Characterization of a novel, stress-responsive sexually dimorphic corticotropin-releasing factor receptor 1 (CRFR1) nucleus in the rostral anteroventral periventricular nucleus of the mouse

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Characterization of a Novel, Stress-Responsive Sexually Dimorphic Corticotropin-Releasing Factor Receptor 1 (CRFR1) Nucleus in the Rostral Anteroventral Periventricular Nucleus of the Mouse

By

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Abstract

Within the United States, women are at double the risk of men to develop a stress-related mood disorder (e.g., anxiety or depression) during their reproductive years (Kornstein et al., 2000; Kessler et al., 2005). Many factors contribute to the potential sex difference in such disorders, including gonadal hormones (hypothalamic-pituitary-gonadal axis; HPG) and how they interact with the stress response system, or hypothalamic-pituitary-adrenal (HPA) axis. Corticotropin-releasing factor (CRF) signals through binding the Gs-coupled receptor, CRF receptor 1 (CRFR1), and activity between CRF/CRFR1 regulates the hormonal and behavioral stress response (Chen et al., 1993; Bale and Vale, 2004; Heinrichs et al., 1995; Smith et al., 1998; Subbannayya et al., 2013). Moreover, dysregulation of CRFR1 has been linked to the onset of stress-related mood disorders (Chrousos 2009; Garcia-Carmona et al., 2015; da Silva et al., 2016; Holly et al., 2016; Schussler et al., 2016). Antagonism or knockout of CRFR1 has been shown to reduce anxiety-like behavior (Webster et al., 1996; Smith et al., 1998; Timpl et al., 1998; Contarino et al., 1999; Tran et al., 2014; Zhao et al., 2007). While knockout or antagonism of CRF receptor 2, increases these same measurements (Bale and Vale, 2003; Bale et al., 2003; Zhao et al., 2007). Importantly, CRF has over a 10-fold higher binding affinity for CRFR1 over R2 (Perrin et al., 1995), and central CRF release is generally considered anxiogenic.

Early life gonadal hormone exposure shapes the way the central nervous system develops, creating structural differences in the organization of neural circuitry between the female and male brain. Development of sexually dimorphic brain structures is largely due to perinatal androgen exposure in males, serving to either increase or decrease the number of cells or to alter aspects of their phenotype (Morris et al., 2004; Zuloaga et al., 2011), and is known to alter stress-related behavior later in life (McEwen et al., 1977). In addition, steroid
hormone exposure in adulthood can maintain or modulate phenotypic expression for select sexually dimorphic nuclei (Morris et al., 2004; Zuloaga et al., 2011, 2012).

Rodent studies show sex differences in adult HPA activity where females have greater baseline and stress-induced adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) secretion. Gonadal hormones contribute to the reported biological and behavioral sex differences at rest and in response to stress; generally, testosterone reduces while estrogen increases HPA axis activation (Burgess and Handa 1992; Burgess and Handa 1993; Mora et al., 1996; Bowman et al., 2002; Rossi et al., 2010; Ramos-Ortolaza et al., 2017). Gonadal steroid receptors play an integral role in modifying the HPA axis tone; their distribution determines the locus of action of circulating hormones. Binding estrogen receptor alpha (ERα) is generally anxiogenic, while activity at ER beta (ERβ) or androgen receptor (AR) is anxiolytic; these findings have been shown in addition to changes in the biological indices of HPA activity (i.e., ACTH, CORT, and CRF increase with ERα and decrease with ERβ or AR activation) (Burgess et al., 1992; Handa et al., 1994; Lund et al., 2005; Lund et al., 2006; Zuloaga et al., 2011). Gonadal hormones also regulate basal diurnal HPA activity and contribute to sex differences in HPA activity in rodents (Atkinson and Waddell, 1997; Seale et al., 2004). The combined influence of hormone receptor distributions, circulating hormone levels, age, and previous stress experience collectively modify the overall tone of the HPA axis, and stress-related behavior.

Our lab has shown that the rostral anteroventral periventricular nucleus of the hypothalamus (AVPV/PeN) is sexually dimorphic in CRFR1 expression at all postnatal ages tested, including adulthood (P0, 4, 21, and P60; Rosinger et al., 2017, 2019a). The female AVPV contains other dimorphic cell populations that are implicated in sex-specific reproductive behaviors (Semaan et al., 2010; Poling and Kauffman, 2013; Kanaya et al., 2014; Scott et al., 2015), and has dense interconnectivity with other brain structures involved with stress and
reproduction in the female rat (Gu and Simerly, 1997). Much like structures traditionally associated with the HPA axis, the AVPV contains high densities of gonadal hormone receptors ERα, ERβ, and AR (Kanaya et al., 2014; Zuloaga et al., 2014), and glucocorticoid receptor (GR), making it a legitimate candidate for the integration of neural and hormonal signals related to stress and sex behavior. Specifically, GR and ERα are highly co-expressed on CRFR1 cells in the female AVPV/PeN (Rosinger et al., 2019a), and we show that a single neonatal injection of estradiol or testosterone masculinizes (eliminates) the female AVPV/PeN CRFR1 cluster, while adult gonadectomy has no influence on the sex difference (Rosinger et al., 2019a). CRFR1 is integral to the stress response, therefore dimorphic expression of CRFR1 within the AVPV/PeN may contribute to observed sex differences in the prevalence of stress-related mood disorders. To test the role in stress adaptations, we exposed female and male mice to chronic stress. After 9 days of chronic variable stress (CVS), CRFR1 doubles the level of expression in only the female mouse AVPV/PeN (Rosinger et al., in preparation), offering more support for AVPV/PeN CRFR1 as a sex-specific site where the HPA and HPG axes integrate and modulate adaptations to stress.

