experimental Wistar rat in a clear plastic rodent restraint (Braintree Scientific) for one hour. Following the one-hour session, Wistar rats were returned to their home cage. The social defeat paradigm consisted of exposure to a larger, adult Long Evans rat. Male retired breeder Long Evans rats were housed with female adult Long Evans rats that were ovariectomized to prevent estrous cycle changes in aggressive behavior. Prior to the social defeat session, one of the Long Evans rats was removed from the home cage, so the experimental Wistar rat only interacted with a same-sex Long Evans rat. On social defeat days, experimental Wistar rats were placed in the home cage of an adult, more aggressive, same-sex Long Evans Rat. They were separated by a clear plastic barrier that allowed both visual and olfactory cues for two minutes to acclimate. The barrier was then removed for the subsequent five minutes, allowing the rats to physically interact. Interaction often resulted in pinning in males and less frequently in females. Injury and biting were very uncommon, and rats were excluded from the study if injury occurred. Following the five minutes of physical interaction, the barrier was replaced with the rats on separate sides for the following twenty-five minutes. Following the session, the experimental Wistar rat was returned to its home cage. Rats were weighed weekly, and pair-housed NS controls were undisturbed throughout the study except for weekly weighing.

Acute stress and collection

In adulthood (at least six weeks following the end of CAS exposure, PND 91-103), rats were exposed to the novel acute stressor of a five-minute forced swim. Rats were acclimated to a room separated from that used for forced swim stressor exposure. Rats were removed for acute stressor exposure and immediately placed in a clear acrylic cylinder (60 cm tall, 22 cm diameter) with 25 °C water for five minutes. Immediately following the forced swim exposure, rats were replaced in their home cage. Rats were rapidly decapitated 15, 30, or 120 minutes following swim exposure, and brain tissue was collected. Brains were frozen on dry ice and stored at -80 °C for future processing. A separate group of rats was collected at baseline, without exposure to the acute novel swim stressor.

Quantitative reverse-transcription polymerase chain reaction

Hippocampal regions were dissected, and the left hippocampal hemisphere was used for quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted with Qiagen RNAeasy mini kits (Germantown, MA) according to manufacturer's instructions in the Yerkes Nonhuman Primate Genomics Core Laboratory. A nanodrop 2000 was used to assess RNA concentration. RNA was standardized to 1µg in 10µl nuclease-free water. Applied Biosystems High-Capacity cDNA Reverse Transcription kit was used to create cDNA (Applied Biosystems by Thermo Fisher Scientific, Vilnius, Lithuania). Quant-IT Pico Green dsDNA Assay kit (Invitrogen) was used to standardize cDNA to 1 ng/ml. qRT-PCR was performed with Thermo Scientific Absolute Blue qPCR SYBR Green ROX Mix and primers (Thermo Scientific, Carlsbad, CA) for *Nr3c1* (Forward: GGA AGG TCT GAA GAG CCA AG; Reverse: GAT GAT TTC AGC TAA CAT CTC TGG), *Fkbp5* (Forward: TGG AGG TGA ACC

CTC AGA AC; Reverse: TCT TGC TCA ATG CTT TGC TG), *Ppid* (Forward:AGA ACC CGC GAG TCT TCT TT; Reverse: GCA GAG GTT TCC CAG TTG TC), *Bag1* (Forward: ATG GAA ACA CCC TTG TCA GC; Reverse: TGC TGG ATG TCA GAA AGC TC), *Fkbp4* (Forward: GGG AAG GAA AGG TTC CAG AT; Reverse: AGT ACA CGG TGC CCC TTT CT), and *Hprt1* (Forward: TGC TGA AGA TTT GGA AAA GG; Reverse: AAT CCA GCA GGT CAG CAA AG) housekeeping gene. qRT-PCR was performed in triplicate and a CV cutoff of 4% was used. If the CV% exceeded the 4% cutoff, one of the triplicates was excluded from analysis. Gene expression was normalized to *Hprt1* housekeeping gene, and the $2^{-\Delta\Delta Ct}$ method was used to calculate and graph fold change (Livak and Schmittgen, 2001). All statistics were performed on ΔCt values.

Proximity ligation assay

Brains were frozen at -80 °C until processing. Brains were cryosectioned on a Leica cryostat at 7 μm thickness. Coronal sections from ~-3.3 mm and ~-5.3 mm Bregma (Kjonigsen et al., 2011; Paxinos and Watson, 1998) were fixed in 10% formalin and washed with 1X phosphate buffered saline (PBS) for five minutes three times. Antigen retrieval was performed at 96 °C, and tissue was permeabilized in 0.2% Triton-X in 1X PBS. Tissue was washed with 1X PBS and blocked in Duolink blocking buffer (Sigma) for one hour at 37 °C. Tissue was incubated overnight at 4 °C with rabbit anti-glucocorticoid receptor (1:600, Bethyl A303-490A) and goat anti-FKBP5 (1 ug/ml, R&D Systems R&D Systems AF4094) primary antibodies in Duolink antibody diluent. A separate section (Bregma ~-3.3 mm) was incubated overnight at 4 °C with only anti-FKBP5 primary antibody as a negative technical control. Proximity ligation assay (PLA) with far red detection reagents was performed according to manufacturer's instructions (Sigma). Briefly, slides were washed 5 x 5 minutes in Duolink Fluorescence Wash Buffer A then incubated with

anti-goat (1:5) and anti-rabbit (1:5) Duolink secondary antibodies in Duolink antibody diluent for one hour at 37°C. Slides were washed in Wash Buffer A and incubated for thirty minutes at 37 °C with ligation solution, consisting of 1:5 ligation stock (5x) and 1:40 ligase (1 U/µl) in nuclease-free water (Duolink). Slides were then washed in Wash Buffer A and incubated with amplification solution consisting of 1:5 Far Red Amplification Stock (5x, Duolink) and 1:80 polymerase (10 U/µl) in nuclease-free water for 100 minutes at 37°C, protected from light. Slides were washed with Wash Buffer B 5 x 5 minutes followed by a single 5 minute wash of 0.01x Wash Buffer B with agitation. Slides were dried overnight and cover slipped with Duolink DAPI mounting media.

Images were acquired on a Leica TCS SP8 confocal microscope. Twenty-one stacks of 0.35 µm step size were acquired as z-stacks (for a total of 7 µm thickness) with a HC PL APO CS2 40x/1.3 OIL objective. Far red signal was acquired at 653 to 691 nm emission spectra on a HyD detector. DAPI counter stain was acquired at 447 to 481 nm emission spectra on HyD detector. All images were acquired with the same acquisition settings. Three z-stacks centered on the pyramidal or granule cells in the CA1, CA3, and dentate gyrus (DG) were acquired from each stained section.

Duolink ImageTool Software was used to quantify number of PLA signals (PLA counts) in acquired images with signal threshold set to 644 and a signal size threshold of 3 pixels. The number of PLA signals for the three images within each region (dorsal and ventral CA1, CA3, and DG) were averaged. If the coefficient of variation (CV) of PLA counts across the three images per region exceeded the 15% CV threshold, one image was removed from analysis. Average PLA counts in treated samples were divided by the average PLA counts in the single antibody negative technical control (only anti-FKBP5 primary antibody) to control for batch to

batch variability. Representative images were adjusted for brightness and contrast in Fiji is just ImageJ (Fiji) image analysis software (Schindelin et al., 2012).

Statistics

Graphpad Prism 7.02 was used for statistical analyses. Two-way ANOVA (with factors adolescent stress x acute stress) test was used for gene expression analyses. An *a priori* t-test was used to assess the impact of CAS on *Fkbp5* gene expression at baseline. Two-way ANOVA (adolescent stress x acute stress) was used to assess significance of PLA analyses with post-hoc Tukey's test when a significant interaction was observed. All analyses were performed with $\alpha = 0.05$.

Results

Chronic adolescent stress increased basal Fkbp5 gene expression in females

Gene expression of *Fkbp5* was elevated with time following novel acute forced swim stressor exposure in both males ($F_{(3,67)}=5.603$, $p=0.0017$) and females ($F_{(3, 71)} = 12.66$, $p<0.001$). CAS females exhibited elevated *Fkbp5* gene expression at baseline ($t_{17}=2.216$, $p=0.0406$). Data are expressed as mean fold change ($2^{-\Delta\Delta Ct}$) ± SEM normalized to same-sex NS baseline (Figure 3.1).

Chronic adolescent stress effects on glucocorticoid receptor co-chaperones

Gene expression of GR co-chaperones was assessed in males and females following CAS and acute stressor exposure (Figure 3.2). Females with a history of CAS exposure exhibited reduced

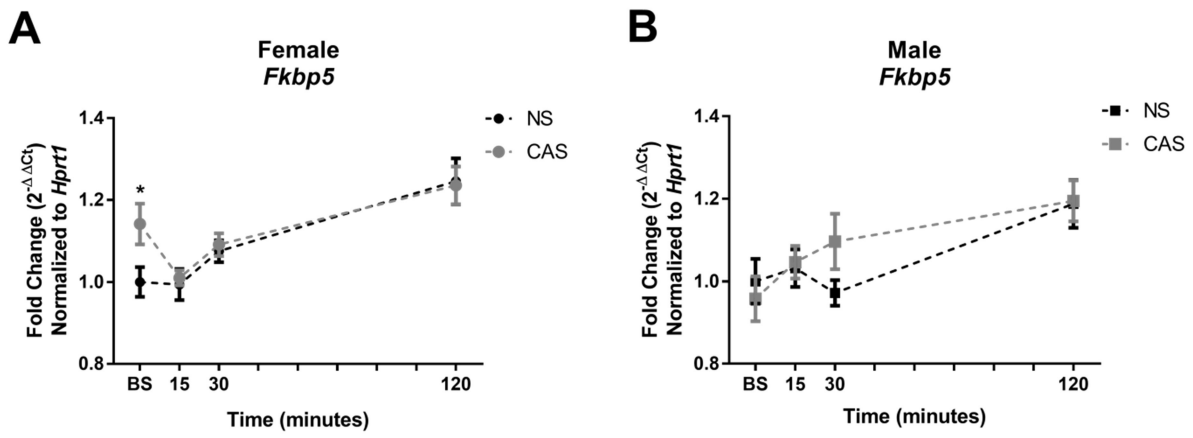

Figure 3.1. *Fkbp5* gene expression in the hippocampus. Male and female rats were exposed to CAS or NS control conditions at baseline or 15, 30, or 120 minutes following exposure to an acute novel stressor in adulthood. Females exposed to CAS exhibited elevated *Fkbp5* gene expression at baseline. There was no significant effect of CAS in males. Both females (A) and males (B) exhibited elevated expression of *Fkbp5* with time following acute novel forced swim stressor challenge. Data are expressed as mean fold change ($2^{-\Delta\Delta Ct}$) ± SEM normalized to same-sex NS baseline group. * indicates a significant difference in CAS to NS comparison with *a priori* t-test, $p<0.05$.

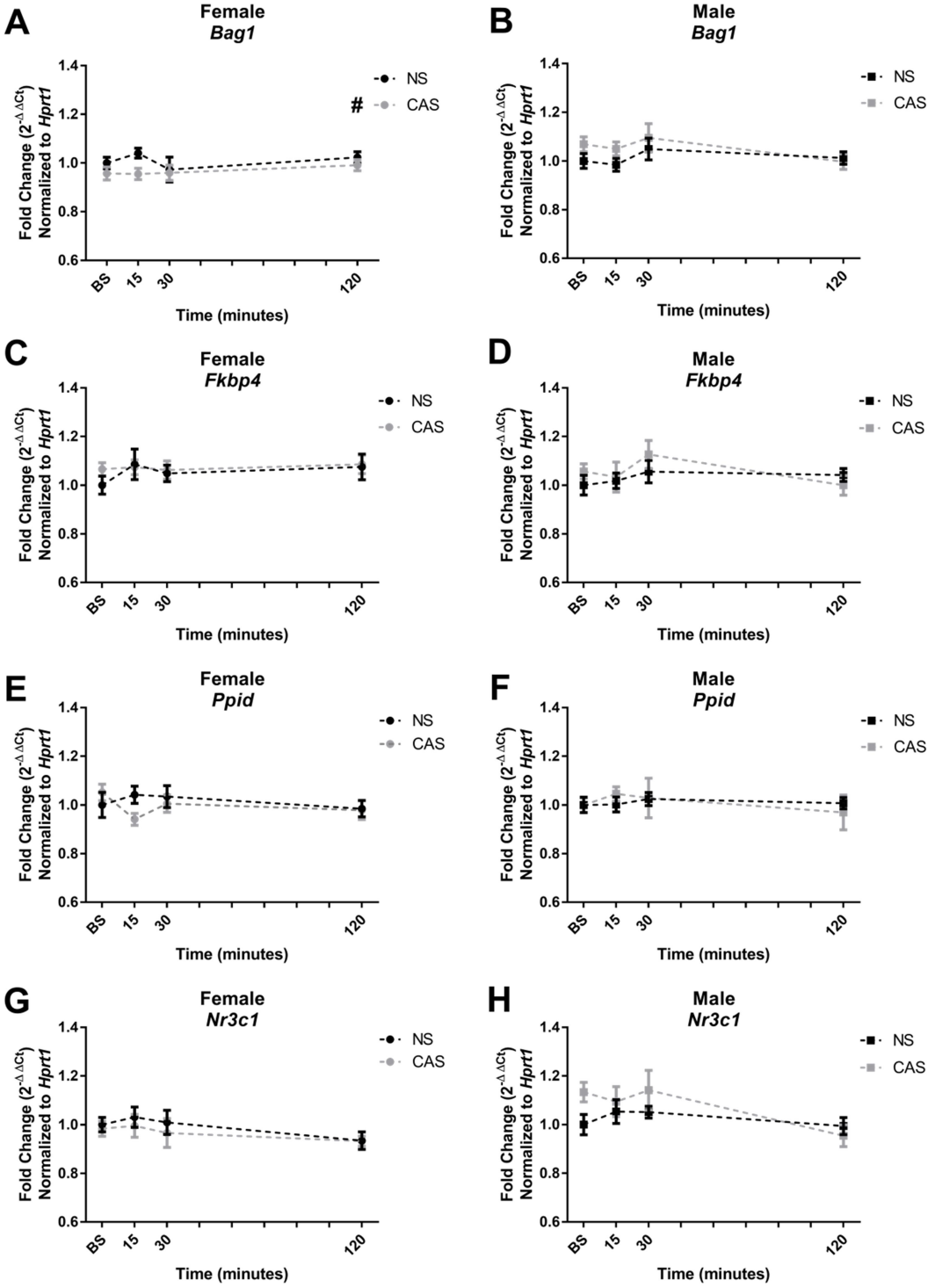

Figure 3.2. Gene expression of GR co-chaperones. Male and female rats were exposed to CAS, and hippocampal tissue was collected at baseline (no acute stress) or 15, 30, or 120 minutes following acute novel forced swim stressor exposure. Gene expression of *Bag1* (A,B), *Fkbp4* (C,D), *Ppid* (E,F), and *Nr3c1* (G,H) are shown for females and males expressed as mean fold change ($2^{-\Delta\Delta Ct}$) ± SEM normalized to the same-sex NS baseline group. # indicates significant main effect of CAS.

Bag1 gene expression (Main effect CAS: $F_{(1, 71)} = 4.207$, p=0.044), but CAS did not impact *Bag1* gene expression in males ($F_{(1, 67)} = 2.633$, p=0.109).

Neither adolescent (Female: $F_{(1, 71)} = 0.452$, p>0.05; Male: $F_{(1, 67)} = 0.6279$, p>0.05) nor acute stress (Female: $F_{(3, 71)} = 0.5895$, p>0.05; Male: $F_{(3, 67)} = 1.092$, p>0.05) impacted *Fkbp4* gene expression in males or females. Adolescent (Female: $F_{(1, 71)} = 0.6453$, p>0.05; Male: $F_{(1, 67)} = 0.005365$, p>0.05) and acute (Female: $F_{(3, 71)} = 0.6371$, p>0.05; Male: $F_{(3, 67)} = 0.4623$, p>0.05) stress also did not impact *Ppid* gene expression.

Nr3c1 gene expression was not altered by adolescent (Female: $F_{(1, 71)} = 0.6715$, p>0.05; Male: $F_{(1, 67)} = 2.312$, p>0.05) or acute stress (Female: $F_{(3, 71)} = 1.359$, p>0.05; Male: $F_{(3, 67)} = 2.333$, p=0.0819, p>0.05) in males or females.

Females exposed to chronic adolescent stress exhibited increased GR-FKBP5 interactions following acute stressor exposure

A proximity ligation assay was used to assess protein-protein interactions between GR and FKBP5 *in situ* in the adult rat hippocampus. Acute stress exposure increased GR-FKBP5 interactions in the dorsal CA1 in adult female rats ($F_{(1, 20)} = 4.627$, p=0.0439), and females exhibited a significant acute by adolescent stress interaction ($F_{(1, 20)} = 6.111$, p=0.0225) (Figure 3.3A). Post-hoc analysis revealed that females with a history of CAS exposure exhibited an increased number of interactions between GR and FKBP5 in the dorsal CA1 following exposure to a novel acute stressor (p=0.0185, Tukey's multiple comparisons test). Males did not exhibit an impact of acute ($F_{(1, 20)} = 1.769$, p>0.05) or adolescent ($F_{(1, 20)} = 1.294$, p>0.05) stress on GR-FKBP5 interactions in the dorsal CA1 (Figure 3.3B). Representative images are shown in Figure 3.4.