We further go on to show that this female-specific cluster of CRFR1 has anterograde and retrograde projections to regions that are consistently implicated in stress- and sex-specific behaviors, such as the paraventricular nucleus of the hypothalamus, bed nucleus of the stria terminalis, arcuate nucleus, medial amygdala, and hindbrain regions, such as the periaqueductal grey, dorsal raphe, and locus coeruleus nuclei. Finally, because ERα is highly expressed within CRFR1 expressing cells in many stress-related brain regions, we tested the hypothesis that gonadal hormone binding of estrogens at ERα/CRFR1 cells is critical for modulating stress-related behaviors. We accomplished this through generating a transgenic mouse line with ERα deleted specifically from CRFR1-expressing cells. Following behavioral analysis of these tests, we report no baseline behavioral differences for either female or male
mice regardless of their genetic background. Further we do not show any differences in baseline, peak, or recovery blood CORT levels within either sex, regardless of genetic background. Overall, these data provide initial evidence that the rostral portion of AVPV/PEN is a sexually dimorphic nucleus. Specifically, the reported studies determine that perinatal gonadal hormones regulate the development but adult gonadal hormones are not required for maintaining dimorphic expression of AVPV/PeN CRFR1. We then show a phenotypic profile of this nucleus, the influence of CVS, the locations with which the AVPV/PeN CRFR1 share afferent and efferent projections, and the role of estrogens acting at CRFR1 in driving the stress response. Based on the information we have collected thus far, we believe this may be a novel sex-specific nucleus that has direct implications in the female-specific pathway for stress-related adaptations, including anxiety- and depression-like behaviors. We believe that these data will contribute to future sex-specific therapeutic interventions for stress-related mood disorders.
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Chapter 1: Overview of Relevant Background

1.1 Sex differences in the prevalence of stress-related mood disorders

While many psychological and physical conditions show a general male over female (M>F) prevalence (e.g., alcoholism, schizophrenia, autism) (NIH, CDC), stress-related mood disorders like generalized anxiety disorder and major depression disorder exhibit a significantly higher prevalence in women. Within the United States, women are at approximately double the risk of men for the onset of a stress-related mood disorder during reproductive years (Kornstein et al., 2000; Kessler et al., 2005). These disorders create a significant economic burden estimated at $210.5 billion annually as of 2010 (Greenberg et al., 2015). Until recently, most of the preclinical research dedicated to such conditions had focused on male subjects, which highlights the importance for understanding female-specific risk factors and treatments. In humans, sex difference in stress-related mood disorders are virtually absent prior to the onset of puberty (Hankin, 2002; Bale & Epperson, 2015). This dramatic change suggests a role for the influx of gonadal steroid hormones (seen during the onset of puberty and throughout reproductive age) on emergence of the observed sex differences. Other work has reported that women are more likely to display pronounced mood disorder symptoms during periods of more extreme hormonal fluctuations, such as during puberty, postpartum, and surrounding menopause (Maeng & Milad, 2015).

Gonadal steroid hormones (e.g., testosterone, estradiol) are generally assumed to influence reported differences between sexes on markers for hypothalamic-pituitary-adrenal axis (HPA) activity (e.g., corticotropin-releasing factor [CRF], adrenocorticotropic hormone [ACTH], corticosterone [CORT]), and mediating behavioral differences in response to stress (female > male). Gonadal steroid hormones are vital for developmental organization and later activation of the HPA axis. This is true for both human and non-human studies, suggesting that
sex differences in human populations are not just an effect of societal influences on typical “gender norms” (e.g., men don't cry). However, little is known about where specifically these hormones and receptors interact to create observed sex differences. Part of why these mechanisms are poorly understood is because until the past few decades, most of the research and pre-clinical advancements for treatment of stress-related mood disorders used male subjects. The clinical treatments currently approved and available are largely designed for a demographic that composes less than half of the affected population. Understanding female-specific vulnerabilities for stress-related mood disorders is vital. Humans exhibit sex differences in depression-like symptoms, where men tend to have more aggression and substance abuse than women (Martin et al., 2013), highlighting potential sex differences in neural and hormonal components of the stress response. We do not know the biological underpinnings for the observed sex differences. However, it is likely that they are due to sex differences in brain organization during embryonic and perinatal development, and how these organized differences are activated later in life. Embryonic sexual differentiation is partially determined from androgen exposure during important perinatal time points, which ultimately masculinize/defeminize the structure and function of various regions of the central nervous system.

1.2 Gonadal steroid hormones and sexual differentiation

Development of sex differences in the rodent central nervous system are attributed to perinatal androgen surges (i.e., testosterone), that occur in males. The perinatal surge on gestational day 18 (GD18) and an additional surge within a small window following parturition are held as the main events that masculinize the rodent brain (McCarthy et al., 2017), including the organization of male-typical reproductive behavior (Phoenix et al., 1959; Raisman and Field, 1971). Perinatal testosterone in the male central nervous system is converted to estradiol (via aromatase enzyme), where it then acts to masculinize various reproductive neural circuits. Typically, androgen pulses either increase or decrease the total volume of cells or certain cell
phenotypes (Morris et al., 2004; Zuloaga et al., 2008). For example, the sexually dimorphic nucleus of the preoptic area (SDN-POA) is located within the medial preoptic area (MPOA) and has been implicated in aspects of male sex behavior (Gorski & Wagner, 1965; Gorski et al., 1981). It is also physically larger in males than females in many species, and requires activation of estrogen receptors to form (Morris et al., 2004). In addition, research has shown that testosterone converted to estradiol is sufficient to masculinize the female anteroventral periventricular nucleus of the hypothalamus (AVPV) through actions specifically at estrogen receptor alpha (ERα), making it physically smaller, resembling the normal male AVPV volume (Kanaya et al., 2014). Gonadal hormone exposure also modifies ERα expression in the principal nucleus of the bed nuclei of the stria terminalis (BSTpr; Ju & Swanson, 1989), which has higher ERα-immunoreactivity in gonadal-intact females (Kelly et al., 2013). In addition, the BSTpr is a hub for many gonadal hormone receptor types (Handa & Weiser, 2014), and key in the stress response (Choi et al., 2007). Apoptosis during perinatal hormone exposure is likely the responsible mechanism of action for many of the observed sex differences within the rodent central nervous system, including the BSTpr, and AVPV (Forger et al., 2004; Kelly et al., 2013). Other structures such as the spinal nucleus of the bulbocavernosus (SNB), are formed and maintained via androgen receptor activation (Nordeen et al., 1985), and without exposure or without functional androgen receptors, the SNB becomes physically smaller. The SNB is vital for male mice to achieve penile erection, and without proper development of the SNB, these mice will not exhibit sex-typical reproductive behaviors (Zuloaga et al., 2011; Raskin et al., 2012).

Following the perinatal androgen surge in males, animals (female and male) experience a period relatively absent of gonadal hormone activity. However, during the adolescent period in humans and in rodents, the gonads begin and continue secreting hormones, which act upon structures that were previously organized in early development. In humans, region-specific development of grey matter is subject to influence by the level of gonadal hormones during
puberty and likely shapes sex-typical grey matter distribution in regions such as the amygdala and hippocampus, which has been shown to be sensitive to variations in circulating testosterone in both female and male subjects (Neufang et al., 2009). The adolescent brain is still developing, and the changes in pubertal hormones alters this development. Some argue that the peripubertal period is just as important as the perinatal period in determining organization of the adult brain and behavior (Schulz & Sisk, 2016).

Schulz and Sisk (2016) discuss how restricting organizational changes to the brain only to sensitive periods such as perinatal and peripubertal, is not beneficial or even fully accurate, stating that “while sensitive periods always involve organizational change, organizational change does not always require a sensitive period.” Overall, the consensus is that gonadal hormones are vital for the development, expression, and maintenance of sex differences in different behaviors. Also, it is important to consider the spectrum of “sex differences” based on genetic/chromosomal sex; more recently researchers have argued that there are different types of sex differences, such as a sexual dimorphism, which is a structure or system that exists in one way for males and another for females, along with the concept of sex convergence or divergence (McCarthy et al., 2012), where female and males express the same ultimate behavior with different neural underpinnings. Stress-related mood disorders are experienced by both females and males, though research continues to suggest that the neural underpinnings are different.

1.3 Hypothalamic-Pituitary-Adrenal (HPA) Axis, CRF/CRFR1, and Stress-related Behaviors

Corticotropin-releasing factor (CRF) is the main positive regulator of adrenocorticotropic hormone (ACTH) for the release of glucocorticoid in response to environmental stressors (Owens and Nemeroff, 1991). When a stressor is detected (physical or psychological) CRF is co-synthesized in the paraventricular nucleus of the hypothalamus (PVN), along with arginine
vasopressin (AVP). From the PVN, CRF and AVP travel through the hypophyseal portal system, through the median eminence and into the anterior pituitary, where they bind to their cognate receptors (CRFR1, V1b, respectively), to induce the synthesis of POMC mRNA, from which ACTH is cleaved, along with several other potential chemical messengers, including β-endorphin. ACTH then travels through the periphery and ultimately binds to the adrenal cortex at the melanocortin 2 receptor (MC2R), and triggers steroidogenesis, leading to the secretion of glucocorticoids such as corticosterone (CORT) in rodents or cortisol in humans. CORT travels back to the anterior pituitary, PVN, and hippocampus, to bind glucocorticoid receptors (GR), stopping the synthesis of CRF, and ultimately ending the release of ACTH, through negative feedback (Viau, 2002; Abel & Majzoub, 2005). Portions of the limbic system, such as the hippocampus, amygdala and prefrontal cortex, contain high densities of GR, and activation throughout these regions is implicated in negative feedback which either turns off the response, or further perpetuates it (Jankord & Herman, 2008). Increases or decreases in HPA activation depend upon the regions and receptors involved. Collectively, this biological cascade in response to stress is called the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is ethologically necessary for survival. When a stressor or threat is detected in the environment, HPA axis activity facilitates necessary vigilance, and recruits resources allowing the organism to combat the stressor, or escape from threat (Sapolsky, 2002). For example, the HPA axis reduces digestion, and increases blood pressure, and oxygen use, in order for the organism to avoid the perceived threat (Sapolsky, 2002). Therefore in the context of acute stress exposure, the HPA axis is adaptive.

CRF is expressed within several brain structures and functions in the central nervous system as a signaling transmitter, and positive regulator of HPA activity. CRF is expressed in the PVN, central nucleus of the amygdala (CeA), bed nuclei of the stria terminalis (BST), Barrington’s nucleus, cerebellum, cingulate cortex, hippocampus, accessory bulb of the nucleus
accumbens, olfactory bulb, and periaqueductal grey (PAG) of both mice and rats (Swanson et al., 1983; Aguilera et al., 2004; Kono et al., 2016). Central release of CRF is anxiogenic, mainly a consequence of CRFR1 binding. CRF signaling at CRFR1 regulates neuroendocrine and autonomic responses to stress, and behavioral response to these stressors (Heinrichs et al., 1995; Smith et al., 1998; Subbannayya et al., 2013). The HPA axis responds to PVN CRF binding CRFR1 in the anterior pituitary through increasing stress-related peptides such as ACTH and CORT (Jankord and Herman, 2008), in addition to central release of CRF, which is partially regulated through amygdala or bed nucleus of the BSTCRF populations (Gilpin et al., 2015). CRF released in the amygdala and/or BST (both limbic) increase anxiety-like behavior in rats (Butler et al., 2016). Normally, CRF release contributes to circadian HPA activity, and in addition to the stress response, is important for the sleep/wake cycle (Buckley & Schatzberg, 2005) in vertebrates. However, chronic CRF release leads to dysregulated function, causing hypervigilance, anxiety, vulnerability to PTSD, sexual dysfunction, and anhedonia. Support for chronic CRF-induced dysregulation has been shown in animal models that use chronic CRF exposure, through genetic manipulation or stereotaxic injections (Stenzel-Poore et al., 1994; Buwalda et al., 1997).