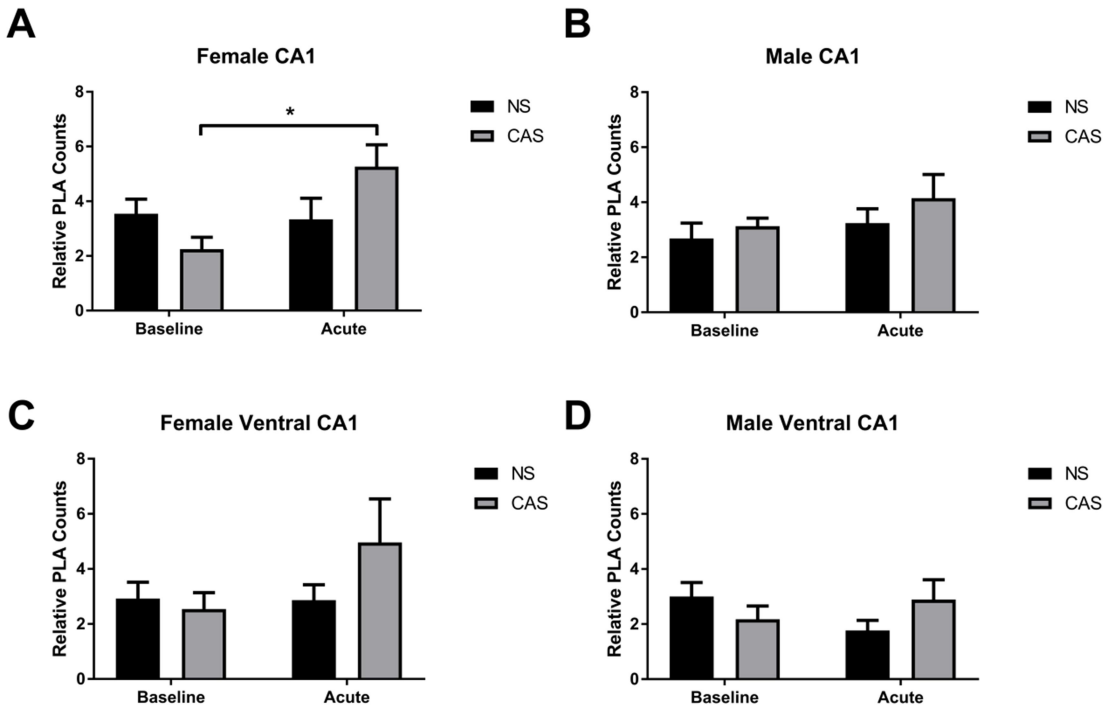

Figure 3.3. Adult female rats exposed to CAS increased GR-FKBP5 interactions following acute novel stressor exposure in the dorsal CA1. Female and male rats were exposed to CAS. In adulthood, tissue was collected at baseline (no acute stressor exposure) or 30-minutes following an acute stressor challenge (acute). A proximity ligation assay was used to assess interactions between GR and FKBP5 protein. Relative PLA counts (compared to negative control) were assessed. PLA signals in the dorsal CA1 in females (A) exhibit a significant main effect of acute stressor exposure ($p<0.05$) and a significant interaction between adolescent stress and time after acute stress ($p<0.05$). Adolescent or acute stress did not significantly impact PLA counts in the male dorsal CA1 (B), female ventral CA1 (C), or male ventral CA1 (D) ($p>0.05$). * denotes significant effect in a post-hoc Tukey's test. Data are presented as mean ± SEM.

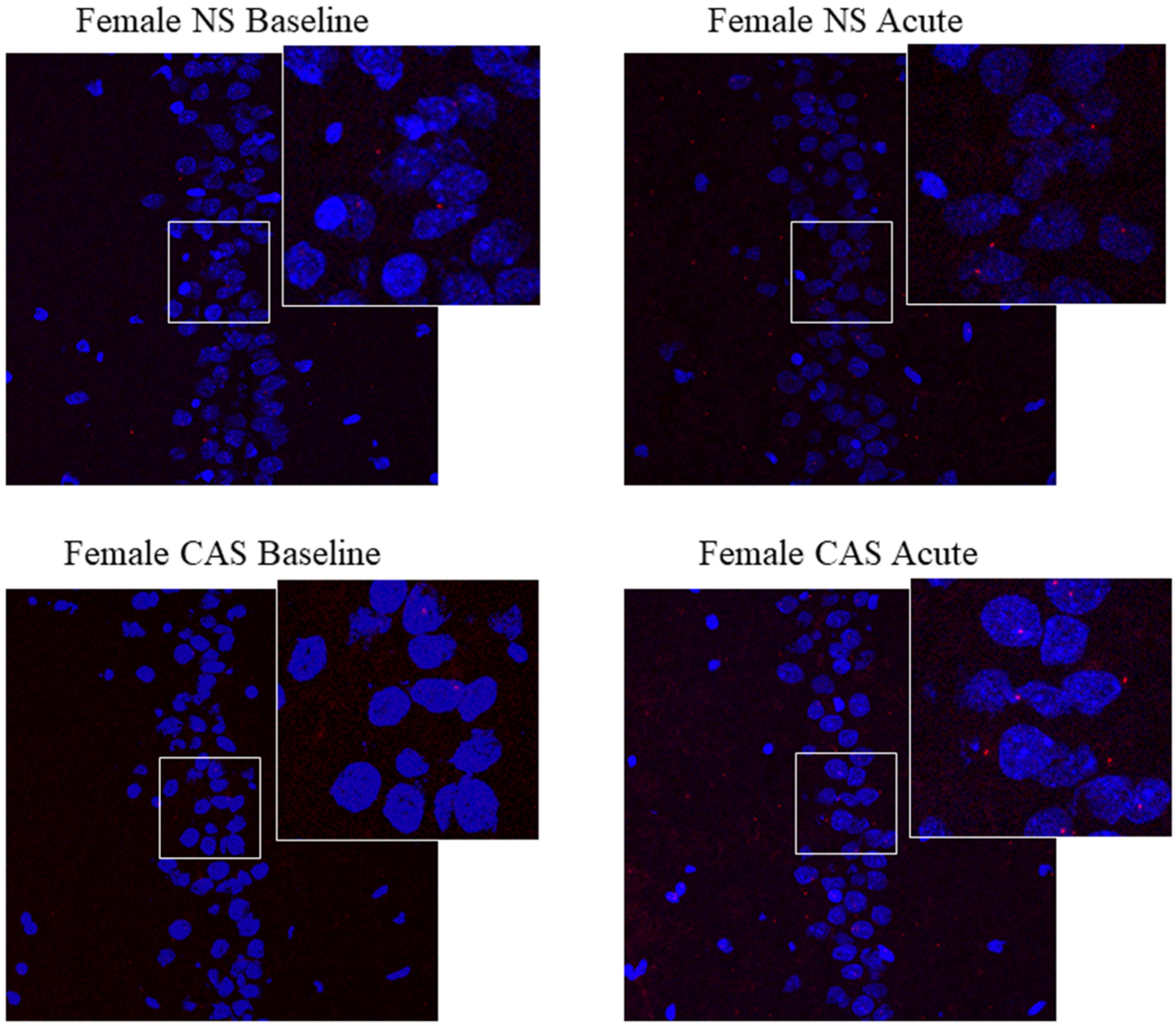

Figure 3.4. Representative images of the dorsal CA1 in female NS and CAS rats at baseline and 30-minutes after exposure to a novel acute stressor. Female rats were exposed to CAS or NS control conditions, and tissue was collected at baseline or 30-minutes following exposure to a novel acute stressor. A proximity ligation assay was used to assess protein-protein interactions between GR and FKBP5 (red puncta). Nuclei were stained with DAPI (blue). Images were acquired in the dorsal CA1 at 40x magnification. Fiji was used to adjust brightness and contrast in representative images.

There were not significant effects of CAS or acute stress in the ventral regions of the CA1. Acute stress (Female: $F_{(1, 20)} = 1.588$, $p>0.05$; Male: $F_{(1, 20)} = 0.2371$, $p>0.05$) or CAS (Female: $F_{(1, 20)} = 0.8465$, $p>0.05$; Male: $F_{(1, 20)} = 0.07515$, $p>0.05$) did not significantly impact GR-FKBP5 interactions in the ventral CA1 in males or females (Figure 3.3C, D). There were also no significant interactions in the ventral CA1 of males or females (Female: $F_{(1,20)} = 1.75$, $p=0.20$; Male: ($F_{(1,20)} = 3.32$, $p=0.0834$)).

Furthermore, GR-FKBP5 interactions were assessed in the dorsal and ventral CA3 and dentate gyrus (DG). A history of CAS or acute stressor exposure did not significantly impact number of GR-FKBP5 interactions in the dorsal or ventral CA3 or DG (Table 3.1). However, adult males did exhibit a non-significant trend in an interaction between history of CAS and acute stress contributing to GR-FKBP5 interactions in the ventral DG ($F_{(1, 20)} = 4.227$, $p=0.0531$, Table 3.1).

	NS Baseline (Mean ± SEM)	NS Acute (Mean ± SEM)	CAS Baseline (Mean ± SEM)	CAS Acute (Mean ± SEM)	Main effect acute stress	Main effect CAS	Interaction
Female CA3	2.905± 0.6986	2.998 ± 0.5219	2.197 ± 0.421	3.304 ± 0.2766	$F_{(1, 20)}$= 1.421, p=0.2473	$F_{(1, 20)}$= 0.1602, p=0.6933	$F_{(1, 20)}$ = 1.013, p=0.3263
Male CA3	2.008 ± 0.2966	2.575 ± 0.661	2.97 ± 1.044	2.311 ± 0.5285	$F_{(1, 20)}$ =0.0044, p=0.9476	$F_{(1, 20)}$ = 0.2574, p=0.6175	$F_{(1, 20)}$ = 0.7936, p=0.3836
Female Ventral CA3	3.232 ± 0.8993	3.544 ± 0.6929	2.451 ± 0.7683	3.7 ± 0.8762	$F_{(1, 20)}$ = 0.9216, p=0.3485	$F_{(1, 20)}$ = 0.1475, p=0.7050	$F_{(1, 20)}$ = 0.3316, p=0.5711
Male Ventral CA3	2.348 ± 0.5879	1.936 ± 0.7305	2.994 ± 1.142	2.464 ± 0.8886	$F_{(1, 20)}$ = 0.2979, p=0.5912	$F_{(1, 20)}$ = 0.4643, p=0.5034	$F_{(1, 20)}$ = 0.004626, p=0.9465
Female DG	3.189 ± 0.636	3.636 ± 0.758	2.613 ± 0.4301	4.503 ± 0.9628	$F_{(1, 20)}$ = 2.612, p=0.1217	$F_{(1, 20)}$ = 0.04065, p=0.8423	$F_{(1, 20)}$ = 0.9963, p=0.3301
Male DG	2.953 ± 0.5925	2.922 ± 0.8478	2.336 ± 0.6693	3.172 ± 0.9978	$F_{(1, 20)}$ = 0.2573, p=0.6175	$F_{(1, 20)}$ = 0.0537, p=0.8191	$F_{(1, 20)}$ = 0.2986, p=0.5908
Female Ventral DG	4.147 ± 1.695	3.454 ± 0.5347	2.394 ± 0.3019	5.922 ± 2.853	$F_{(1, 20)}$ = 0.7056, p=0.4109	$F_{(1, 20)}$ = 0.04483, p=0.8345	$F_{(1, 20)}$ = 1.564, p=0.2256
Male Ventral DG	3.145 ± 0.5909	1.804 ± 0.57	1.825 ± 0.3843	3.42 ± 1.103	$F_{(1, 20)}$ = 0.03145, p=0.8610	$F_{(1, 20)}$ = 0.04314, p=0.8376	$F_{(1, 20)}$ = 4.227, p=0.0531

Table 3.1 Relative PLA counts in the dorsal and ventral CA3 and DG. Mean ± SEM is shown for PLA signals (relative to negative technical control) in the dorsal and ventral CA3 and DG. Statistics of main effects and interactions from a two-way ANOVA (adolescent x acute stress) are shown.

Discussion

These experiments assessed the extent to which prior exposure to CAS altered adult expression of co-chaperones of the GR and interactions between GR and its co-chaperone FKBP5. Here, we show that females exposed to CAS exhibited elevated basal gene expression of *Fkbp5* and increased GR-FKBP5 interactions in the hippocampus. These data are consistent with data shown in Chapter 2 in which a history of CAS reduced nuclear GR in females but not males.

Adult female rats with a history of CAS exposure exhibited elevated *Fkbp5* gene expression at baseline compared to NS controls. Elevated basal *Fkbp5* expression is consistent with findings from other groups using a longer chronic mild stress model in adulthood and assessment shortly after the end of the chronic stress paradigm (Guidotti et al., 2013). Furthermore, another group showed that chronic corticosterone treatment in mice increased hippocampal *Fkbp5* expression when assessed shortly after the treatment paradigm, but the increased gene expression did not persist following four weeks of recovery (Lee et al., 2010). Here, in contrast with that observed by Lee et al., we report elevation of *Fkbp5* in a lasting manner, such that elevated gene expression is apparent months removed from stressor exposures. Furthermore, females exhibited elevated *Fkbp5* gene expression but males did not, suggesting sex-specific enhanced vulnerability to the lasting effects of CAS on adult *Fkbp5* expression. One mechanism that could underlie elevated transcription of *Fkbp5* in females exposed to CAS may be altered epigenetic regulation at the *Fkbp5* gene. Chronic administration of corticosterone reduced DNA methylation in specific regions of the hippocampal *Fkbp5* gene (Lee et al., 2010), suggesting that reduced methylation could be an underlying mechanism promoting elevated basal expression of *Fkbp5*; the extent to which CAS alters epigenetic modification in adulthood should be addressed in future studies.

In previous studies, adolescent female rats exposed to CAS exhibited increased elevations of *Bag1, Nr3c1,* and *Fkbp5* after acute stressor exposure compared to NS controls (Bourke et al., 2013), a pattern that is no longer apparent in adulthood, suggesting that transcriptional regulation of these co-chaperones does not exist in a lasting manner. Adult females exposed to CAS did exhibit subtle reductions in *Bag1* gene expression though the fold change was much less than that observed in adolescents (Bourke et al., 2013) or the differences observed in adulthood in *Fkbp5*. However, there were no significant effects on expression of *Nr3c1* or other GR co-chaperones.

The impact of CAS on *Fkbp5* expression was more pronounced than the CAS effect on other co-chaperones, and whether altered *Fkbp5* gene expression has functional implications on interactions with the GR was then assessed with a proximity ligation assay. While others have shown that chronic corticosterone treatment (Zhang et al., 2016) and chronic mild stress (Chen et al., 2016) increase protein expression of FKBP5, how interactions of FKBP5 and the GR are impacted by the interaction of chronic and acute stress had not been shown.

In females, chronic stress during adolescence and acute stress interacted to alter GR-FKBP5 interactions of the GR and its co-chaperone FKBP5 in the hippocampus. While previous work has shown that the GR and FKBP5 interact *in vitro* (Davies et al., 2002), these current experiments show that CAS and acute stress interact to increase GR and FKBP5 interactions in the rat brain *in situ*. Adult female rats with a history of CAS exposure had increased GR-FKBP5 interactions following exposure to an acute novel stressor in the hippocampal dorsal CA1, an impact of CAS that is long-lasting into adulthood and is consistent with the elevated basal *Fkbp5* gene expression shown in Figure 3.1 and impaired GR nuclear protein shown in Chapter 2. These studies show that interactions between GR and FKBP5 quickly increase following a novel

acute stressor (within 30-minutes), providing new information about the time scale of the "ultra-short negative feedback loop" involved in regulating GR activity (Binder, 2009). A visual, but not statistical trend toward increased interactions in the ventral CA1 in females was also apparent (Figure 3.3C), suggesting that the elevated interactions may not be unique to the dorsal CA1. The absence of a significant effect in the dorsal CA1 males suggests that CAS impacts FKBP5 interaction with the GR in females to a greater extent than in males.

FKBP5 is expressed in the hippocampus, and dexamethasone treatment has been found to increase *Fkbp5* gene expression in the mouse CA1 and DG, but not the CA3 (Scharf et al., 2011), suggesting that there may be hippocampal region-specific control of *Fkbp5* expression. Because dexamethasone is a synthetic GR-agonist, the influence of dexamethasone on *Fkbp5* gene expression further substantiates the role of the GR in mediating *Fkbp5* gene expression in the brain, and consistent with the current results, FKBP5 regulation following GR stimulation in the CA1 may be more pronounced than in other hippocampal regions.