Like CRF, CRFR1 is widely distributed throughout the rodent central nervous system, having been reported throughout the olfactory bulb, cerebral cortex, hypothalamus, hippocampus, amygdala, brainstem regions, and cerebellum (Van Pett, 2000; Justice et al., 2008; Rosinger et al., 2017). As mentioned in the abstract, CRFR1 activation is generally anxiogenic (Webster et al., 1996; Smith et al., 1998; Timpl et al., 1998; Contarino et al., 1999; Tran et al., 2014; Zhao et al., 2007), while CRFR2 activity is generally anxiolytic (Bale and Vale, 2003; Bale et al., 2003; Zhao et al., 2007), and CRF has a 10-fold higher binding affinity for CRFR1 over R2 (Perrin et al., 1995). In general, central CRF release is classified as anxiogenic. Global, non-selective knockout of both CRF receptors in mice causes differential sex-specific
impacts on anxiety-like behavior (Bale et al., 2002), which suggests an important role for the CRF receptor system in regulating sex differences in anxiety-like behavior. While CRF/CRFR1 activity is capable of modulating the HPA axis activation, the extent of activation and behavioral display of anxiety depends upon the anatomical locations being activated. For instance, infusion of a CRFR1-specific agonist into the medial prefrontal cortex attenuates c-Fos activity in the amygdala (Pentkowski et al., 2013). Moreover, CRFR1 activation in the globus pallidus reduces HPA activity and anxiety-like behavior (Sztainberg et al., 2011). Infusion of a CRFR1 agonist (CP376395) into the basolateral amygdala increases anxiety-like behavior (Cipriano et al., 2016). Therefore, it depends upon where CRFR1 activation occurs, and what portions of the limbic system are being co-engaged. However, global CRFR1 knockouts show anxiolytic effects (Smith et al., 1998; Contarino et al., 1999), while knockouts or antagonists for CRFR2 show anxiogenic effects (Bale and Vale, 2003; Bale et al., 2003; Zhao et al., 2007). Regardless, the interplay between these systems is complex, warranting further investigation. An added note, investigators have shown possible polymorphisms on the CRFR1 promoter that are associated with increased basal HPA activity; the reports show higher CRFR1 expression in animals with high HPA activity than animals with lower HPA activity, the reported difference was eliminated by using a CRFR1 antagonist (Labermaier et al., 2014). It is important to keep in mind that increased serum ACTH and CORT don't guarantee increased anxiety, they increase the probability for experiencing anxiety. As an example, some have shown that increased CORT facilitates the efficacy of antidepressants such as fluoxetine (aka Prozac) in mice (Robinson et al., 2016).

1.4 Known Sex Differences within the Corticotropin-Releasing Factor System

Sex differences in CRF have been reported with male rats having more expression in the central amygdala than females (Karanikas et al., 2013), while female rats have more CRF in the preoptic area and BST (McDonald et al., 1994; Funabashi et al., 2004). The preoptic area is
vital for the expression of maternal behavior, established for decades now, especially the medial portion of the preoptic area (Numan, 1974; Fahrbach and Pfaff, 1986), as is the BST (Bayerl et al., 2016). In addition, portions of the BST (anterior and posterior) project directly to CRF-expressing cells of the PVN, which increase HPA axis activity (Viau, 2002). These regions, and other hypothalamic/limbic structures, all express dense volumes of gonadal hormone receptors such as ERα, ERβ, and AR (Simerly et al., 1990; Shughrue et al., 1997; Mitra et al., 2003). At present there is far less known about sex differences in the distribution of CRFR1. Research indicates that CRFR1 binding is higher in the adult female rat nucleus accumbens, olfactory tubercle, piriform cortex, and anterior cingulate cortex (Wealthington et al., 2014). In adult voles, another group (Lim et al., 2005) found increased CRFR1 in the female BST, when comparing with male vole counterparts. However, more information is needed regarding sexually dimorphic CRFR1 expression patterns, and how these contribute to observed sex differences in HPA activity, and stress-related behavior (e.g., anxiety- and depression-like behaviors).

The currently established sex differences in the CRF/CRFR1 system do not necessarily explain the sex differences in stress-related mood disorders. However, there is a strong body of research pointing to CRF/CRFR1 signaling as an integral part of both protection (Ramot et al., 2017) or ultimately dysregulation of the stress-response (Chrousos 2009; Garcia-Carmona et al., 2015; da Silva et al., 2016; Holly et al., 2016; Schussler et al., 2016). It depends upon where within the central nervous system the signaling occurs. Regardless, because both men and women develop stress-related mood disorders, it is very likely that different neural circuits underlie the sex differences and similarities following stress. The current problem is a lack of information about these specific circuits. Further, there are relatively few human studies (mentioned below), but most suggest that gonadal hormones affect these circuits similarly to rodent studies. However, we know very little about these sex-specific circuits in either humans
or rodents. Increasing our understanding of the CRF/CRFR1 systems in female and male rodents will help push the field of stress research forward.

1.5 Interactions between the Hypothalamic-Pituitary-Gonadal (HPG) and HPA axis

The hypothalamic-pituitary-gonadal (HPG) axis involves gonadotropin-releasing hormone (GnRH)-expressing neurons that send projections to the median eminence, signaling GnRH to the anterior pituitary, triggering the release of LH (luteinizing hormone) and follicle stimulating hormone (FSH) (Handa and Weiser, 2014). LH precedes gonadal hormone release in females and males. Specifically in males, LH stimulates testosterone and FSH stimulates spermatogenesis. In females, LH and FSH act to increase estradiol release from the ovaries and lead to ovulation. In addition, male rodent androgen surges are daily and pulsatile, and female rodents go through 4-day estrous cycles; depending upon the day and timing of any given day, a different balance of estrogen/progesterone is present (Plant, 2015). Typically, higher levels of estradiol and lower progesterone levels are associated with increased basal and stress-induced HPA activity (Ramos-Ortolaza et al., 2017). This has been demonstrated with gonadectomy/hormone replacement studies (Weiser and Handa, 2009), showing that estradiol increases activation of the HPA axis, measured via serum CORT and ACTH levels.

Gonadal hormone receptors such as AR, ERα, and ERβ are important for modulating HPA activity (for comprehensive reviews: Viau 2002; Klein and Romeo, 2013; Handa and Weiser, 2014). Research has shown that hormone activity at AR and ERβ reduce, while ERα increases HPA axis activation (Burgess et al., 1992; Handa et al., 1994; Zuloaga et al., 2011). The female HPA axis shows higher basal and stress-induced activity assessed through ACTH and CORT (Seale et al., 2004); estradiol interferences with GR-mediated negative feedback in females (Burgess and Handa, 1992), through impaired GR function (Weiser and Handa, 2009). In ovariectomized (OVX) female rats, there is a reduced/blunted ACTH and CORT response to
repeated foot-shock (Burgess and Handa, 1992) suggesting estradiol increases HPA activation in this context, considering the females without circulating estrogens had lower indications of HPA activity. In contrast, the male HPA axis has lower in basal and stress-induced levels of ACTH and CORT, due to testosterone and testosterone metabolite 5α-dihydrotestosterone (DHT) (Handa et al., 1994; Lund et al., 2006). In addition, DHT, 3β-diol (a DHT metabolite), and testosterone are all capable of reducing HPA activation through binding ERβ and/or AR (Lund et al., 2006; Handa et al., 2011; Zuloaga et al., 2011).

Sex differences in HPA axis response to stress is likely related to the different evolutionary needs for female versus male organisms (Glover & Hill, 2012). Female and male rodents experience different forms of stressors in the wild, which require appropriate stress responses. The HPA axis may therefore need to respond differently based on sex, and ethological relevance for optimal survival (Palanza, 2001; Palanza & Parmigiani, 2017). For example, female rodents spend more time nursing or keeping their pups warm, while being vigilant for any potential predators near the nest site. Male rodents are more likely to be seen by predators while away from the nest and require a different type of environmental awareness.

Behavioral differences in rodent models are somewhat mixed: there are reports of estradiol replacement following OVX as anxiolytic, while others reported it anxiogenic following stress (Burgess and Handa, 1992; Burgess and Handa 1993; Mora et al., 1996; Bowman et al., 2002; Figueiredo et al., 2003; Lagunas et al., 2010; Mahmoud et al., 2016). On the other hand, male gonadectomy (GDX) increases anxiety-like behavior, and replacement of testosterone decreases anxiety-like behavior (Zuloaga et al., 2008). Behavioral results depend on individual experimental and methodological designs, with mixed findings overall. Regardless, the general consensus is that both androgens and estrogens have important roles in mediating anxiety- and depression-like behavior (Toufexis, Myers, & Davis, 2006; Maeng & Milad, 2015). For the most part, ERβ and AR reduce anxiety-like behavior, while ERα increases anxiety-like behavior. This
has been shown with increased anxiety in animals with ERβ-knockout (Krezel et al., 2001) or AR-knockout (Chen et al., 2014), and reduced anxiety with ERα antagonists, which can be reversed if ERα is activated (Weiser and Handa, 2009). In rodents, perinatal gonadal hormone exposure also shapes later anxiety-like behavior (McEwen et al., 1977). Inhibiting the male aromatase enzyme during the neonatal period increases anxiety-like behavior in adulthood, while giving a single injection of testosterone in females on the day of birth reduces adult anxiety-like behavior (McEwen et al., 1977; Goel & Bale, 2008).

Basic research has been more commonly performed in rodents than humans. However, human research has shown that genes encoding for CRF within the PVN contain androgen responsive elements on the promoter region, potentially facilitating subsequent sex differences in CRF activity (Bao et al., 2006), where reports have shown testosterone as potentially protective against HPA activation. There are other studies that have investigated HPA tone through ACTH/CORT in the context of human stress exposure. Much of the information regarding human sex differences in HPA tone, via ACTH/CORT levels, involve the androgen testosterone and its metabolite DHT. These studies show an androgen presence as protective against HPA axis dysregulation (Rubinow et al., 2005; Lee et al., 2012). Men with exogenous testosterone administration have been shown to suppress CRF-stimulated CORT better than those without exogenous testosterone (Rubinow et al., 2005). In addition, reports indicate that negative feedback in women may be slower/delayed compared with men (Kudielka and Kirschbaum, 2005; Gallucci et al., 1993; Heuser et al., 1994), a finding that also lines up with literature on sex differences in the rodent HPA axis. In general, gonadal steroid regulation of the HPA axis likely contributes to the observed sex difference in the prevalence for stress-related mood disorders (Altemus et al., 2014).

While it is generally assumed that sex-specific hormone exposure contributes to differences in prevalence rates for stress-related mood disorders, the neural circuitry that
mediates these differences is still largely unknown. Gonadal hormones act in conjunction with the nervous system to affect the overall activity of the HPA axis and stress-related behaviors, but further information is needed regarding sex differences in specific cell phenotypes, neural circuits, and brain structures as they relate to the etiology of stress-related mood disorders, and sex differences observed in prevalence rates.

1.6 Preoptic Area (POA) and Rostral Anteroventral Periventricular Nucleus (AVPV/PeN)

The preoptic area (POA) is implicated in parental care in rodents (Numan et al., 1974; Lee et al., 2002). The medial POA (MPOA) in particular, has been consistently implicated in maternal behavior (Numan et al., 1974; Terkel et al., 1978; Fahrbach and Pfaff, 1986). The “parental” pathway involves signaling from the POA to other limbic and hypothalamic structures (i.e., prefrontal cortex, amygdala, BST, and ventral tegmental area) to cause the new parent to approach and care for offspring, thereby inhibiting the natural “avoid” or stress response seen in virgin rodents without pup experience. Therefore, the POA is implicated in the stress response as well as parental behavior. The POA projects to the PVN, which is the major orchestrator of the stress response, while the POA also receives inputs from the amygdala, ventral subiculum, and medial PFC (Jankord and Herman, 2008). POA signaling to the PVN modifies the tone of the HPA axis at baseline and following stress exposure (Herman & Cullinan, 1997), and the MPOA has high glucocorticoid receptor binding, functioning as an important structure in the HPA axis negative feedback (Viau & Meaney, 1996). In addition, there is a body of evidence suggesting that stress can have a negative influence on female fertility/reproduction, through CRF signaling and the subsequent influence on GnRH neuron activity, which is located in the POA. CRF signal reduces GnRH firing rate and as consequence significantly reduces/eliminates LH surges (Dobson et al., 2003). Various stressors have also been shown to downregulate kisspeptin and kisspeptin receptor expression in the MPOA, concurrent with limited/inhibited LH
surges (Kinsey-Jones et al., 2009), while testosterone in the male MPOA enhances HPA axis negative feedback (Viau & Meaney, 1996). Further, chronic CRF in the MPOA can significantly delay the onset of puberty in the female rat (Kinsey-Jones et al., 2010). In addition, activation of CRFR1 within the female MPOA has been shown to impair maternal behavior (Klampfl et al., 2018). Overall, research shows that the POA is involved in sex-specific parental- and stress-related behaviors.