We were unable to detect a significant impact of acute stress or CAS on GR-FKBP5 interactions in the dorsal or ventral CA3 or DG in males or females (Table 3.1). Males did exhibit a non-significant trend towards an interaction between CAS and acute stress in the ventral DG such that males exposed to acute stress and CAS exhibited increased GR-FKBP5 interactions. This group also exhibited higher variability than the others, potentially indicating a bimodal distribution in response to CAS and acute stress. Recently, Ritov et al. classified the behavioral responses to stress in a PTSD model to group animal into separate behavioral phenotypes. They found behavioral phenotype-specific effects in regional brain activity and suggest that identifying behavioral sub-groups of rodents can be informative (Ritov et al., 2016). Experiments could be conducted to determine if specific behavioral characteristics in the acute

response to stress predict altered GR-FKBP5 interactions, and future studies could assess whether differing phenotypes of behavioral responses to stressors in adult males exhibit different patterns in GR-FKBP5 interactions in the ventral DG.

Though there was not a significant impact of acute stress or CAS on GR-FKBP5 interactions in the dorsal or ventral CA3 or DG, we are unable to conclude that interactions between GR and FKBP5 are only impacted in the CA1 as we did not distinguish among cell types in the staining and quantification. In addition to neurons, microglia (Wohleb et al., 2011) and astrocytes (Carter et al., 2012) express GR and FKBP5. Differences in distribution of microglia density in the mouse hippocampus have been found across dorsal and ventral hippocampal regions (Jinno et al., 2007), and it is therefore possible that differential contribution of different cell types to PLA interactions could dilute an effect in a single cell type.

Because there were no differences in *Fkbp5* gene expression between CAS and NS female rats in the time period after acute stressor exposure, it is unlikely that differences in the rate of transcription (between CAS and NS females) after the acute stressor exposure drives the increased protein interactions in CAS females. Rather, it is possible that the elevated basal gene expression of *Fkbp5* allows greater availability of basal FKBP5 protein to interact with the GR after challenge with an acute stressor. However, these experiments did not directly assess protein availability of FKBP5 at baseline though others have shown that chronic stress paradigms or chronic corticosterone paradigms increase FKBP5 protein in the hippocampus (Chen et al., 2016; Zhang et al., 2016).

These experiments highlight the role of FKBP5 in participating in the sex-specific consequences of CAS. Collectively, these experiments show that CAS alters adult expression of

Fkbp5 and its interaction with the GR in the CA1 region of the hippocampus in a sex-specific manner. These findings are consistent with the female-specific blunted nuclear localization of the GR observed in Chapter 2, suggesting that increased interactions between GR and FKBP5 may impair translocation of the GR following acute stressor exposure. These experiments show that acute and chronic stress interact to increase interactions between the GR and FKBP5 in the rat hippocampus in females and furthermore, that the effects of adolescent exposure to chronic stress impacts interactions between these two proteins in a lasting manner. These results provide evidence that GR and FKBP5 interact in the rodent hippocampus and that these interactions are susceptible to disruption by chronic stress.

We have shown that nuclear localization and co-chaperone interaction with the GR are impacted by CAS in a sex-specific manner, but as the GR is a transcription factor that can have widespread impact on gene transcription, global assessment of hippocampal transcription is necessary to determine the extent to which CAS modifies the hippocampal transcriptome. To that end, Chapter 4 will assess the extent to which CAS alters the hippocampal transcriptome in male and female rats in adulthood and assess the involvement of the GR on global hippocampal transcription.

Chapter 4

Transcriptional effects of chronic adolescent stress in the hippocampus are prolonged and sex-specific

Adapted from:

SA Rowson, M Bekhbat, SD Kelly, E Binder, D Weinshenker, G Tharp, M Bent, G Shaw, G Hodes, Z Qin, GN Neigh. Transcriptional effects of chronic adolescent stress in the hippocampus are prolonged and sex-specific

In preparation

Introduction

In Chapters 2 and 3 we showed that nuclear localization of the glucocorticoid receptor (GR) and its interaction with the co-chaperone FKBP5 were altered in a sex-specific manner. Given the role of the GR as a transcription factor, it is important to assess the global transcriptional consequences of CAS exposure and the extent to which the GR may be involved in the transcriptional impact of CAS. Furthermore, it is not likely that GR is the only

transcription factor impacted by CAS, and assessment of the hippocampal transcriptome will identify other important transcription factors that may be involved in driving the sex-specific consequences of CAS. Here, we used the model of chronic adolescent stress (CAS) exposure in rats that has been found to alter adult behavior and adolescent regulation of the hippocampal GR (Bourke and Neigh, 2011b; Bourke et al., 2013) as in Chapters 2 and 3.

Despite recognition of sex differences in humans and animal models, the global effects of CAS are incompletely understood. The hippocampus has been implicated as a site of sex-specific alterations to the transcriptome following acute stressor exposure (Marrocco et al., 2017), but whether there are sex differences that persist into adulthood following CAS exposure in the hippocampus and more specifically, whether exposure to stressors during the adolescent period alter the adult transcriptional response to acute stressor exposure, has not yet been established. Though previous studies have focused on the effects of CAS in specific selected gene targets (Bourke et al., 2013; Pyter et al., 2013), the impact of CAS is likely widespread, impacting numerous transcriptional networks. Here, we used the discovery-based method RNA sequencing to assess the global impact of CAS on the adult hippocampal transcriptome in male and female rats and assessed the extent to which the transcriptional effects of CAS are sex-specific. Previous studies have established that specific effects of prior chronic stress exposure must be revealed by acute stressor challenge (Bourke et al., 2013), so we probed hippocampal reactivity to an acute novel forced swim stressor, in addition to assessing global transcription at baseline, to assess the extent to which prior exposure to CAS impacts the future adult transcriptional response to stress. The discovery-based method RNA sequencing allows assessment of global transcriptional patterns as well as the opportunity to map altered genes to biological pathways to identify networks that are most profoundly impacted by CAS exposure in the hippocampus.

These studies assess the extent to which CAS modifies the adult hippocampal transcriptome in a sex-specific manner. Furthermore, these studies seek to understand the extent to which prior exposure to chronic adolescent stress modifies the adult response to an acute stress challenge and identify upstream transcriptional regulators that are impacted by CAS exposure.

Methods and Materials

Animal husbandry

Pups from Wistar dams from Charles River (Durham, NC) were housed on a 14:10 reverse light:dark cycle with standard rat chow and water available *ad libitum*. Litters were culled to eight pups, and rats were weaned on postnatal day 21. Forty-eight male and female Wistar rats were used for the RNA sequencing studies at Emory University, resulting in n=6 for each experimental group. Throughout all experiments, rats were housed in AALAC approved facilities at Emory University, and all studies were approved by the Institutional Animal Care and Use Committee of Emory University.

Mixed-modality chronic adolescent stress paradigm

On postnatal day (PND) 35, CAS Wistar rats were isolation-housed. Non-stress (NS) controls continued to be paired with same-sex littermates. From PND 38-49, the CAS groups were exposed to twelve alternating days of social defeat (six total days) and restraint (six total days) exposures as previously performed (Bourke and Neigh, 2011b; Bourke et al., 2013). For the restraint paradigm, each Wistar rat was placed in a clear plastic restraint (Braintree Scientific, Braintree, MA) for one hour. For the social defeat paradigm, the adolescent Wistar rat was

placed in the home cage of an adult Long Evans same-sex rat for a two-minute habituation period separated by a barrier that allows visual and olfactory cues, followed by five minutes of physical interaction and an additional twenty-five minutes separated by the barrier. Male retired Long Evans rats were housed with ovariectomized female Long Evans rats as previously established (Bourke and Neigh, 2011b; Bourke et al., 2013). In adulthood (PND 94), tissue and trunk blood were collected via rapid decapitation at baseline (no acute stressor exposure) or thirty-minutes following the start of exposure to a novel five-minute forced swim stressor (acute time point) (Bourke et al., 2013). Brains were extracted and frozen on dry ice and stored at -80 ˚C.

RNA isolation and sequencing

The left hippocampus hemisphere was dissected and used for RNA extraction. RNA-Sequencing analysis was conducted at the Yerkes NHP Genomics Core Laboratory. Total RNA was prepared using QIAGEN RNeasy kits (Germantown, MD). The quality of total RNA was assessed on the Agilent bioanalyzer instrument (Santa Clara, CA). Available RIN values were >8. 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 (San Diego, CA). Clustered flowcells were sequenced to achieve target read depth of 15 million reads per sample on an Illumina HiSeq 1000 in 100-base paired end-read reactions.

RNA sequencing analysis

Sequenced reads were aligned to the rat RefSeq rn5 genomic reference using the

STAR aligner (v2.4.0g1) (Dobin et al., 2013). Counts of reads that uniquely map to genes in the rn5 reference annotation were accumulated using "htseq-count" (HTSeq 0.6.1p1) (Anders et al., 2015). Read counts were library size normalized with DESeq2 (Love et al., 2014) to provide normalized abundance estimation of transcripts. Deseq2 was used to assess normalized reads for each gene.

EdgeR (McCarthy et al., 2012; Robinson et al., 2010) was used to assess differential gene expression of paired comparisons. One set of comparisons contrasted gene expression in adulthood of CAS vs. NS groups at either baseline or after an acute stress challenge in adulthood (30-minutes following forced swim). Acronyms used for each RNA sequencing group are detailed in Table 4.1, and the groups compared for each paired comparison are detailed in Table 4.2. A second set of contrasts compared gene expression at baseline to expression 30-minutes after the acute stress adult challenge. Significance cutoffs for differentially expressed genes (DEGs) were set at a fold change (FC) of 1.3 (or 1/1.3) and uncorrected p-value of 0.05 to assess global patterns in gene expression changes as previously performed (Bagot et al., 2017; Hodes et al., 2015). Fold changes (FC) were expressed as $\log_2$(FC) for analyses and figures. Heatmap.2 in R was used to generate heat maps of differential gene expression. GeneOverlap function in R (Shen and Sinai, 2013) was used to perform Fisher's Exact test of significance in R with the total number of 17,089 background genes. Qiagen Ingenuity Pathway Analysis (IPA) (Mootha et al., 2003; Subramanian et al., 2005) was used to perform Upstream and pathway analyses. Upstream Analysis predicts upstream transcriptional regulators that could mediate the transcriptional effects observed in each paired comparison. The significance threshold for the Upstream Analysis overlap p-value is 0.01 according to Qiagen's manufacturer's instructions. Activation z-

Group	Acronym
Female Non-stress Baseline	F-NS-B
Female CAS Baseline	F-CAS-B
Female Non-stress 30-minute acute	F-NS-A
Female CAS 30-minute acute	F-CAS-A
Male Non-stress Baseline	M-NS-B
Male CAS Baseline	M-CAS-B
Male Non-stres 30-minute acute	M-NS-A
Male CAS 30-minute acute	M-CAS-A

Table 4.1 Groups assessed in RNA sequencing experiments. Male and female rats were exposed to CAS or NS control conditions during adolescence. In adulthood, hippocampal tissue was collected at baseline or 30-minutes following exposure to a novel acute stressor challenge.

Comparison included in Figure	Paired comparison details	Figure notation
Figure 4.1	F-NS-B vs F-CAS-B	Female Baseline
Figure 4.1	F-NS-A vs. F-CAS-A	Female Acute
Figure 4.1	M-NS-B vs M-CAS-B	Male Baseline
Figure 4.1	M-NS-A vs. M-CAS-A	Male Acute
Figure 4.2	F-NS-B vs. F-NS-A	Female NS
Figure 4.2	F-CAS-B vs. F-CAS-A	Female CAS
Figure 4.2	M-NS-B vs. M-NS-A	Male NS
Figure 4.2	M-CAS-B vs. M-CAS-A	Male CAS

Table 4.2 Paired comparisons used in Figures 4.1 and 4.2.

scores that are higher than 2 or lower than -2 can be used to determine significance of predicted activation z-scores according to Qiagen's IPA manufacturer's instructions.

Uterine Weight

GraphPad Prism 7.02 was used for statistical analysis of uterine weight. Uterine weights (mg) from rats used in RNA sequencing analyses were normalized to terminal body weight (g). Two-way ANOVA was used to assess statistical significance of uterine weight with factors of CAS and acute stress. $\alpha=0.05$ for all analyses.

Results

Impact of CAS exposure on hippocampal gene expression: Direct CAS to NS comparison at baseline

Direct comparison of CAS to NS groups within collection time point (baseline, post-acute stress) and sex were performed to assess the impact of CAS exposure on hippocampal transcription, detailed in Table 4.1 and 4.2 (F-NS-B vs F-CAS-B; M-NS-B vs M-CAS-B). Genes were divided into up- and down-regulated differentially expressed genes (DEGs) (Figure 4.1A). There is little to no overlap of DEGs between males and females at baseline (Figure 4.1A). At baseline (Figure 4.1B), different genes are altered in males and females as evidenced by lack of significant overlap of up and downregulated DEGs with a Fisher's Exact Test of overlap between males and females ($p>0.05$). Upstream Analysis in IPA was used to predict upstream regulators that may cause the measured transcriptional patterns (Figure 4.1C). Upstream analysis predicts overlap between lists of genes known to be regulated by an upstream regulator and the list of significant DEGs in the dataset. P-value of overlap indicates significance of overlap between lists with a

significance cutoff of 0.01. The activation z-score can be used to predict activity of the upstream regulator (if the z-score is greater than 2 or less than -2). Upstream Analysis reveals significant overlap with ESR1-regulated genes in females at baseline (bias corrected z= -2.750; z= -4.588, p=4.44 e-15) and predicts reduced ESR1 activity in females with a history of CAS exposure.

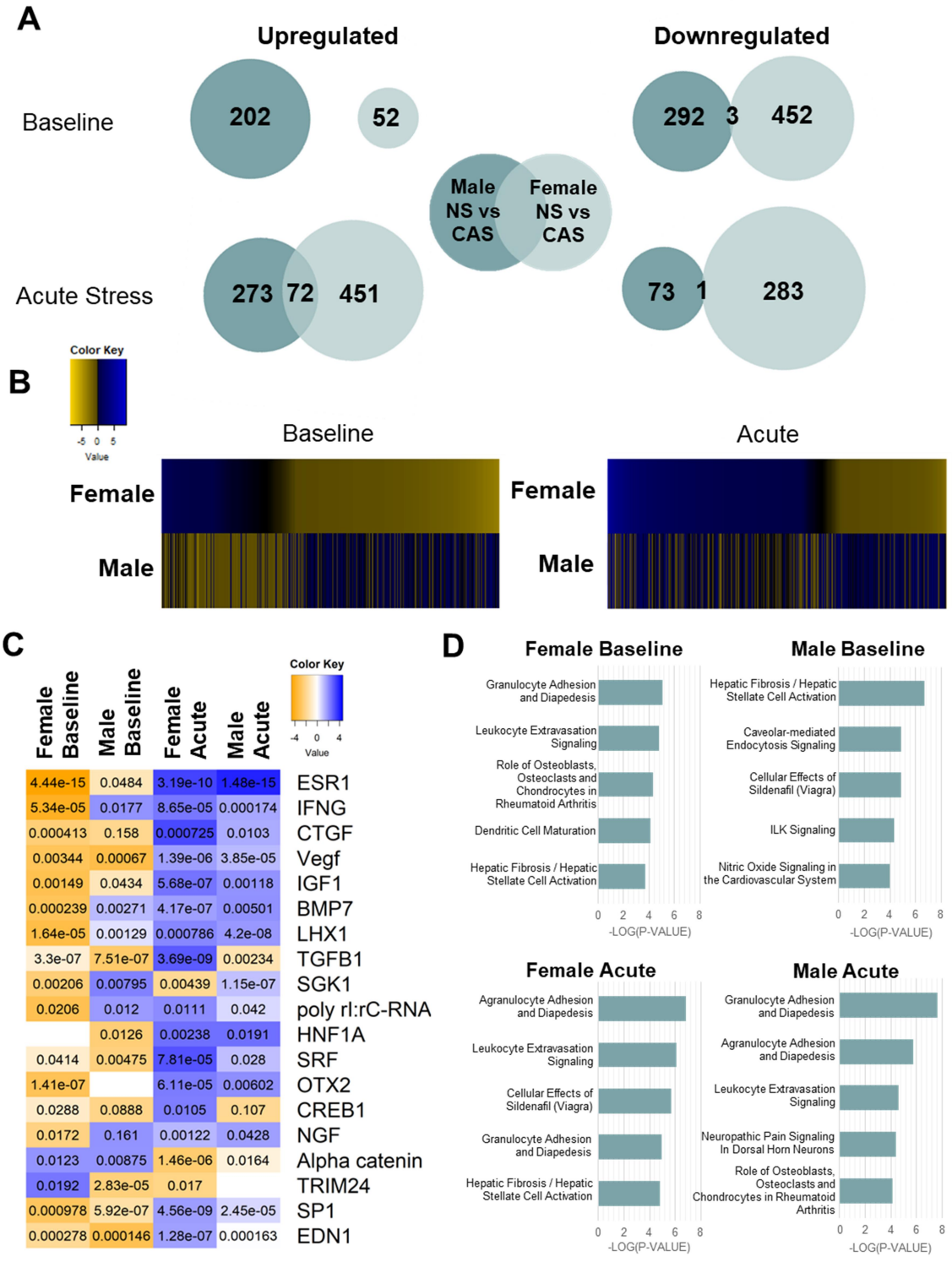

Figure 4.1. Transcriptional effects of CAS. Paired comparison of gene expression data between rats exposed to chronic adolescent stress (CAS) and non-stressed (NS) controls within each sex and acute stress time point was assessed. Differentially expressed genes (DEGs) were

divided into groups that were up or down regulated in the CAS vs NS condition. A) Number of DEGs in the NS vs CAS comparison in males and females at baseline or 30-minutes (acute) following exposure to a novel acute stressor are shown in Venn diagrams. B) DEGs in either paired comparison are represented in a heat map using $\log_2$(FC). A Fisher's exact test of overlap was used to determine that up and down regulated DEGs in females and males do not significantly overlap ($p>0.05$). At the acute stress time point, upregulated genes significantly overlap ($p = 1.3$ e-39) between males and females, but down-regulated DEGs do not ($p > 0.05$). C) Qiagen Ingenuity Pathway Analysis (IPA) was used to predict upstream regulators of transcriptional changes. The heat map color represents uncorrected Activation Z-score, the predicted activity of transcription factors for each of the four paired comparisons (CAS vs NS). P-value of significance of overlap of DEGs with lists of genes known to be altered by each upstream regulator are notated within each cell. IPA sets a significance cutoff of $\alpha= 0.01$ for significance of upstream regulators. The heat map is sorted top to bottom by z-score. D) IPA was used to assess enrichment of pathways in each of the four paired comparisons. –log(p-value) of pathway enrichment is shown for the top 5 enriched pathways for each paired comparison.