The rostral anteroventral periventricular nucleus (AVPV/PeN) is situated within the POA, has been shown to express various sex differences, most of which are biased toward females within rodent models. This includes tyrosine hydroxylase and kisspeptin, which are both more highly expressed in the female (Simerly, 1989; Simerly et al., 1997; Semaan et al., 2010; Brock, et al. 2015; Scott et al., 2015), in addition a general volumetric difference following the same pattern (F>M). Moreover, the AVPV/PeN changes expression of TH based on sexual and maternal experience, and is involved in regulating ovulation (Simerly et al., 1997; Scott et al., 2015). Our findings show a sex difference (F>M) in CRFR1 within the AVPV/PeN, present at P0 and remains expressed throughout development and into adulthood (Rosinger et al., 2017; more information in chapter 2). The sex difference we found at P0 is expressed earlier developmentally than other known sex differences like TH or KISS (Waters and Simerly, 2009; Poling and Kauffman, 2013). The early, sexually dimorphic appearance of CRFR1-expressing cells may influence the development of other dimorphic cell phenotypes within the AVPV/PeN or within regions to which these cells project. For example, others have shown the importance of CRFR1 activity on the development of structures like the olfactory bulb (Garcia et al., 2016), which is integral for male- or female-typical displays of sexual and parental behavior in rodents (Heimer and Larsson, 1967; Rowe and Edwards, 1972; Jakupovic et al., 2008). It is also possible that the female-specific expression of CRFR1 in the AVPV/PeN is contributing to sex differences in the HPA axis activity and mediating the behavioral response to stress exposure.
Gonadal steroid hormone receptors (ERα, ERβ, and AR) are highly expressed in the AVPV/PeN, (Kanaya et al., 2014; Zuloaga et al., 2014; Rosinger et al., 2019a) suggesting these CRFR1 cells may be influenced by circulating gonadal hormones. Additionally, others have shown that natural variations in ERα expression within the MPOA are associated with differences in maternal care (Champagne et al., 2003). The female rat AVPV contains neurons that project to a number of brain areas that regulate reproductive and stress-related behaviors as well as hormone release (vascular organ of the lamina terminalis, medial preoptic area, ventral lateral septum, arcuate nucleus, PVN, and periaqueductal gray) (Gu and Simerly, 1997). It is possible that AVPV/PeN CRFR1-expressing projections to these regions in the female mouse may regulate sexually dimorphic patterns of behavior and hormonal functions, though this information is currently unavailable. Previous work has shown that the AVPV also expresses GR mRNA (Morimoto et al., 1996), and our findings further demonstrate that GR is co-expressed specifically on CRFR1-expressing cells in the female AVPV/PeN, indicating that this specific cell phenotype might also be modified by glucocorticoid exposure. Moreover, ER expression has been shown to modify the stress response in hypothalamic regions, such that ERα increases the HPA activity while ERβ reduces the activity (Burgess et al., 1992; Handa et al., 1994; Zuloaga et al., 2011). Our data show a high degree of co-expression between CRFR1 and ERα within the sexually dimorphic CRFR1 nucleus of the AVPV/PeN.

Therefore, CRFR1 in the female AVPV/PeN may provide a sex-specific site at which stress acts to influence HPA axis activity and mediate stress-related behaviors. The overarching goal of this series of investigations is to answer important questions surrounding the dimorphic expression of CRFR1 we have reported in the female mouse AVPV/PeN. The studies within this dissertation probe the chemical/phenotypic composition of these cells, the role of gonadal hormones on development and maintenance of these cells, investigate afferent and efferent projections, determine if these cells are altered following chronic variable stress, and Lastly, test
the role of the ERα/CRFR1 cell phenotype in driving sex differences in the biological and behavioral response to acute stressors.

Chapter 2: Phenotypic/chemical characterization and gonadal hormone regulation of AVPV/PeN

Introduction

Our laboratory is interested in the interplay between the endocrine and central nervous systems, as they relate to stress-related mood disorders (e.g., anxiety, depression, addiction). Reproductive-aged women are significantly more likely to develop anxiety or depression, compared with age-matched men (Kessler et al., 2005). Though many factors contribute to these differences, our lab focuses on the interplay of gonadal hormones and CRFR1, in mice. We focus on CRFR1 because dysregulation at these receptors has been linked to psychiatric disorders including anxiety and depression (Chrousos 2009; Garcia-Carmona et al., 2015; da Silva et al., 2016; Holly et al., 2016; Schussler et al., 2016). Compared to CRF receptor 2 (CRFR2), CRFR1 is expressed at higher levels in the brain (Van Pett et al., 2000; Lein et al., 2007), and has a greater binding affinity to CRF (~10x greater, Perrin et al., 1995). Based on previous research, we know that CRFR1 mRNA is expressed throughout the mature rodent brain, and during rodent and human development (Van Pett, 2000; Korosi and Baram, 2008; Sandman and Glynn, 2009). Developmental studies on the distribution of CRFR1 are largely in the rat, however, and have primarily investigated the hippocampus, amygdala, cortex, and the paraventricular nucleus of the hypothalamus (PVN) (Avishai-Eliner et al., 1996).

CRFR1 exerts lasting effects on the development of several brain regions both during normal development and following early life stress. Neonatal stress induces alterations in dendritic development, spinogenesis, and synapse formation in the mouse hippocampus and cerebral cortex and these effects are reversed by pharmacological antagonism of CRFR1 (Liao et al., 2014; Yang et al., 2015). Work by Garcia and colleagues (2014) investigating the role of
CRFR1 in normal development of the mouse olfactory bulb suggests that CRF signaling through CRFR1 drives synaptic and dendritic formation required for functional olfaction. Furthermore, early life stress can produce lasting effects on CRFR1 expression in several brain regions including the rat amygdala (Grundwald and Brunton, 2015).