Uterine weight was used as a proxy for estrous cycle staging. Uterine weight did not differ among groups, suggesting that differences in ovarian hormones did not drive changes in predicted estrogen receptor activity ($p>0.05$) (Table 4.3) (Harrell et al., 2014).

Because previous studies found alterations in GR (NR3C1[1]) activity in females with a history of CAS exposure (Bourke et al., 2013), and we observed alterations in GR localization and regulation (Chapters 2 and 3), we examined IPA Upstream predicted NR3C1 activity in this dataset (Table 4.4, 4.5). At baseline, females exposed to CAS (compared to NS controls) exhibit a non-significant reduction in NR3C1 activity (bias corrected z-score= -0.39, z-score=-0.724, p= 0.036, Table 4.4, 4.5). The activation z-score can be used to predict activity of the upstream regulator, but neither the z-score nor the overlap p-value reached statistical significance according to the cutoffs recommended by Qiagen.

Enriched pathways determined through IPA also differ between males and females (Figure 4.1D). Males exhibit enrichment in pathways relating to hepatic fibrosis, endocytosis, ILK signaling, and nitric oxide. Females exhibit enrichment of pathways relating to granulocyte adhesion, leukocyte extravasation, dendritic cell maturation, and hepatic fibrosis, among others (Figure 4.1D). Largely pathways were non-overlapping between males and females with the exception of the hepatic fibrosis pathway.

[1] In this chapter the glucocorticoid receptor will be referred to as NR3C1 in order to remain consistent with naming conventions used by Qiagen Ingenuity Pathway Analysis software for Upstream Analysis. Non-italicized NR3C1 will refer to the glucocorticoid receptor protein. *NR3C1* (human) or *Nr3c1* (rat) formatting will be used to reference the gene.

	NS-B	CAS-B	NS-A	CAS-A	Main Effect CAS	Main Effect Acute Stress	Interaction
Mean ± SEM	1.9 ± 0.062	2.274 ± 0.247	1.91 ± 0.077	2.552 ± 0.5314	$F_{(1, 19)}$ = 2.81, p=0.11	$F_{(1, 19)}$ = 0.224, p=0.64	$F_{(1, 19)}$ = 0.197, p=0.66

Table 4.3 Uterine Weight. Uterine weight at collection was normalized to terminal body weight (mg/g). Uterine weights per group are expressed as mean ± SEM. Uterine weights did not differ among groups used in RNA sequencing experiments.

Comparison	Expr Log Ratio	Predicted Activation State	Bias-corrected z-score	Activation z-score	Bias Term	p-value of overlap
F-NS-B vs F-CAS-B	-0.248		-0.387	-0.724	-0.104	3.55E-02
M-NS-B vs M-CAS-B	0.061		0.839	0.78	-0.021	2.29E-02
F-NS-A vs. F-CAS-A				n.s.		
M-NS-A vs. M-CAS-A	-0.012	Activated	2.296	2.559	0.084	1.22E-03
M-CAS-B vs. M-CAS-A	-0.077		0.096	0.524	0.106	2.98E-07
F-CAS-B vs. F-CAS-A	0.123	Activated	2.695	2.99	0.088	3.52E-07
M-NS-B vs. M-NS-A	-0.008		0.019	0.187	0.052	7.39E-07
F-NS-B vs. F-NS-A	-0.047		1.304	1.04	-0.078	2.29E-02

Table 4.4. Upstream analysis predicted activity for NR3C1 (GR). IPA Upstream Analysis was used to assess predicted activity for NR3C1. Significance cutoff for p-value of overlap for this analysis is $p<0.01$. A z-score over 2 or under -2 was considered significant and able to predict activity of the upstream regulator.

Comparison	Target molecules in dataset
F-NS-B vs F-CAS-B	ABCA1,AQP1,AQP5,AVP,F3,GRIN2A,GRIN2B,GRM3,GRM5, MYOC,OCLN,POMC
M-NS-B vs M-CAS-B	CGA,FN1,GBP2,GHRHR,GRIN2A,GRIN2B,GRM3,ISG15,LYST, NOS3,NOTCH4,TLR2
F-NS-A vs. F-CAS-A	
M-NS-A vs. M-CAS-A	AQP1,C1R,CXCR4,CYR61,GRIN2A,GRIN2B,GRM5,HCAR2, LYST,NOS3,PIGR,PPARA,SLC10A2,SLC1A6
M-CAS-B vs. M-CAS-A	AQP1,CDKN1A,CGA,CYR61,DDIT4,DUSP1,EGR1,F2RL1,FN1, FOS,IER2,IFIT2,ITGB6,LHB,MFSD2A,NFKBIA,NOS3, NOTCH4,OASL,PER1,PIGR,PTGR1,SGK1,VDR
F-CAS-B vs. F-CAS-A	CDKN1A,CYR61,DDIT4,DUSP1,EDN1,EGR1,ERRFI1,FOS, GRIN2A,HSD11B2,IER2,NFIL3,NFKBIA,PER1,POMC, SERPINE1,SGK1,SLC2A5
M-NS-B vs. M-NS-A	CDKN1A,CYR61,DUSP1,EGR1,F2RL1,FOS,GRIN2A,GRIN2B, HCAR2,IER2,IFIT2,LYST,NFKBIA,PER1,SERPINE1,SGK1, SLC1A7
F-NS-B vs. F-NS-A	AQP1,AVP,CXCL10,CYR61,DUSP1,EGR1,FOS,GBP2,IER2, IL1A,ITGB6,LYST,NFKBIA,OCLN,PDPN,SGK1

Table 4.5 Genes mapped to NR3C1 (GR) Upstream Analysis. Genes that were differentially expressed that mapped to the NR3C1 Upstream list for each paired comparison. Table 4.4 corresponds to statistics in Table 4.3.

Impact of CAS exposure on hippocampal gene expression: Direct CAS to NS comparison following acute stressor exposure

At the acute stress time point (F-NS-A vs. F-CAS-A; M-NS-A vs. M-CAS-A), only genes that are upregulated by CAS significantly overlap between males and females (p=1.3 e-39). Genes that have reduced expression in rats with a history of CAS exposure do not significantly overlap between males and females (p>0.05). At the acute stress time point, females (p=3.19 e-10) and males exhibit significant overlap (p=1.48 e-15) with ESR1[2]- regulated genes and increased predicted activity in CAS-exposed rats. There is no significant difference between CAS and NS females in predicted NR3C1 activity at the acute stress time point (Table 4.4, 4.5). Males at the acute stress time point exhibit enhanced NR3C1 activity compared to NS controls (bias corrected z-score= 2.30, z-score= 2.56, p= 0.001, Table 4.4, 4.5).

Transcriptional response to acute stressor exposure: Direct 30-minute to baseline comparison in NS rats

In order to assess DEGs that are altered during the acute transcriptional response to novel stressor exposure with or without a history of CAS, the acute stress time point was directly compared to baseline (F-NS-B vs. F-NS-A; M-NS-B vs. M-NS-A). Though the majority of DEGs do not overlap between males and females, genes that are upregulated following acute stressor exposure overlap between NS males and NS females to a greater degree (9% overlap, Fisher's Exact p = 4e-28) than downregulated genes (0.6%, Fisher's Exact p>0.05) (Figure 4.2A,B). Furthermore, NS females exhibit enhanced repression of genes following acute stressor

[2] ESR1 refers to estrogen receptor α. ESR1 will be used in this chapter to refer to ERα to remain consistent with naming conventions used by Qiagen Ingenuity Pathway Analysis software.

exposure (548 genes) compared to males (94 genes). IPA Upstream Analysis (Figure 4.2C) reveals overlap with genes regulated by multiple transcription factors including interferon alpha, IL5, Pkc(s), ERK, and SP1. NS females exhibit a non-significant increase in predicted NR3C1 activity (z=1.304, overlap p-value = 0.0229) following acute stressor exposure (Table 4.4, 4.5). NS males exhibit significant overlap with NR3C1 regulated genes (p=7.39 e-7). IPA (Figure 4.2D) was used to assess pathway enrichment.

Transcriptional response to acute stressor exposure: Direct 30-minute to baseline comparison in CAS rats

Rats with a history of CAS (F-CAS-B vs. F-CAS-A; M-CAS-B vs. M-CAS-A) exhibit more upregulated genes in the acute stress response and reduced repression of gene expression relative to NS controls (Figure 4.2A). Interestingly, males and females with a history of CAS exhibit increased similarity in their acute transcriptional response to stressor exposure indicated by significant overlap between genes with increased (Fisher's Exact p=2.3e-76) and decreased (Fisher's Exact p=0.011) expression though there was a greater number of overlapping upregulated genes (14%) compared to downregulated DEGs (2%) (Figure 4.2A). CAS females exhibit a significant increase in predicted NR3C1 activity (bias corrected z-score = 2.695, z-score= 2.99, overlap p-value= 3.52 e=-7), suggesting a more transcriptionally active NR3C1 response following acute stressor exposure in CAS females (Table 4.4, 4.5). CAS males also exhibit significant overlap with NR3C1 regulated genes (p= 2.98e-7) but do not exhibit a significant predicted activation state.

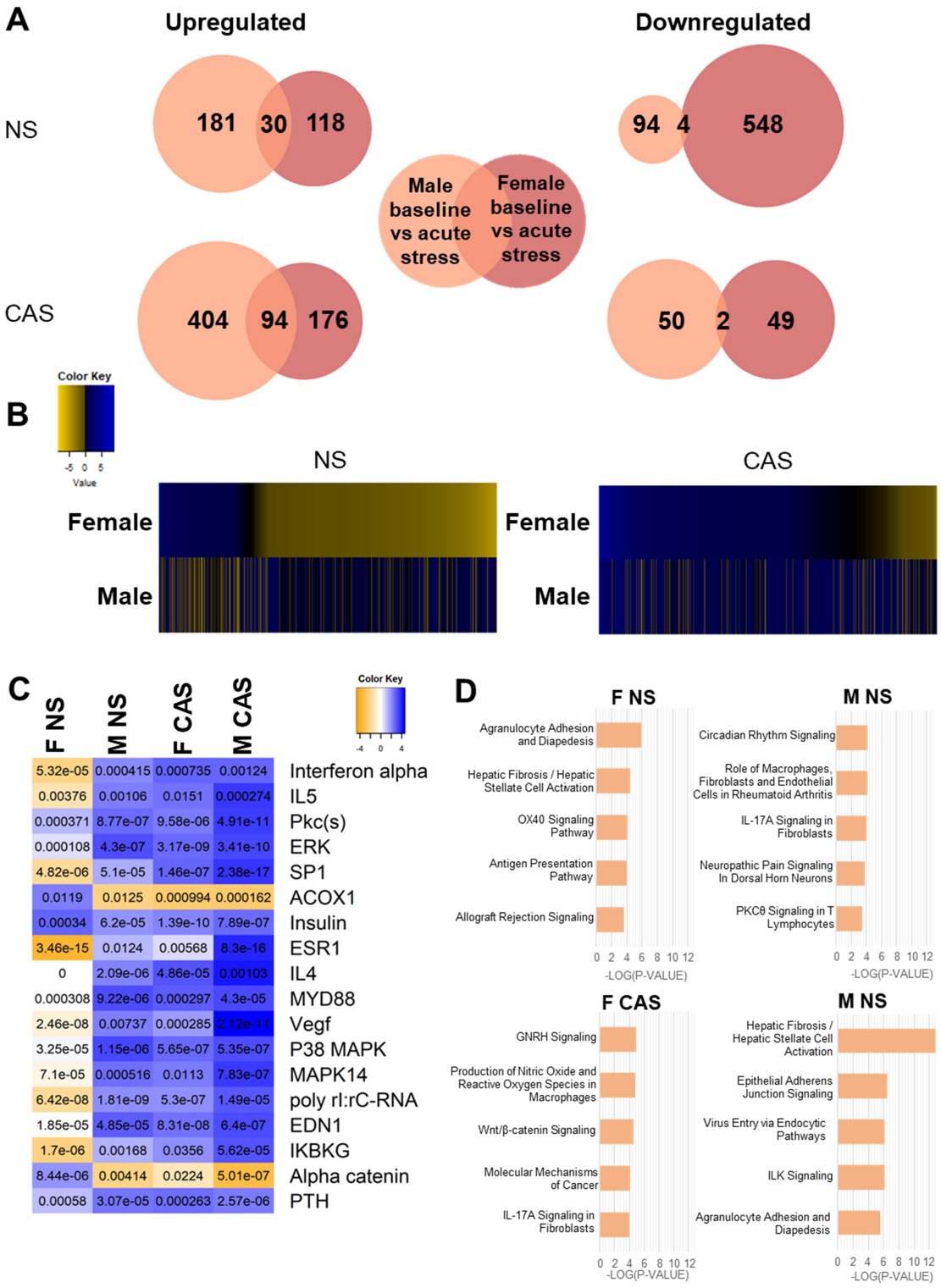

Figure 4.2. Transcriptional effects of acute stress. Paired comparison of gene expression between acute stress and baseline time points was assessed within sex and adolescent stress history group (non-stress (NS) and chronic adolescent stress (CAS)). A) Differentially expressed genes (DEGs) were split into up and downregulated genes and shown in Venn diagrams. B)

Heatmaps show direction of gene expression change using log_2(FC) following acute stressor exposure in male and female rats with a history of CAS or NS control conditions. Genes that were significantly up or downregulated in either males or females were included in heatmaps. A Fisher's Exact test of overlap shows that upregulated genes in NS males and females significantly overlap (p = 4e-28) but down regulated genes do not (p >0.05). In animals with a history of CAS, both up (p = 2.3e-76) and downregulated (p=0.011) genes significantly overlap between males and females. C) Qiagen Ingenuity Pathway Analysis (IPA) upstream analysis was used to assess upstream regulators of transcriptional changes. The heatmap represents Activation Z-score in each cell. P-value of overlap between significant DEGs in each paired comparison (acute stress vs baseline) and genes known to be regulated by each upstream regulator are notated within each cell. α= 0.01 for IPA Upstream Analyses. D) IPA Pathway analysis was used to assess pathways that are enriched following acute stressor exposure (acute vs. baseline) within each sex and adolescent stress history.

Discussion

These studies establish that chronic adolescent stress exposure profoundly alters transcriptional regulation in the adult hippocampus in a sustained and sex-specific manner. Effects of CAS on the transcriptome were assessed in adulthood, months removed from exposure to the adolescent stressors. These data provide new evidence that chronic stress exposure during adolescence is sufficient to confer transcriptional changes in the hippocampus that persist into adulthood.

Previous work has assessed the effect of adolescent stress exposure on expression of specific targeted genes and found a gene-specific impact of adolescent stress in adolescence (Bourke et al., 2013; Wulsin et al., 2016) and adulthood (Kelly et al., 2014; Pyter et al., 2013; Wulsin et al., 2016) at baseline or following acute stress or lipopolysaccharide challenge. We extend the appreciation of the lasting consequences of CAS exposure on gene transcription to the global scale and show that CAS-induced alterations to gene transcription are widespread and sex-specific. In this dataset, there were global shifts in gene expression such that CAS altered the global direction of gene transcription in opposite directions in males and females at baseline and following exposure to a subsequent novel acute stressor in adulthood. Together, these data show that CAS has a lasting impact on adult transcriptional regulation of the hippocampus in a distinctly sex-specific manner. Previous work has observed sex differences in the nucleus accumbens transcriptome following subchronic variable stress exposure (Hodes et al., 2015) or in the ventromedial prefrontal cortex and nucleus accumbens following chronic variable stress (Labonte et al., 2017) in mice. However, these sequencing studies occurred in adults shortly after stressor exposure, in contrast to the lasting effect observed in the current data. Therefore, we extend the previous findings to show that exposure to stressors during adolescence alters the

transcriptome in a lasting manner, months removed from stressor exposure. We furthermore identify the hippocampus as a site of sex differences in chronic stress susceptibility. These studies establish that adolescence is a susceptible developmental period in which chronic stress is sufficient to substantially change global transcriptional regulation into adulthood.

We assessed the extent to which CAS impacts the transcriptional response to a novel acute stress challenge by directly comparing global gene expression following exposure to a novel acute stressor to baseline gene expression (Figure 4.2). Males and females without a history of CAS exhibited little overlap in the transcriptional response to an acute stressor, indicating widespread sex differences in the transcriptional response to acute stressor exposure (Figure 4.2). Sex differences in the global transcriptional response following an acute stressor exposure have been previously reported in adult mice in the CA3 region of the hippocampus in which acute stress alters a greater number of genes in females than males (Marrocco et al., 2017). There were a greater number of downregulated genes in females, however, there were not similarly a greater number of upregulated genes in females as observed by Marrocco et al. (Marrocco et al., 2017). These differences in transcription pattern may be brain region-specific or diluted by our assessment of the whole hippocampus.

We then assessed the extent to which prior exposure to CAS modifies the adult acute stress response (Figure 4.2). Males and females with a history of CAS exposure upregulated a greater number of genes following acute stressor exposure than same-sex NS controls and downregulated fewer genes following acute stressor exposure than NS same-sex controls. Interestingly, males and females with a history of CAS exhibited more similar transcriptional profiles relative to NS controls following acute stressor exposure despite sex differences in the transcriptional stress response in NS animals. While NS females suppressed expression of many

genes after acute stressor exposure, CAS females, and to a lesser degree CAS males, suppressed fewer genes relative to their NS controls. Females with a history of CAS exposure exhibited a reduced ability to suppress gene expression following novel acute stressor exposure (Figure 4.2A,B). Collectively, this pattern of gene expression is more consistent with the pattern of gene expression observed in males following acute stressor exposure. Developmental stress has previously been reported to induce masculinizing effects on behavior and on the endocrine system, and in altering sex hormone receptors in the hippocampus in female guinea pigs (Kaiser et al., 2003).

IPA was used to assess upstream regulators and pathways that were impacted by CAS exposure to extend the global transcriptional findings to biologically relevant networks of genes (Figure 4.1C). IPA Upstream Analysis identified genes regulated by the estrogen receptor alpha (ESR1) to be significantly altered by CAS exposure (compared to NS controls). Adult females with a history of CAS exposure exhibited reduced predicted ESR1 activity at baseline. Conversely, both males and females exposed to CAS exhibit enhanced predicted ESR1 activity compared to NS controls at the acute stress time point. The protective effects of estrogen on mood disorders have been extensively studied (Schmidt et al., 2000; Walf and Frye, 2010; Walf et al., 2009), and sex differences have been reported in the specific role of each estrogen receptor in the hippocampus (Oberlander and Woolley, 2016). More specifically, a deficiency in estrogen signaling was implicated in sex differences in affective behavior following chronic stress exposure in mice (Hu et al., 2012). Furthermore, following chronic social defeat stress, susceptible mice exhibit reduced predicted upstream activity of ESR1 in the nucleus accumbens compared to resilient mice (Lorsch et al., 2018). We show here that CAS exposure altered ESR1 in the hippocampus with a pattern consistent with the pro-susceptible activity pattern observed

previously. As a proxy for estrous cycle staging, we measured uterine weight at collection (Harrell et al., 2014). There was no significant effect of CAS or acute stress on uterine weight (Table 4.3), suggesting that there were not group-specific differences in estrous cycle stage. The absence of a group effect on uterine weight suggests that the effect observed of CAS on predicted ESR1 activity is not a function of different estrous cycle stages among groups.

Due to the established role of the GR in the adolescent effects of CAS (Bourke et al., 2013) and adult effects of CAS in Chapters 2 and 3, we examined predicted GR (NR3C1) activity. At baseline, females exposed to CAS exhibited a non-significant trend in reduced predicted NR3C1 activity but not at the acute stress time point (Table 4.4). Females with a history of CAS exhibited a significant increase in predicted NR3C1 activity following acute stressor exposure while NS females exhibit a non-significant increase, suggesting that females with a history of CAS may have a more transcriptionally active GR following exposure to a novel stressor relative to females that were not exposed to developmental stressors (Table 4.4). This difference may be in part impacted by the trend in reduced activity of NR3C1 in CAS females at baseline, and the absence of a difference in the direct comparison of CAS to NS females at the acute stress time point should be noted. The transcriptional impact of the GR may be finely tuned to specific genes and may be unable to be comprehensibly assessed with global upstream analysis. The increased predicted NR3C1 activity of females exposed to CAS following acute stressor exposure will be discussed in greater detail in Chapter 5. However, examination of altered NR3C1 pathway genes revealed reduced expression of glutamate receptor or receptor subunit genes in females exposed to CAS at baseline (*Grin2a, Grin2b, Grm3, Grm5*). Changes to glutamate-related genes were previously observed following acute stressor exposure

in a sex specific manner in the CA3 such that expression of glutamate system genes were increased following acute stress exposure (Marrocco et al., 2017).

Both the estrogen receptor (Wei et al., 2014) and SGK1 (Yuen et al., 2011), a glucocorticoid-sensitive transcription factor also identified with IPA Upstream Analysis that has been implicated in stress-induced changes in hippocampal neurogenesis (Anacker et al., 2013), have been implicated in glucocorticoid and stress-related changes to the glutamate system. Males and females exhibit directionally opposite predicted activity of SGK1 at baseline and following acute stressor exposure (Figure 4.1C), suggesting that SGK1 may be involved in promoting sex differences in gene expression. SGK1 promotes NR3C1 translocation (Anacker et al., 2013), and reduced basal SGK1 activity in females is in line with the trend in reduction in predicted basal NR3C1 activity in females. Reduced activity of SGK1 may be a sex-specific adaptive mechanism to promote a reduction in NR3C1 activity following repeated exposure to elevated glucocorticoids during the CAS paradigm and is consistent with the reduced GR translocation observed in Chapter 2. Collectively, these results suggest that CAS may dysregulate a balance among transcription factors including ESR1, SGK1, and NR3C1, and it will be important to assess transcription factor activity to elucidate the specific role of these transcription factors in mediating the impact of CAS exposure in the future.

These studies provide new evidence that the effects of CAS exposure are prolonged and sex-specific. Furthermore, prior exposure to CAS alters the adult transcriptional response to novel acute stressor exposure in a sex-specific manner. It will be important to understand the consequences of adolescent exposure to stressors and in developing treatment strategies, how targets and pathways are altered differently in males and females. More complete understanding of the global and specific effects of adolescent stress exposure can provide insight into

developing new targets for therapy that account for sex differences in the impact of chronic stress exposure.

Chapter 5

General Discussion

Introduction

Chronic stress exposure is a risk factor for the development of disease, and adolescents may be particularly vulnerable to the disruptive impact of chronic stress exposure. The glucocorticoid receptor (GR) is a regulator of the stress response that is sensitive to disruptions by chronic stress. The experiments in this dissertation assessed the extent to which exposure to chronic stress during adolescence impacts the hippocampal GR in adulthood. Experiments in Chapter 2 showed that females exposed to chronic adolescent stress (CAS) exhibited reduced nuclear GR following acute stressor exposure. Furthermore, Chapter 3 detailed experiments that showed elevated basal *Fkbp5* gene expression and increased interactions between GR and

FKBP5 protein in the hippocampus adult of female rats exposed to CAS. Finally, CAS induced long-term changes in gene expression in the hippocampus, detailed in Chapter 4. And furthermore, the GR may be more transcriptionally active in females exposed to CAS following acute stressor exposure. Together, these data indicate that CAS induces lasting changes to hippocampal GR regulation in adulthood and furthermore, that the lasting consequences of adolescent exposure to stressors is sex-specific.

CAS alters hippocampal GR regulation in a sex-specific manner

Together the data discussed in this dissertation (Figure 5.1) suggest enhanced FKBP5- mediated negative feedback on GR localization within the hippocampus, consistent with the model described by Binder et al. (Binder, 2009). Female rats exposed to CAS exhibited higher basal expression of *Fkbp5*, which could result in increased available FKBP5 protein to interact with the GR complex following an acute stressor exposure (Chapter 3). Following exposure to an acute novel stressor, females with a history of CAS exhibited increased interactions of FKBP5 and GR in the hippocampus (Chapter 3) which may prevent efficient translocation of the GR to the nucleus because FKBP5 inhibits efficient GR nuclear translocation (Tatro et al., 2009; Wochnik et al., 2005). CAS females exhibited blunted GR-nuclear protein following acute stressor exposure (Chapter 2), consistent with the established role of FKBP5 in inhibiting GR translocation. Ultimately, rats exposed to CAS exhibit a sex-specific altered transcriptional profile in the whole hippocampus with a predicted increase in transcriptional activity of GR following acute stressor exposure and a reduced influence of ESR1 signaling at baseline. Furthermore these effects are more pronounced in females.

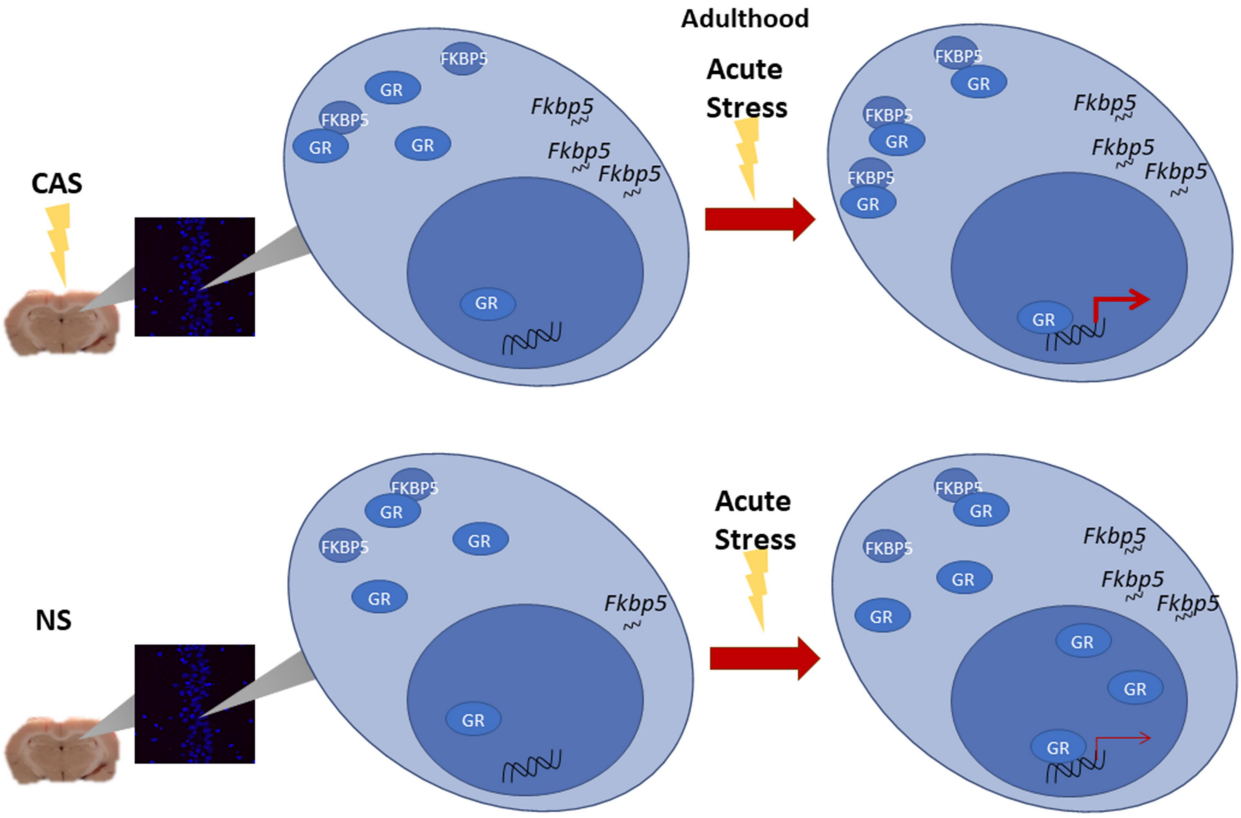

Figure 5.1 Effects of CAS on GR regulation in adult female rats. Female rats exposed to CAS exhibited elevated expression of *Fkbp5* (Chapter 3). Following exposure to an acute novel stressor, females exposed to CAS exhibited reduced nuclear GR protein (Chapter 2) and increased interactions of GR and FKBP5 (Chapter 3). Female rats exposed to CAS exhibited increased predicted transcriptional activity of the GR following acute stressor exposure (Chapter 4).

Global impact of CAS: Insights from RNA sequencing

RNA sequencing analysis revealed that adult female rats with a history of CAS exhibited a significant increase in predicted GR activity after acute stressor exposure (compared to baseline transcription) while females without adolescent exposure to stressors (NS) did not. These results suggest that following acute stressor exposure, the GR is more transcriptionally active in adult female rats exposed to CAS relative to NS females. Results from Chapters 2 and 3 suggested reduced activity of the GR through increased interactions with FKBP5 and reduced nuclear localization. These data initially appear to be contradictory with the predicted increased activity of the GR in the RNA sequencing experiments (Chapter 4), but translocation of the GR is only one level of potential GR regulation. A mechanism that enhances nuclear activity of the GR could compensate for reduced nuclear content by promoting a more transcriptionally active GR in females exposed to CAS. CAS could enhance nuclear GR activity through multiple possible mechanisms. A reduction in DNA methylation at GR target genes could promote enhanced transcriptional activity of the nuclear GR. Others have suggested that DNA methylation may exhibit sex-specific differences (Uddin et al., 2013), and chronic stress can alter DNA methylation (Matosin et al., 2017). Others have reported reduced methylation at the GR-target gene *Fkbp5* (Lee et al., 2010), and it is possible that CAS could induce a sex-specific impact on DNA methylation of other GR target genes. Future studies should assess global DNA methylation and determine if effects of CAS on DNA methylation patterns are sex-specific and if sets of genes corresponding to specific transcription factors are differentially impacted. Altered methylation patterns could shift GR transcriptional activity if distribution of CAS-induced methylation is uneven among GR-target genes. In the RNA sequencing data (Chapter 4), there was a global pattern of gene expression such that more genes were increased in the acute stress

response in CAS relative to NS rats, suggesting that there could be reduced methylation throughout the genome. Gassen et al. recently reported that FKBP5 interacts with and reduces activity of DNMT1, a DNA methyltransferase that participates in *de novo* DNA methylation (Gassen et al., 2015). Elevated *Fkbp5* expression, if it increased FKBP5 protein, could also interact with and reduce DNMT1 activity as shown in Gassen et al. to reduce DNA methylation (Gassen et al., 2015). CAS could also increase expression of GR transcription coregulators which could enhance GR's nuclear activity despite reduced nuclear content of GR protein. Additionally, post-translational modifications of the GR could also alter transcription at specific GR-target genes which could alter the transcriptional profile of the GR (Chen et al., 2008; Galliher-Beckley et al., 2008). Altered rates of expression of specific GR-target genes could drive the altered predicted activity of GR. Chromatin immunoprecipitation (ChIP) sequencing could be used to assess the sites of GR interactions with DNA to determine if CAS alters GR-DNA interactions differently in males and females and whether this activity at specific target genes corresponds with the transcriptional patterns of CAS shown in the current RNA sequencing results (Chapter 4).

Because the IPA Upstream Analysis is a predictive analysis based on the transcriptional effects observed in an RNA sequencing dataset, it will be important to confirm the predicted increased activity of the GR in CAS females. A luciferase reporter assay and ChIP sequencing could be used to confirm enhanced nuclear activity of the GR and GR-DNA interactions to further test the hypothesis that adult females exposed to CAS exhibit a more transcriptionally active GR as suggested by the IPA Upstream Analysis results. A luciferase reporter assay has been useful in assessing the impact of chronic and acute stress on hippocampal activity of the GR (Lee et al., 2016) and could be used to assess the effect of CAS on nuclear GR activity in adult

male and female rats. However, ChIP sequencing would provide more detailed information about the sites of GR-DNA interactions.

Whether the predicted increased activity of the GR can be attributed to increased DNA interactions with the GR or due to reduced co-repression by other transcription factors is an interesting question that should be addressed in the future. Interactions with transcription factors known to be inhibited by and to inhibit the GR, such as NFκB could be investigated with proximity ligation assays. Assessment of the transcriptome at multiple time points could also provide information about the time-dependent impact of CAS on the transcriptome. We chose to examine the 30-minute time point because previous reports in adolescent animals observed an impact of CAS in target gene transcription 30-minutes following stressor exposure (Bourke et al., 2013), and to investigate the time point at which reduced GR protein localization was observed (Chapter 2). But interestingly, GR-mediated transcription varies with time for hours after stimulation in a gene-specific manner (John et al., 2009). Assessment of the impact of CAS and sex on the time course of GR-mediated transcription will provide essential details about how prior exposure to CAS could alter the time course of GR-mediated gene transcription following activation.

In addition to the effects of CAS on the GR, Upstream Analysis also predicted reduced activity of the ESR1 (ERα) at baseline in females, an attractive target for future study that will be discussed later in this chapter.