CRF producing cells are expressed in several regions throughout the brain, including the PVN, central amygdala, bed nucleus of the stria terminalis (BST), Barrington’s nucleus, cingulate cortex, hippocampus, accessory bulb of the nucleus accumbens, olfactory bulb, and periaqueductal grey of both mice and rats (Swanson et al., 1983; Aguilera et al., 2004; Kono et al., 2016). CRF has been described in the mouse PVN as early as embryonic day 13.5 (E13.5) and undergoes dynamic fluctuations over perinatal development marked by decreased levels around the time of birth (Keegan et al., 1994). By contrast, in the amygdala there are low levels of CRF during the prenatal period with levels rising during the neonatal period (~P3) (Keegan et al., 1994). Other areas such as the cerebral cortex express no CRF mRNA until P3 (Keegan et al., 1994). Distribution of CRF receptors in adult rats and mice is vast; CRFR1 expression has been reported throughout the olfactory bulb, cerebral cortex, hypothalamus, hippocampus, amygdala, brainstem, and cerebellum (Van Pett, 2000; Justice et al., 2008). CRFR2 is also expressed throughout the brain with the highest levels found in the lateral septum, BST, medial portion of the amygdala, and dorsal raphe nucleus of mice and rats (Chalmers et al., 1995; Van Pett et al., 2000; Aguilera et al., 2004). Less is known about CRFR1 distribution during development, particularly in the mouse. However, in the rat, large fluctuations in brain CRFR1 have been reported throughout the neonatal period (Avashi-Eliner et al., 1996). A more comprehensive description of CRFR1 distribution in the neonatal brain will enhance our understanding of the neural circuitry that regulates early life stress. In humans, specific CRFR1 polymorphisms have been linked to the onset of adult depression following early life adversity.
(Grabe et al., 2010; Laucht et al., 2013). This supports the role for early life CRFR1 expression and function in the development of adult mood disorders.

Sex differences in rats have been reported with males having more CRF expressing cells than females within the central amygdala (Karanikas et al., 2013). On the contrary, CRF positive cells are more abundant in the female rat preoptic area and BST (McDonald et al., 1994; Funabashi et al., 2004). Sex differences in CRFR1 have also been reported and depend upon the region and species of interest. Wealthington et al. (2014) reported greater CRFR1 binding in adult female compared to male rats within the nucleus accumbens, olfactory tubercle, piriform cortex, and the anterior cingulate, while Lim and colleagues (2005) found greater CRFR1 expression in the BST of female voles. To our knowledge, prior to our investigation, potential sex differences in CRFR1 expression had not been explored in mice. Such sex differences in the distribution of CRF and its receptors may contribute to observed differences in a variety of stress-related behavioral and hormonal responses reported in both rats and mice (Handa et al., 1994; Jasnow, et al., 2006; Zuloaga et al., 2008). Deletion of CRF receptors in mice results in differential effects on anxiety-like behavior in male and female mice (Bale et al., 2002), further supporting the importance of CRFR1 in regulating sex differences in anxiety. In humans, sex differences in distribution and function of CRF and CRFR1 expression may potentially contribute to the etiology of stress-related psychiatric disorders such as anxiety and depression (Bao et al., 2006; Valentino et al., 2012), both of which are more prevalent in women than men (Weissman et al., 1993; Kornstein et al., 2000; Kessler et al., 2005; Seney and Sibille, 2014).

In the current set of studies, we first report CRFR1 expression differences based on sex, and across development through the use of a validated CRFR1 reporter mouse, bacterial artificial chromosome (BAC) transgenic reporting expression of CRFR1, with green fluorescent protein (BAC transgenic CRFR1-GFP mice; Justice et al., 2008). Our findings indicate that
CRFR1 expression is dynamic throughout neonatal development, with sex- and age-dependent differences. For example, CRFR1 expression increased with age in several regions including the medial amygdala, arcuate nucleus, paraventricular hypothalamus, medial septum, CA1 hippocampal area, and the lateral habenula. Regions showing decreased CRFR1 expression later in development (i.e., by P21) include the intermediate portion of the periventricular hypothalamic nucleus, and CA3 hippocampal area. The AVPV/PeN was the most dramatic and earliest dimorphic structure we observed, which held constant from P0 through adulthood (Rosinger et al., 2017, 2019a). We felt that further understanding the phenotypic composition of these cells was a logical and vital next step toward understanding the female AVPV/PeN CRFR1, and any implications it may have in female-specific stress response.

In the cluster of CRFR1 that is found almost exclusively in the female AVPV/PeN, we describe the chemical profile of these cells compared to other previously reported sexually dimorphic AVPV sub-populations, including tyrosine hydroxylase and kisspeptin (Simerly, 1989; Simerly et al., 1997; Semaan et al., 2010; Brock, et al. 2015; Scott et al., 2015). Gonadal hormone receptors are important for regulating the central effects of HPA axis activity, and we show a high level of co-expression with ERα, which is important for sexual differentiation of the brain (McCarthy et al., 2017; Wu & Tollkuhn, 2017), and is known to increase the HPA axis response and anxiety-like behavior (Burgess & Handa, 1992; Weiser & Handa, 2009). We performed immunohistochemical labeling for GR, to probe the potential for these cells to respond to circulating glucocorticoids, and show significant co-expression of GR/CRFR1 in the female mouse. We then aimed to establish the organization of this nucleus, and following perinatal injections of either testosterone or estradiol, we show that the sexually dimorphic expression of CRFR1 in the AVPV/PeN is sensitive to perinatal androgen surges for the sex difference to surface, but is not dependent upon maintained adult gonadal hormone circulation as evidenced by our adult gonadectomy study (Rosinger et al., 2019a). The presence of these
CRFR1 cells suggest this may be a female-specific site at which circulating stress hormones and transmitters could influence adaptations to stress. We thus tested the impact of acute psychological stress and report that the CRFR1 cells of the AVPV/PeN are activated (pCREB/CRFR1+) following acute 30-minute restraint, not seen in unstressed females or in males of either treatment condition. Taken together, the evidence from these preliminary investigations suggest that the AVPV/PeN CRFR1 population is unique to females from early postnatal life, co-expresses other receptors that are known to regulate the HPA axis, is organized by perinatal hormone surges, resilient to changes following ovariectomy in adulthood, and is only activated in the female response to acute restraint stress.