Potential mechanisms contributing to altered GR-FKBP5 interactions

One mechanism through which CAS could modify the interactions of GR and FKBP5 is through increased availability of FKBP5 driven by increased basal expression of *Fkbp5* mRNA,

as observed in Chapter 3. Altered epigenetic regulation of the *Fkbp5* gene could promote differences in gene expression, and specifically, reduced *Fkbp5* methylation could promote increased *Fkbp5* transcription. Chronic treatment with corticosterone reduces DNA methylation in specific regions of the *Fkbp5* gene (Lee et al., 2010). Furthermore, a polymorphism of the human *FKBP5* gene interacts with childhood-trauma to exhibit reduced DNA methylation of the *FKBP5* gene, and increased methylation of this region is associated with reduced dexamethasone-stimulated activity at the *FKBP5* gene in HeLa cells (Klengel et al., 2013). Regulation of methylation of the *Fkbp5* gene in our rat model could be susceptible to the disruptive effects of CAS. Reduced methylation at the *Fkbp5* gene could promote increased transcription which could result in increased FKBP5 protein availability and interactions of GR and FKBP5 in adult female rats exposed to CAS and acute stress (Figure 5.1). Increased transcription of *Fkbp5* may be an adaptive protective mechanism following repeated exposure to stressors in adolescence to prevent further excessive GR signaling in the hippocampus. However, this adaptation is likely not without consequence, and altered FKBP5 interactions with GR may disrupt GR's transcriptional activity.

We assessed interactions of GR and FKBP5 *in situ* with a proximity ligation assay in the hippocampus but did not distinguish among different cell types present in the analysis. In the future, the contributions of different cell types in the hippocampus to the measured GR-FKBP5 interactions should be assessed. Cell-type specific markers could be used to label neurons, astrocytes, or microglia, all of which express GR and FKBP5 (Carter et al., 2012; Wohleb et al., 2011) to assess whether specific cell types exhibit altered interactions between the two proteins. Altered interactions in glial cells could promote future study of the stress-induced role of GR and FKBP5 interactions in non-neuronal cell types. Images acquired in the current studies were

centered on the pyramidal or granule cell layer, so it is likely that the majority of the currently assessed effects are neuronal. However, without counterstaining for specific cell types it is not possible to completely rule out contribution from other cell types. Microglia do express GR and FKBP5, and previous reports have found reduced *Fkbp5* in microglia following social defeat exposures, suggesting that regulation of the GR and FKBP5 could be vulnerable to changes in regulation following exposure to our stress paradigm (Wohleb et al., 2011). However, to assess the effects of CAS on interactions between GR and FKBP5 in non-neuronal cell types, the imaging location within hippocampal regions should be adjusted to acquire a region less densely population by neurons.

The GR chaperone machinery is a complex system, and there are other mechanisms that may impact interaction of GR with FKBP5. Post translational modifications of FKBP5 alter its interactions with Heat Shock Protein 90 (Hsp90), FKBP5's binding partner in the GR cytosolic complex (Antunica-Noguerol et al., 2016). Altered ability to interact with HSP90 could impact GR activity, and the role of CAS in modifying post-translational modification of FKBP5 protein should be assessed in the future. Additionally, an altered transcriptional landscape (Chapter 4) could alter expression of other chaperones and co-chaperones of the GR. However, targeted PCR experiments assessing expression of the co-chaperones *Fkbp4* and *Ppid* found no difference in gene expression of these co-chaperones, suggesting that these specific co-chaperones do not drive altered localization of the GR. However, there could be differences in protein expression or activity of these co-chaperones that are not reflected by their gene expression, and protein expression of co-chaperones could be investigated further. Chapter 3 experiments also showed subtle reductions in *Bag1* gene expression in CAS females. Reduced Bag-1 protein would be expected to increase translocation activity of the GR which would not be consistent with the

nuclear localization data in Chapter 2. However, the effects of CAS on *Bag1* were subtle, though gene expression may not reflect protein expression or activity, and to better understand the potential role of Bag-1 in CAS females, it would first be necessary to assess whether these transcriptional effects impact expression of Bag-1 at the protein level.

FKBP5 interactions with GR may not be the only contributor to impaired GR nuclear localization (Chapter 2). The GR can also be phosphorylated at serine residues which are able to influence GR protein localization (Itoh et al., 2002). Interestingly, alterations in GR phosphorylation can differentially alter the expression of target genes, increasing the complexity of the consequences of GR post translational modifications (Blind and Garabedian, 2008; Chen et al., 2008; Galliher-Beckley et al., 2008). Assessment of the GR phosphorylation state could further illuminate the role of CAS on GR regulation as specific phosphorylation sites have been associated with differences in nuclear localization and transcriptional activity (Blind and Garabedian, 2008; Itoh et al., 2002).

Increases in transcriptional activity of the GR following acute stress (Chapter 4), despite impaired nuclear translocation following acute stress and no difference in localization at baseline, could in part explain the increased transcription of *Fkbp5* at baseline since *Fkbp5* is a target gene of the GR. Furthermore, with time following exposure to an acute stressor, there was no difference in *Fkbp5* transcripts in CAS compared to NS females even though GR nuclear localization was reduced in females exposed to CAS. This suggests that the reduced nuclear content of GR in CAS females may indeed be more transcriptionally active to exhibit no difference in *Fkbp5* transcription despite reduced nuclear content, consistent with the RNA sequencing results (Chapter 4). Whether this increased activity of GR is due to enhanced activity of the GR through increased interaction activity with DNA or association with transcription

coregulators or a priming of the *Fkbp5* gene via reduced methylation should be assessed to better understand consequences of chronic stress exposure on regulation of the *Fkbp5* gene. Increased nuclear activity of the GR is also consistent with the absence of impact of CAS on circulating corticosterone. With reduced nuclear localization and increased interactions between GR and FKBP5, we would predict to see an impaired negative feedback on circulating corticosterone, similar to the pattern observed in adolescent females exposed to CAS (Bourke et al., 2013). However, that there is not a prolonged corticosterone response and impaired negative feedback on the HPA axis is consistent with RNA sequencing results that suggest enhanced transcriptional activity of the GR. Alternatively, the absence of a prolonged corticosterone response could be in part due to enhanced activity in other sites of HPA-axis negative feedback: the hypothalamus and pituitary.

Potential for a role of ESR1

The GR may not be the only transcription factor that mediates the sex-specific effects of CAS. In Chapter 4, ESR1 was identified as a predicted Upstream Regulator of the observed transcriptional changes. Specifically, adult female rats with a history of CAS exposure exhibited predicted reduced basal activity of ESR1. Estrogen has been found to be protective in animal studies (Yuen et al., 2016), and a recent study by Lorsch et al. observed that following a chronic stress paradigm, resilient animals exhibited predicted increased activity of ESR1 in the nucleus accumbens while susceptible rats (compared to resilient rats) exhibited reduced activity of ESR1 (Lorsch et al., 2018). The reduced ESR1 activity predicted in CAS females in the current studies may be reflective of a pro-susceptible phenotype mediated by reduced influence of ESR1 signaling. Lorsch et al. further showed that overexpression of ESR1 protein was sufficient to shift the transcriptome to exhibit increased similarity to the earlier observed pro-resilient

transcriptional profile by using rank-rank hypergeometric overlap (RRHO) analysis to assess similarity of transcriptional profiles (Marrocco et al., 2017; Plaisier et al., 2010).

The current studies identified reduced influence of ESR1 (ERα) as a potential driver of the CAS-induced transcriptional landscape. To assess whether the global impact of CAS is driven predominantly by reduced ESR1, similar to the experiments performed by Lorsch et al., overexpression of ESR1 in the hippocampus in adult female rats previously exposed to CAS could be used to assess whether ESR1 overexpression normalizes the transcriptional profile to become more similar to the transcriptome of NS female rats. Similarity of transcriptional profiles could be assessed by using rank-rank hypergeometric overlap analysis (Plaisier et al., 2010). These experiments could be used to assess whether reduced ESR1 is indeed a unique driver of the global transcriptional profile of CAS.

Estrogen receptor and FKBP5

Though interactions of FKBP5 have been more extensively studied with the GR, studies have shown that the estrogen receptor (ER) also interacts with FKBP5 (Dhamad et al., 2016; Nair et al., 1996). Estrogen replacement potentiates the corticosterone response after stressor exposure in ovariectomized rats (Burgess and Handa, 1992), and estradiol facilitates transcription of *Fkbp5* in the presence of corticosterone in hippocampal cultures (Malviya et al., 2013). ERα protein has been found to associate with FKBP5 (Dhamad et al., 2016; Nair et al., 1996), and FKBP5 overexpression enhanced ERα activity in a luciferase assay (Shrestha et al., 2015). Interestingly, FKBP5 also enhances activity of the androgen receptor and reduces activity of the progesterone receptor (Hubler et al., 2003; Ni et al., 2010; Periyasamy et al., 2010). It is possible that increased availability of FKBP5 protein could have functional effects on ER signaling. However,

given FKBP5's role of facilitating ER activity, is unlikely that increased FKBP5 interactions with ER would contribute to the predicted reduced activity of ESR1 observed at baseline in females exposed to CAS.

Sex and CAS effects

The CAS effects on adult localization and regulation of the GR are more pronounced in females than males in these studies. Female-specific effects on the GR and its regulator FKBP5 in adulthood in the current studies are consistent with the GR effects observed in adolescent females with a history of CAS (Bourke et al., 2013) and with the enhancement of depressive-like behavior observed in adolescent and adult female rats previously (Bourke and Neigh, 2011a). Sex-specific elevation of *Fkbp5* transcripts could be impacted by a sex-specific reduction of DNA methylation. DNA methylation of the *Fkbp5* and other relevant genes could occur in a sex-specific manner as others have reported sex-specific methylation of the *NR3C1* and other genes (Kosten et al., 2014; Uddin et al., 2013). Furthermore, DNMT3a, an enzyme that participates in maintenance of DNA methylation has been found to be involved in sex-specific transcriptional effects of chronic stress and associated behaviors (Hodes et al., 2015).

Following acute stressor exposure, adolescent female rats exhibit an elevated corticosterone response relative to males (Bourke et al., 2013), and it is therefore possible that the differences in exposure to corticosterone during the CAS paradigm in males and females promotes differential methylation at the *Fkbp5* gene or promotes adaptations in GR regulation as observed in adult females in these experiments. Furthermore, we (Chapter 4) and others (Marrocco et al., 2017) have observed sex differences in the acute transcriptional response in the hippocampus to an acute stressor exposure. The sex-specific transcriptional profile induced

following acute stress could differentially predispose females to the adaptations observed in these experiments. Because the GR can be regulated at many levels, an altered transcriptional profile at baseline or following stressor exposure could promote differences in expression of GR regulators including that promote sex-specific alterations in GR function.

Furthermore, in Chapter 4, basal transcriptional activity of ESR1 was predicted to be reduced in females, and whether altered ESR1 activity drives the sex-specific alterations in GR regulation through altered expression of co-chaperones or post-translational modifications or alternatively, if altered ESR1 activity is a consequence of the altered GR, should be explored in future studies.

FKBP5 targeted therapeutics: Implications for neuropsychiatric disease

Activity of the GR has been implicated in neuropsychiatric diseases and therapeutics (Anacker et al., 2011; Raison and Miller, 2003), and the ideal balance and fine-tuning between insufficient and excessive glucocorticoid signaling is complex (Anacker et al., 2011). Co-chaperones and specifically FKBP5 are potent regulators of GR activity. In recent years, the role of FKBP5 has become a focus in many preclinical and clinical studies of stress-related disorders (Binder, 2009; Binder et al., 2004; Fries et al., 2017; Klengel et al., 2013). Specifically, functional polymorphisms of the *FKBP5* gene that result in increased expression of FKBP5 protein are associated with increased number of depressive episodes in humans (Binder et al., 2004).

FKBP5 participates in an "ultra-short negative feedback loop" on GR activity (Binder, 2009). A female-specific impact of CAS on increased expression and interaction of FKBP5 with the GR in the hippocampus was observed in these studies. These studies suggest a functional role

of FKBP5 in regulation of the GR as a consequence of CAS and acute stress exposure. The GR localization (Chapter 2) and GR-FKBP5 interaction (Chapter 3) data suggest reduced sensitivity of the GR which initially appears to be contradictory with the increased predicted transcriptional activity of the GR in CAS females with RNA sequencing (Chapter 4). The GR is regulated at many levels, and it is possible that though CAS females exhibit altered localization and co-chaperone regulation that a more active nuclear GR compensates for these processes. There was not a significant impact of CAS on the corticosterone response, which would be expected if CAS indeed induced impaired FKBP5-mediated negative-feedback on the GR. As others have discussed, the balance of GR activity is complex (Anacker et al., 2011), and fine-tuning of interactions of GR with its co-chaperone FKBP5 could fine-tune its transcriptional activity and the profile of genes it regulates.

Recently, a specific-inhibitor of FKBP5 was created that increases corticosterone suppression in the dexamethasone suppression test and reduces floating and increases struggling behavior in the forced swim test (Gaali et al., 2015), indicating a behavioral impact of reduced FKBP5 activity in animals even without a history of stress exposure. The FKBP5 inhibitor also increases neurite outgrowth (Gaali et al., 2015), reduces anxiety-like behavior (Hartmann et al., 2015), and reduces pain severity (Maiaru et al., 2016). Whether the FKBP5-specific inhibitor disrupts GR-FKBP5 interactions in the hippocampus of CAS female rats and whether GR nuclear localization and transcription could be normalized with hippocampal FKBP5 inhibition should be assessed. It would be particularly interesting to assess whether pharmacological inhibition of FKBP5 alone could reduce GR-FKBP5 interactions and shift the hippocampal transcriptional landscape to produce a transcriptional profile similar to that of NS females without developmental exposure to stressors or whether overexpression of FKPB5 produces a

similar transcriptional landscape to females exposed to CAS. Rank-rank hypergeometric overlap could be used to assess similarity of transcriptional profiles of different comparisons (Plaisier et al., 2010). It would furthermore be necessary to assess the behavioral impact of FKBP5 inhibition in adult rats exposed to CAS in the sucrose preference test and forced swim test as earlier studies found that adult female rats exposed to CAS exhibited reduced sucrose preference and decreased struggling and reduced latency to float in the forced swim test (Bourke and Neigh, 2011a), and others have observed reduced depressive-like behaviors with FKBP5 inhibition (Gaali et al., 2015). Furthermore, viral vector-mediated overexpression of *Fkbp5* in the hippocampus in non-stressed rats could be used to determine if hippocampal overexpression of *Fkbp5* in non-stressed rats is sufficient to reduce GR nuclear localization following acute stressor exposure and increase GR-FKBP5 interactions as we observed in female rats exposed to CAS. These studies would clarify whether increased basal *Fkbp5* expression could be the driver for increased GR-FKBP5 interactions and impaired nuclear localization. If this is indeed the case, why these effects of CAS on *Fkbp5* expression are sex-specific could be further investigated, and these results could promote future study into mechanisms that could contribute to increased basal transcription of *Fkbp5* such as reduced DNA methylation. The previously observed behaviors in females exposed to CAS, the forced swim and sucrose preference tests (Bourke and Neigh, 2011a), should also be assessed in non-stressed rats with hippocampal overexpression of FKBP5 to assess whether the FKBP5 expression alone is able to replicate the CAS-induced behavioral patterns.

Dorsal and ventral hippocampus

The role of GR-FKBP5 interactions in the dorsal and ventral hippocampus should be further delineated in the future. There may be a disconnect between increased GR-FKBP5

interactions observed specifically in the dorsal CA1 and the predicted increased GR activity in the whole hippocampus assessed with whole hippocampal RNA sequencing. Increased FKBP5 inhibition of the GR may take place primarily in the CA1 region which could then be normalized with pharmacological FKBP5 inhibition. Region-specific RNA sequencing could potentially reveal differences in predicted GR activity in the dorsal CA1 compared to other hippocampal regions. Though the dorsal hippocampus is thought to primarily participate in cognitive processes, dorsal hippocampal treatment that increases neurogenesis reduces depressive-like behaviors (Dow et al., 2015), indicating a relevance of the dorsal hippocampus to affective disorders. Future studies should tease apart the relative contributions of GR activity in the dorsal and ventral hippocampus on affective and cognitive behaviors. A recent study investigated dendritic morphology at multiple time points through adolescence and found dorsal and ventral hippocampal-specific alterations in dendritic morphology through time in singly housed rats, suggesting that these differences in timing could be differentially disrupted by stress exposure during adolescence. Furthermore, pair-housing relative to single housing had opposite effects on branching of dendrites in the dorsal and ventral CA1, indicating the single housing alters dendritic morphology differently in dorsal and ventral regions (Chen et al., 2018), in line with the dorsal-specific effects on GR regulation observed in the current studies, suggesting that the dorsal and ventral hippocampus are not impacted by stressors in the same manner. The dorsal CA1, relative to the ventral hippocampus, may be particularly susceptible to the chronic exposure to stressors during adolescence in altering regulation of the GR.

Furthermore, the relative impact of increased FKBP5 expression in the dorsal and ventral hippocampus could be further studied with viral vector-medicated FKBP5 overexpression with site-specific infusion in dorsal and ventral hippocampal regions to assess whether there are

behavioral effects in the forced swim and sucrose preference tests specific to FKBP5 overexpression in the ventral or dorsal hippocampus.

Potential effects of CAS in additional brain regions

In these studies we only assessed regulation of the GR in the hippocampus, however, other brain regions could also be impacted by CAS exposure. A chronic mild stress paradigm during adolescence has been found to elevate FKBP5 protein in the basolateral amygdala (Xu et al., 2017), consistent with the current patterns in *Fkbp5* expression, suggesting that similar mechanisms of *Fkbp5* regulation in the hippocampus could also take place in the basolateral amygdala. Others have shown that overexpression of FKBP5 in the basolateral amygdala increased anxiety-like behavior (Hartmann et al., 2015), illustrating behavioral consequences of increased FKBP5 expression in this region. These studies suggest that chronic stress could induce similar patterns to FKBP5 regulation in the basolateral amygdala though futures studies would need to specifically assess the impact of CAS on *Fkbp5* expression and GR-FKBP5 interactions in the basolateral amygdala to confirm this. Furthermore, a chronic mild stress paradigm in adulthood has been found to increase *Fkbp5* gene expression in the hippocampus and prefrontal cortex but not the hypothalamus (Guidotti et al., 2013), suggesting that the hippocampus and prefrontal cortex may be vulnerable to the effects of chronic stress on alterations in *Fkbp5* expression and that patterns of *Fkbp5* regulation do not similarly occur in all brain regions involved in the stress response. An absence of an impact of CAS on GR and FKBP5 regulation in the hypothalamus could in part explain why there is no impact of CAS on the resolution of the corticosterone response following acute stressor exposure (Figure 2.4). If both the hippocampus and hypothalamus experienced elevations of FKBP5, disrupted feedback on the plasma corticosterone response would be expected. In the current studies, we did not

assess the impact of dexamethasone suppression on plasma corticosterone, and this test could better address the role of the GR on HPA axis negative feedback. It is likely that we would not observe impaired suppression, in line with the absence of a prolonged corticosterone response following acute stressor exposure. However, alternatively, if CAS females do exhibit impaired dexamethasone suppression, this finding could promote investigation of the role of the GR in other sites of HPA axis feedback: the hypothalamus and pituitary.

Consequences of altered GR: Implications for cognitive behaviors

While the ventral hippocampus is commonly thought to participate in negative feedback on the HPA axis, the dorsal hippocampus has primarily been associated with its role in memory-related activity (Jankord and Herman, 2008; Moser and Moser, 1998). It is therefore interesting that the most pronounced impact of acute stress and CAS on GR-FKBP5 interactions were observed in the dorsal CA1, and these effects of CAS could have relevance to cognitive behavior in adult female rats. In humans, depression has been associated with cognitive decline at a subsequent follow up assessment (Paterniti et al., 2002), suggesting similar processes may underlie affective and cognitive decline.

Other labs have observed an impact on cognitive behaviors and related processes in various stress models. Exposure to stressors can disrupt spatial memory performance (Warner et al., 2013), reduce hippocampal cell proliferation and survival (Barha et al., 2011), and reduce the number of synapses in the dorsal CA1 (Maras et al., 2014). Furthermore, exposure to glucocorticoids specifically may negatively impact hippocampal function; corticosterone treatment has been found to suppress long term potentiation (LTP) in the hippocampus (CA3-CA1 projections) (Ooishi et al., 2012). Due to the important role of the dorsal hippocampus in

cognitive behaviors and the disruptive impact of excessive glucocorticoid exposure, the effects of CAS on GR and FKBP5 regulation may be an adaptive response to the chronic adolescent stressors to enhance negative feedback on the GR to protect from the disruptive effects of excessive glucocorticoid signaling. However, these alterations in GR-FKBP5 interactions in the hippocampus may not be without consequence. In a despair-associated memory experimental design using the forced swim test, GR inhibition reduced LTP in the CA1 (Jing et al., 2015), indicating a functional impact of disrupted GR signaling. It is therefore possible that disruption of GR activity through increased interactions with FKBP5 following CAS and acute stress could disrupt CA1 LTP. Furthermore, whether the altered basal transcriptional state following CAS alters cognitive processes should be further explored because transcriptional effects of chronic stress have been implicated in altered Morris water maze performance (Jung et al., 2017). Adaptations to protect from excessive glucocorticoid exposure could ultimately result in impaired cognitive processes because of dysregulated GR activity through altered co-chaperone composition, shifting towards increased interactions with FKBP5. A thorough assessment of the impact of CAS on spatial memory related tasks such as the Morris water maze and the object in place recognition task (Barker and Warburton, 2015) could provide important details about the cognitive impact of CAS. If CAS causes sex-specific behavioral alterations in spatial memory, the FKBP5 inhibitor (Gaali et al., 2015) could be used to assess whether disruption of GR-FKBP5 interactions alters spatial memory tasks. Furthermore, estrogen signaling may also play an important role in cognitive processes as estrogen has been found to reverse cognitive impairments following chronic stress exposure in the prefrontal cortex (Wei et al., 2014), suggesting that reduced basal ESR1 activity predicted from the RNA sequencing analysis may also have cognitive consequences.

A thorough assessment of the impact of CAS and GR transcription on neurotransmitter systems is also necessary. Glutamate receptors may be impacted by CAS in the hippocampus. In the RNA sequencing experiments, *Grm5,* the gene encoding mGluR5, was reduced in CAS compared to NS females at baseline (Table 4.4). Others have reported a functional impact of mGluR5 in the hippocampal CA1; specifically, positive allosteric modulators of mGluR5 increased LTP and LTD in the CA1 (Ayala et al., 2009). Furthermore, chronic corticosterone has previously been observed to reduce mGluR5 protein (Iyo et al., 2010), indicating a specific role of exposure to elevated glucocorticoids on receptor expression. Whether expression of glutamate receptors in the CA1 is a potential driver of cognitive impact of CAS and whether GR-FKBP5 interactions play a role in the transcriptional regulation of this process could be assessed in future studies. Furthermore, at baseline CAS also reduces expression of *Grin2a, Grin2b,* and *Grm3* in females (Table 4.4). Interestingly, *Grin2a* was increased following acute stress in the CA3 in female mice (Marrocco et al., 2017), suggesting that the opposite direction observed in our chronic stress paradigm is a down regulatory compensatory mechanism for acute stress induced increases. In order to determine if there is a functional role of reduced gene expression of glutamate receptors and receptor subunits, examination of the impact of CAS on glutamatergic signaling and LTP in the hippocampus and the extent to which these effects are a consequence of increased GR-FKBP5 interactions should be performed. Furthermore, whether inhibition of FKBP5 impacts these processes should be assessed because GR regulates the expression of multiple glutamate receptor or receptor subunit genes.

Finally, because estrogen has been found to be protective in reversing stress-induced alterations in glutamatergic transmission associated with cognitive deficits (Wei et al., 2014), the role of the estrogen receptor in CAS effects on cognitive behaviors should also be assessed.

Whether hippocampal overexpression of ESR1 or if treatment with estradiol reverses CAS-induced cognitive impairments should be assessed to determine the role of ESR1 signaling in the dorsal hippocampus.

Conclusions

Chronic exposure to stressors in adolescence alters regulation and activity of the GR into adulthood in a sex-specific manner. In Chapter 2 we showed that CAS altered GR nuclear localization in a lasting and sex-specific manner, such that females, but not males, exposed to CAS exhibited reduced nuclear localization following exposure to an acute novel stressor. These findings highlight the persistent and sex-specific nature of the effects of CAS exposure that lasts months removed from exposure to stressors. Furthermore, these studies show that the GR, a transcription factor with wide-spread actions is impacted in the hippocampus by CAS. In order to understand a potential regulatory mechanism through which GR activity could be modulated, we assessed hippocampal gene expression of *Fkbp5* (Chapter 3) and found elevated levels of *Fkbp5* in females at baseline. Furthermore, in Chapter 3 we showed a functional impact of FKBP5 such that history of CAS and exposure to acute stress interacted in females to increase interactions between the GR and FKBP5, consistent with FKBP5-mediated impairment of GR nuclear translocation. Finally, in Chapter 4, due to the role of the GR as a transcription factor, we assessed the global transcriptional impact of CAS on the hippocampal transcriptome. CAS altered the hippocampal transcriptome in a sex-specific manner into adulthood, and the GR was predicted to be more transcriptionally active in CAS relative to NS adult female rats. Collectively, these studies show that the effects of CAS persist into adulthood in a sex-specific manner, and specifically that adult regulation and activity of the hippocampal GR is altered in a sex-specific manner. These studies advance our understanding of how adolescent stressor

exposure impacts the GR and its co-chaperone FKBP5 with implications for interventions in affective and cognitive consequences of chronic stress exposure.

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Appendix

Appendix I

Locomotor sensitization to cocaine in adolescent and adult female Wistar rats

Adapted from:

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Locomotor sensitization to cocaine in adolescent and adult female Wistar rats
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Introduction

Chronic stress is an important risk factor in the development of addiction (Burke and Miczek, 2014). Due to the extensive neuronal maturation that occurs during the adolescent period, exposure to stress during adolescence may result in more severe drug abuse outcomes (Burke and Miczek, 2014; Lepsch et al., 2005; Yajie et al., 2005). Rodents have been useful in studying the impact of adolescent stress exposure on the behavioral response to drugs of abuse, but the

majority of these studies have focused on males (Frantz et al., 2007; Lepsch et al., 2005). Given the sex differences observed in the behavioral and molecular response to chronic adolescent stress (CAS) exposure, it is essential to include females in studies examining the interaction between adolescent stress and addictive drugs (Bourke and Neigh, 2011; Bourke et al., 2013).

Locomotor sensitization is considered a behavioral representation of drug-induced plasticity, and it is well established that cross-sensitization between stress and drug responses occurs (Burke and Miczek, 2014). While previous studies have examined locomotor sensitization to cocaine in adult females, there are far fewer studies that have assessed adolescent female rats, and the majority of these employed the Sprague Dawley or Long Evans strain (Franke et al., 2007; King et al., 2009; Kozanian et al., 2012; Laviola et al., 1995; Mohd-Yusof et al., 2014; Serafine et al., 2015; Serafine et al., 2016; Wiley et al., 2011). By contrast, the Wistar strain has been essential in experiments evaluating the impact of CAS exposure in adolescents (Bourke and Neigh, 2011), but cocaine sensitization in female Wistar rats has only been evaluated in adults (Dow-Edwards et al., 1989; Souza et al., 2014; van Haaren and Meyer, 1991). Because a number of studies report variable sensitization responses in adolescents and adults, assessing the behavioral response to cocaine in adolescent female Wistar rats is important for integrating the adolescent stress, sensitization, and sex differences literature (King et al., 2009; Laviola et al., 1995).

Methods

Timed-pregnant Wistar rats were purchased from Charles River (Raleigh, NC) and housed on a 12:12 light:dark cycle with food and water available *ad libitum*. All experiments were performed in an AAALAC approved facility, and experiments were approved by the

Institutional Animal Care and Use Committee of Emory University and were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Litters from timed-pregnant females were culled to 5 female and 3 male (or 6 female and 2 male) pups and weaned on postnatal day (PND) 21 into same-sex pairs. Only females were used for the reported experiments. On PND 35, consistent with the established paradigm (Bourke and Neigh, 2011; Bourke et al., 2013), rats in the CAS group were individually housed, while non-stress controls (NS) remained pair-housed. All rats were weighed weekly throughout the study. On PND 38-49, rats in the CAS group were exposed to 12 days of a mixed-modality CAS paradigm as previously described (Bourke and Neigh, 2011; Bourke et al., 2013). Briefly, CAS consisted of a pseudorandom alternating schedule of 6 days of social defeat and 6 days of restraint. For each social defeat session, adolescent female Wistar rats were individually placed in the home cage of an ovariectomized female Long Evans rat (Charles River) for 2 min, separated by a clear plastic barrier that allowed both visual and olfactory cues. The barrier was then removed, and the rats were allowed to physically interact for 5 min. The barrier was then replaced for an additional 25 min before the Wistar rat was returned to its home cage. Each female Long Evans rat was housed with a male retired breeder Long Evans rat that was removed prior to each session. This housing arrangement is consistent with previous experiments (Bourke and Neigh, 2011; Bourke et al., 2013). For the restraint paradigm, each rat was placed in a clear plastic rodent restraint for 60 min.

Separate groups of rats were used for sensitization testing in adolescence and adulthood. On the day following the final day of the CAS paradigm or during adulthood (PND 92) (Figure I.1), female rats were habituated to the locomotor chamber (8 x 17 inches) for 2 h. Consecutive beam breaks (ambulations) in automated locomotor chambers (8 y-axis beams and 4 x-axis

beams separated by 1 and 15/16", San Diego Instruments, La Jolla, CA) were recorded in 5-min bins to assess the locomotor response to novelty. Rats were habituated to locomotor chambers on the second day for 2 h. On the third day and recurring every day for 5 days, rats were habituated to the locomotor chamber for 30-60 min prior to administration of saline or cocaine (15 mg/kg, i.p.), and ambulations were assessed for the following 2 h. In adult rats, an additional cocaine challenge was assessed after 7 days of abstinence (PND 105). One adolescent rat included in novelty testing did not complete the sensitization paradigm and was excluded from sensitization analysis. Estrous cycle was tracked in adult females from PND 85-105. Adolescents were not tracked because they would not exhibit regular estrous cycles at the age of testing (Gabriel et al., 1992). Total ambulations from x and y beams from 5-min bins were summed over the 2-h testing session following cocaine or saline administration. Two-way ANOVA was used to assess statistical significance ($\alpha=0.05$). Adjusted p-values from Dunnett's post-hoc multiple comparison tests were used to assess post-hoc differences between days for NS and CAS groups, and a Sidak's test was used to assess differences between CAS and NS groups within bins. GraphPad Prism Version 7.02 was used for all statistical analyses.

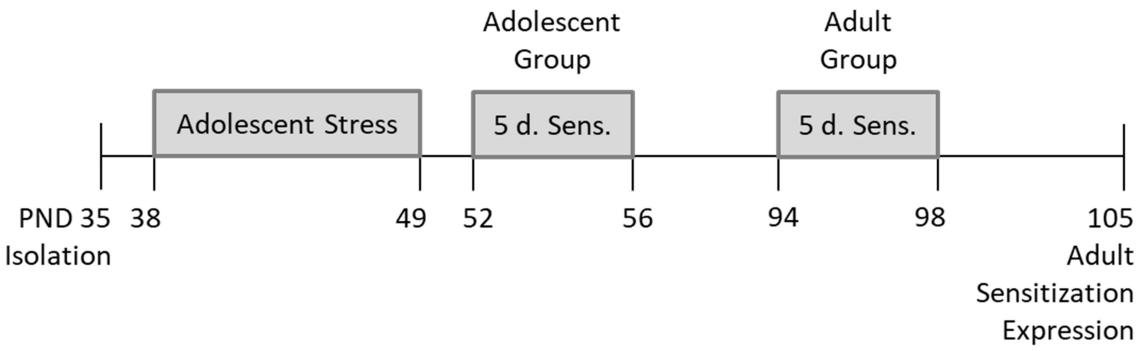

Figure I.1. Timeline for CAS and testing. On PND 35, female CAS rats were isolation-housed, exposed to social defeat and restraint from PND 38-49, and tested for cocaine sensitization from PND 52 to 56 (adolescents) or PND 94 to 98 (adults). Expression of sensitization following abstinence was assessed on PND 105 in adults.

Results

We first assessed the impact of CAS on the locomotor response to a novel environment in adolescent females (Figure I.2A) and adult females (Figure I.2B). A history of CAS and bin number significantly interacted to impact locomotor activity in both adolescents ($F_{(23,276)} = 2.37$, $p < 0.001$) and adults ($F_{(23,138)} = 1.87$, $p=0.014$), although there was only a trend towards a main effect of CAS history alone (adolescents: $F_{(1,12)} = 4.68$, $p =0.052$; adults: $F_{(1,6)} = 2.30$, $p = 0.18$). Post-hoc Sidak's multiple comparisons test revealed that CAS reduced initial locomotor activity (i.e. the first 5-min bin) after being placed in the locomotor chamber in adolescents ($p<0.001$) and adults ($p=0.008$) compared NS controls. As expected, locomotor activity habituated over time in both groups (adolescents: $F_{(23,276)} = 61.2$, $p<0.001$; adults: $F_{(23,138)} = 21.91$, $p <0.001$).

We next assessed whether age of testing impacted locomotor sensitization to cocaine. Adolescents sensitized to cocaine across the 5 day regimen (main effect of treatment day, $F_{(4,20)} = 5.74$, $p=0.003$) (Figure I.3A), and Dunnett's post-hoc tests revealed that locomotor activity was significantly higher on day 5 compared to day 1 in both NS and CAS exposed rats (NS: $p=0.0094$, CAS: $p=0.0162$). To confirm that the observed sensitization was due to the pharmacological properties of cocaine rather than sensitization to repeated injections, we repeated the experiment but administered saline instead of cocaine. As expected, there was no effect of testing day on locomotor activity in saline-treated adolescent female rats ($F_{(4,16)} = 0.74$, $p > 0.05$). In contrast to adolescents, adults did not sensitize to cocaine; a significant main effect of test day was observed ($F_{(5,70)} = 2.50$, $p = 0.038$), but Dunnett's post-hoc testing did not reveal a significant effect on locomotor activity when comparing any two days, including day 4 or day 5 vs. day 1.

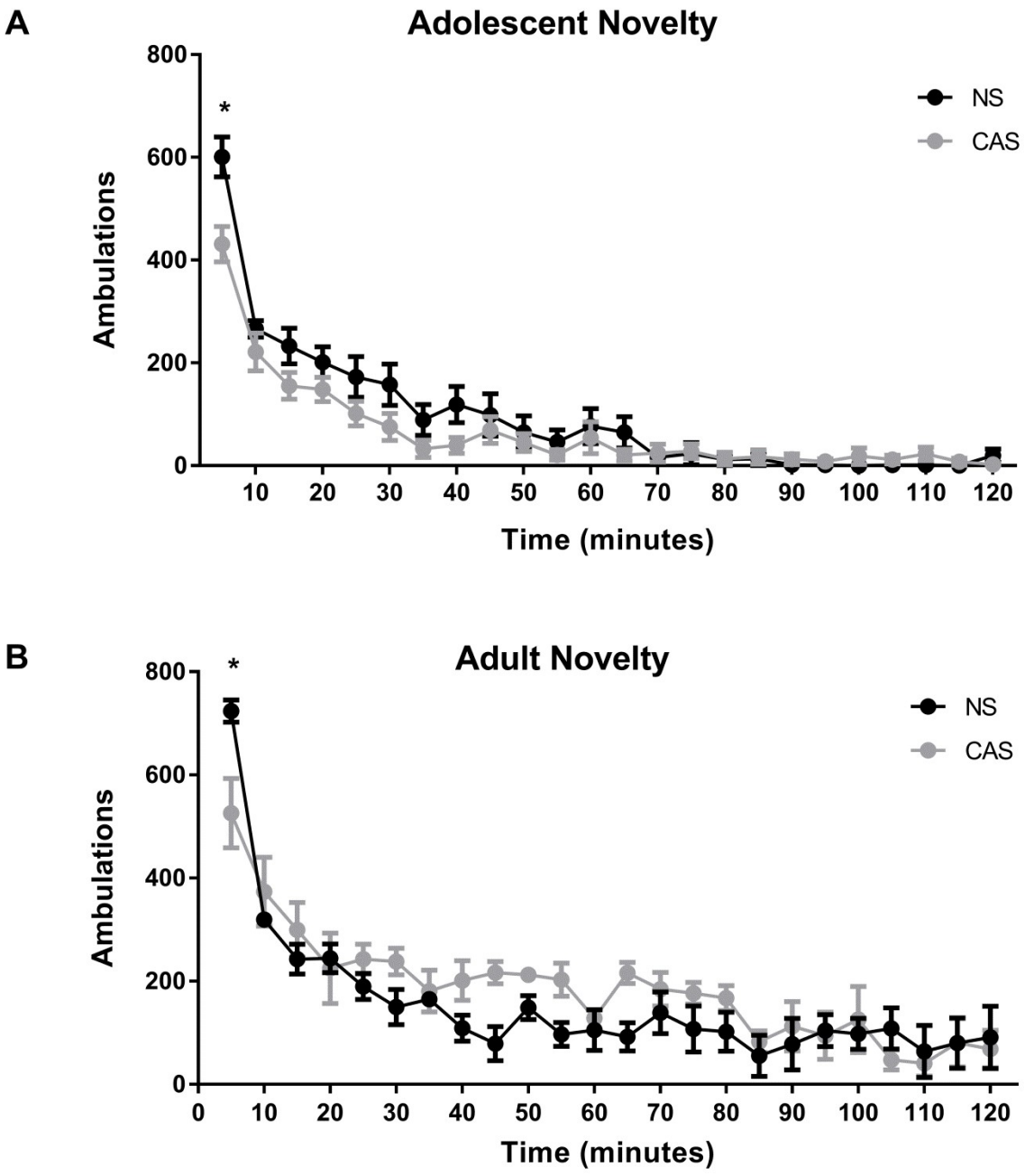

Figure I.2. CAS attenuates novelty-induced locomotion in adolescent and adult female Wistar rats. Locomotor activity following exposure to a novel environment was assessed in adolescent, n=6-8 (A) and adult, n=4 (B) female Wistar rats with a history of CAS or NS for 2 h. Locomotor activity was significantly impacted by an interaction between CAS history and bin in both adolescents (A) and adults (B). Data are presented as mean ± SEM. $\alpha = 0.05$, *denotes significant effect in Sidak's post-hoc test (CAS vs NS).

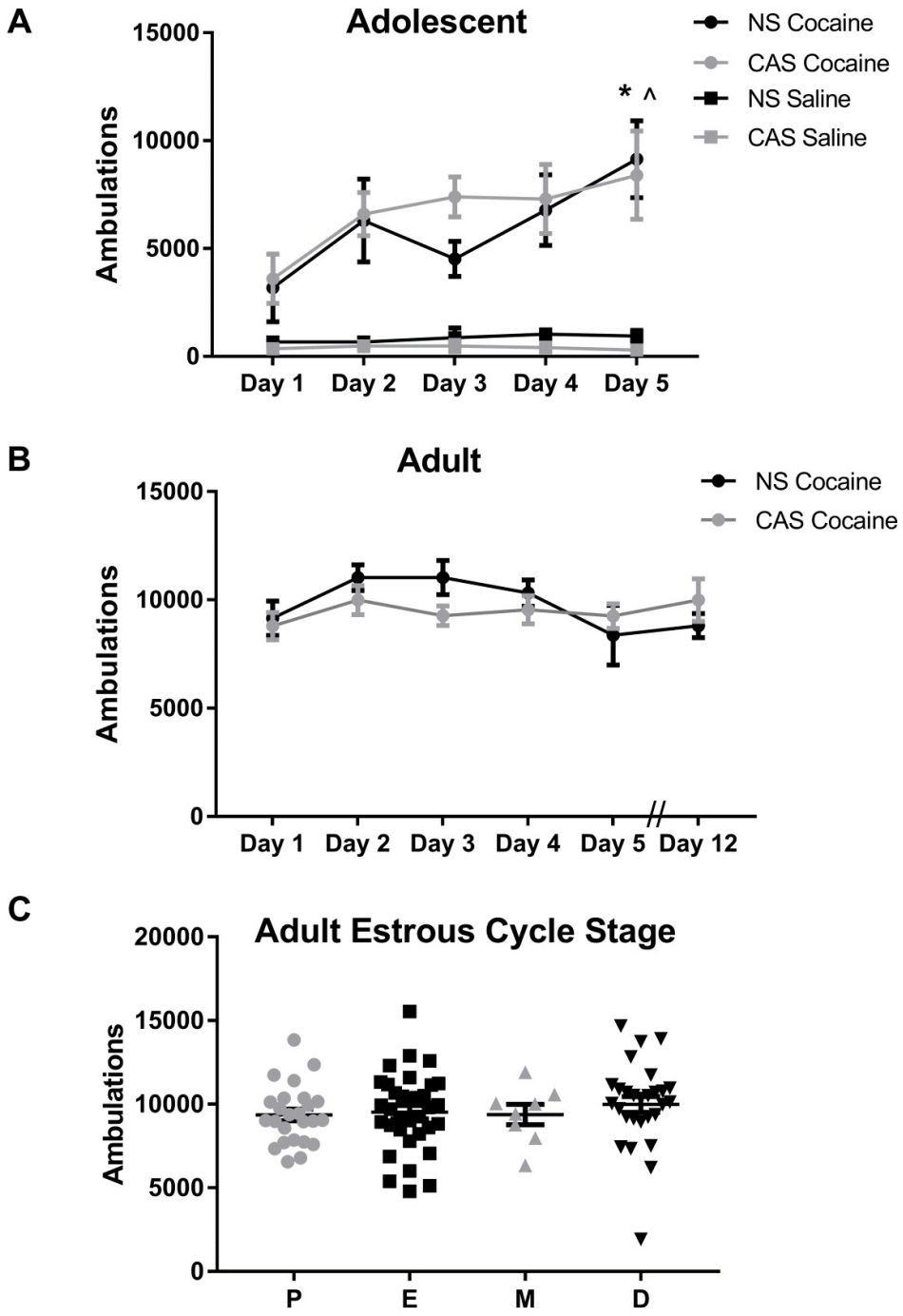

Figure I.3. Adolescent, but not adult female Wistar rats sensitize to cocaine, with no effect of CAS. Locomotor activity (summed ambulations) was assessed for 2 h following cocaine administration (15 mg/kg/d, i.p., for 5 days) in adolescent, n=3-4 (A) and adult, n=8 (B) female Wistar rats or saline (once/day for 5 days) in adolescent females, n=3 (A). Adolescent rats exhibited enhanced locomotor activity on Day 5 compared to Day 1. Adult rats did not exhibit sensitized locomotor activity (Days 4, 5, or 12 compared to Day 1). Data in (A) and (B) are presented as mean ± SEM. $\alpha = 0.05$, a significant effect in Dunnett's multiple comparisons post-

hoc test compared to Day 1 for the NS group is denoted with ^ and for the CAS CAS group with *. Estrous cycle was tracked in adult female Wistar rats (C), and locomotor activity (summed ambulations) was graphed by cycle stage (Proestrus (P), Estrus (E), Metestrus (M), Diestrus (D)) pooled across stress group and test day. Estrous cycle stage did not impact locomotor activity ($p>0.05$).

Because CAS exposure has been shown to impact behavior in female Wistar rats, we assessed whether a history of CAS exposure altered the locomotor response to cocaine in both adolescent and adult females. We found no effect of CAS exposure on sensitization in adolescents ($F_{(1,5)} = 0.19$, $p > 0.05$) (Figure I.3A) or adults ($F_{(1,14)} = 0.18$, $p > 0.05$) (Figure I.3B). We also confirmed that there was no effect of CAS history ($F_{(1,4)} = 2.55$, $p > 0.05$) on locomotor activity in saline-treated adolescent female rats (Figure I.3A). Finally, we found no effect of estrous cycle stage on locomotor activity ($F_{3,91}=0.42$, $p>0.05$, Figure I.3C) in adults pooled across stress group and test day.

Discussion

Here, we report that adolescent female Wistar rats sensitized to 15 mg/kg cocaine across 5 days of testing, but that adult female Wistars did not. While most sensitization studies have employed males, we focused on females given the sex differences observed in the behavioral and molecular response to CAS exposure (Bourke and Neigh, 2011; Bourke et al., 2013). In addition to sex, there are 3 important variables that must be considered in sensitization paradigms: age, strain, and dosing regimen (Table I.1).

Studies concerning whether locomotor activity following psychostimulant exposure is higher in adults or adolescents have yielded inconsistent results. Following acute cocaine exposure, some report higher locomotor activity in adults (King et al., 2009; McDougall et al., 2015) while others find similar activity levels across ages (Wiley et al., 2011). We observed higher locomotor activity in the adult group, consistent with the work by McDougall et al (McDougall et al., 2015). In chronic treatment paradigms, adolescent female rats may exhibit locomotor sensitization to cocaine more readily than adult females (King et al., 2009; Laviola et

al., 1995), although in other studies, adult females have been found to exhibit similar sensitization to adolescents (Wiley et al., 2011). It is unlikely that a ceiling effect prevented adult sensitization in the current studies because a moderate dose of cocaine was used (15 mg/kg) that is still on the ascending limb of the dose-response curve (Kalivas et al., 1988; Lau et al., 1991; Witkin and Goldberg, 1990). It is important to note that the adult rats used in the study by King et al. (King et al., 2009) had previous adolescent exposure to cocaine which is not the case in our current study. Although the mechanisms underlying these age differences are not clear, one possibility is that adolescence is a period of intense synaptic remodeling in the brain, and thus provides a particularly hospitable environment for cocaine-induced plasticity (Crews et al., 2007). Although the sample sizes we used in the adolescent sensitization experiment were on the low side compared with some other studies, the results were very consistent and robust, and statistical analysis confirmed that the study was adequately powered to detect significant sensitization in both the CAS and NS groups.

Rat strain also plays a critical role in determining the magnitude of sensitization. Few studies have examined cocaine sensitization in adult Wistar female rats, though sensitization in adult females across other rat strains has been more widely studied. Long Evans rats may sensitize more readily than Fischer rats (Wiley et al., 2011; Zhou et al., 2016). For example, in a five-day (15 mg/kg/d) treatment paradigm, Wiley et al. reported sensitization on days 4 and 5 in adult female Long Evans rats (Wiley et al., 2011), while Zhou et al. failed to observe sensitization after five days with a similar regimen in female Fischer rats (Zhou et al., 2016). Strain-specific changes in dopamine receptor binding (Zamudio et al., 2005) and dendritic spine morphology (Selvas et al., 2017) may also contribute to differences in behavioral response to cocaine.

Cocaine dose and length of administration schedules vary considerably between sensitization studies (Table I.1), and in general, higher cocaine doses and more administration days produce greater sensitization. Three previous papers reported sensitization in Wistar females, but all of them used either a higher cocaine dose (e.g. 20-40 mg/kg) (Dow-Edwards et al., 1989) or a longer sensitization period (e.g. 8-10 days) (Souza et al., 2014; van Haaren and Meyer, 1991) than in the current study.

CAS did not impact the locomotor response to acute cocaine (Day 1, NS vs. CAS), or cocaine sensitization in adolescent or adult female Wistar rats. Previous studies have shown that adolescent stress exposure increases cocaine self-administration in male Long- Evans rats (Burke et al., 2016) and cocaine-induced locomotor activity in male Wistar rats (Lepsch et al., 2005), but a pilot study did not reveal an effect of CAS on cocaine self-administration in female Wistar rats (our unpublished data). These results suggest that the ability of adolescent stress to alter behavioral responses to drugs of abuse may be more potent in males than females, but additional studies are needed to confirm.

While previous work has indicated a facilitating effect of estradiol and an impact of estrous cycle stage on cocaine-induced locomotor activity and sensitization (Sell et al., 2000; Sell et al., 2002), we did not observe an effect of estrous cycle stage on locomotor activity in our data pooled across day and stress group. Other studies report locomotor sensitization in adult female rats that are normally cycling (Thomas et al., 2009), and some report that only normal cycling females, rather than females with ovariectomy and estradiol replacement, exhibit locomotor sensitization (Souza et al., 2014). Thus, the influence of estrous cycle is unlikely to explain the failure of our adult cohort to exhibit cocaine sensitization.

Age on First Day of Treatment Paradigm	Strain	Dose	Treatment Paradigm	Reference	Result
Adult ~PND 71	Wistar	15 mg/kg	8 days cocaine, day 19 challenge	(Souza et al., 2014)	Sensitization
Adult 175-200g	Wistar	20, 40 mg/kg	5 days cocaine	(Dow-Edwards et al., 1989)	Sensitization
Adult ~PND 111	Wistar	10 mg/kg	10 days cocaine	(van Haaren and Meyer, 1991)	Sensitization
PND 41	Sprague Dawley	3, 7.5, 15 mg/kg	1 day saline, 6 days cocaine, day 11 challenge	(King et al., 2009)	Sensitization on day 7 (7.5mg/kg), day 11 (7.5, 15 mg/kg)
PND 33	Sprague Dawley	10, 20 mg/kg	4 days cocaine, day 6 challenge with 10 mg/kg cocaine	(Laviola et al., 1995)	Sensitization on day 6
PND 20	Sprague Dawley	30 mg/kg day 1, 20 mg/kg day 2	2 days cocaine	(Mohd-Yusof et al., 2014)	Sensitization on day 2 compared to 20 mg/kg first cocaine exposure group
PND 25	Sprague Dawley	1-17.8 mg/kg	4 cocaine doses every 15 min on 6 test days once weekly	(Serafine et al., 2015)	Sensitization trend; stats not available
70-80g (~PND25-27)	Sprague Dawley	1-17.8 mg/kg	4 cocaine doses every 15 min on 6 test days once weekly	(Serafine et al., 2016)	Sensitization
PND 34	Sprague Dawley	30 mg/kg day 1, 20 mg/kg day 2	2 days cocaine	(Kozanian et al., 2012)	No sensitization; Significant in first 10-min bin
PND 32	Sprague Dawley	5 and 15 mg/kg	5 days cocaine, day 20 challenge	(Franke et al., 2007)	Sensitization trend, no significance on day 20
PND 27	Long Evans	7 and 15 mg/kg	two rounds of 5 days cocaine separated by 2 days	(Wiley et al., 2011)	Sensitization on day 4, 5, and 9 to 15 mg/kg

Table I.1. Summary of studies assessing locomotor sensitization to repeated cocaine administration in adolescent and adult female rats.

We also assessed the locomotor response to novelty in adolescent and adult female Wistar rats (Figure I.2) with or without a history of CAS and found that initial exploratory activity (in the first 5-min bin) following exposure to a novel environment was attenuated in both adolescent and adult CAS rats. One study found that males exposed to prenatal stress exhibited an enhanced locomotor response to a novel environment compared to NS controls (Deminiere et al., 1992), while another reported that adolescent female isolation-housed rats traveled an increased distance following exposure to a novel environment compared to pair-housed females (Zakharova et al., 2012). Our finding of reduced exploratory activity suggests a unique effect of the mixed-modality stress paradigm during adolescence, and that isolation housing alone is unlikely to drive the altered initial response to novelty. The reduced locomotor response to novelty in CAS rats was surprisingly long-lasting; we observed a reduction in locomotor activity in adulthood, weeks removed from exposure to the stressors.

Combined with the existing literature, these data demonstrate that careful consideration should be given to age and strain when designing and interpreting the results of cocaine sensitization experiments. In addition, the findings reported here contribute evidence of age-dependent sensitization to the collective understanding of cocaine-induced behavioral plasticity in adolescent and adult female Wistar rats.

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