**Methods**

**Animals:** Male and female mice on a C57BL/6J background, using a validated bacterial artificial chromosome (BAC) identified green fluorescent protein specific for CRF receptor 1 (CRFR1) (BAC GFP-CRFR1) were used for all portions of chapter 2. Animals were maintained under a 12/12 L/D cycle (lights on at 0700), with food (phytoestrogen free) and water available *ad libitum*. Mice were genotyped via PCR; using the nucleotide sequences: CCT ACG GCG TGC AGT GCT TCA GC forward and CGG CGA GCT GCA CGC TGC GTC CTC reverse EGFP350 primers. Actin470 was used as a control gene.

**Developmental Distribution of CRFR1:** We used 10 BAC CRFR1-GFP+ animals per age group (N = 5 per sex; P0, 4, 21), comprising the three developmental time points. Animals were maintained in their home cages until euthanasia. Pregnancies were monitored daily, when there was a litter, the litter was noted and randomly assigned to one of the three developmental time points. If it was assigned to the P0 group, the litter was removed and euthanized via rapid decapitation, with brains extracted and transferred to 4% paraformaldehyde for 24 hours, and then transferred to a 30% sucrose solution and stored at 4° C until sectioning. For developmental points P4 and P21, animals were left undisturbed with the mother and removed...
for sacrifice at the assigned time point. All animals were sacrificed with brain tissue extracted for immunohistochemistry before 1200h to remove any variation diurnal CORT fluctuations may have had on the investigation. Additional figures illustrating developmental distributions of CRFR1 in regions aside from the AVPV/PeN can be seen in the supplementary materials (S1-S3).

**P0 Hormone Supplement:** To investigate the role of perinatal androgen surge in masculinization of the AVPV/PeN, each morning the animal vivarium was monitored for new litters. When new litters were born, offspring were given a subcutaneous injection of either testosterone propionate (TP; 100µg dissolved in oil, N = 6 female, 7 male), estradiol benzoate (EB; 20µg dissolved in oil; N = 6 female, 6 male), or sesame oil (VEH; N = 7 female, 6 male) as controls. A hypodermic needle (27gauge) was inserted subcutaneously (sc) at the lower portion of the back, and the needle was inserted gently until it was seen roughly between the shoulder blades, where it was then injected, and slowly withdrawn. If there was a significant amount of leaking at the injection site, the animal was re-injected. Animals were returned to their home cage, masked with the scent of home cage bedding, and left undisturbed until weaning. On P21, pups were weaned and immediately euthanized via rapid cervical dislocation and decapitation, with brain tissue extracted for histological analyses.

**Adult Gonadectomy (GDX):** Adult mice (P60; N= 24, 8 per treatment per sex) were gonadectomized (GDX). Briefly, adult male mice were put under anesthesia via inhaled isoflurane/oxygen mixture (~2.5% isoflurane) and incision areas were cleaned with ethanol and betadine. The testes were externalized via bilateral incisions made in the scrotum. The vas deferens were tied off with dissolvable silk suture prior to cutting, and following successful removal of the testes, incisions were closed with Vetbond brand surgical glue. For removal of ovaries (OVX), female mice were anesthetized with isoflurane and the incision areas were cleaned with ethanol and betadine. Small bilateral incisions were made through the skin and
muscle wall overlying the ovaries. The ovaries were retracted using forceps, ligated using
dissolvable sutures between the ovary and uterine horn, and removed using surgical scissors.
Muscle walls were closed using dissolvable sutures and skin sealed via surgical staple. Sham
male and female GDX were performed in the same fashion as mentioned above, however the
gonads were simply visualized, leaving gonads intact, before closing the incision sites. The
analgesic carprofen (0.05 mg/kg) was injected (sc) for two days post operatively. The animals
were given a 6 week period without gonadal hormones, undisturbed in their home cages before
they were sacrificed. Of note: in this study, the estrus cycle of females was not monitored. The
six week time point post-GDX was chosen based on another study reporting a significant
reduction of kisspeptin expression in the AVPV 6 weeks post-GDX (Brock and Bakker, 2013).

Restraint Stress: Gonadal intact adult mice (N = 7 female, 5 male) ranging in age from P60 to
80 were placed into a restraint tube (L: 6-4/5", W: 3-9/10", H: 2-3/5") and left inside their home
cage for 30 minutes, after which they were removed from the tube and left undisturbed in their home
cage until sacrificed (90 min after the onset of restraint stress). All animals were
restrained and euthanized before 1200 to avoid circadian fluctuations in ACTH and CORT.

Fluorogold, Assessing Neurosecretory Function: Female BAC CRFR1-GFP mice (N = 4)
received a peripheral sc injection using 50µL of 5% fluorogold (Fluorochrome, Denver, CO) in
normal sterile saline (0.9%). Animals were sacrificed 5 days later, for immunohistochemistry
using anti-GFP antibody (protocol within dual-label immunohistochemistry section below). The
injection concentration and volume were established from previous investigations (Kriegsfeld et
al., 2003; Oyola et al., 2017).

Tissue collection for immunohistochemistry (IHC): All animals underwent cervical
dislocation followed by rapid decapitation at the end of each experiment. Brains were removed
and immersed in 4% paraformaldehyde (PFA), and stored overnight at 4° C. The following day,
brains were transferred into a 30% sucrose cryoprotectant solution, where they remained at 4°C until sectioning. For immunohistochemistry, brains were coronally sectioned at 40 μm, into 3 series using a cryostat (Microm HM505E, MICROM international GmbH, Walldorf, Germany). Tissue was placed in cryopreservative, and stored at 4°C until we performed immunohistochemistry. For the development study P0 brains were sectioned through the coronal plane at 50μm into 2 alternate series, P4 was sectioned at 40 μm into 2 series, and P21 was sectioned at 40 μm into 3 series. P0 tissue is fragile, which is why we sectioned at 50 instead of 40 μm, preserving tissue integrity.

**Perfusion (of fluorogold-injected animals):** Mice were injected with an overdose of ketamine (100 mg/kg)/xylazine (10 mg/kg)/acepromazine (3 mg/kg) (KXA) 5 days following fluorogold injection and, once anesthetized, intra-cardially perfused with 20 ml phosphate buffered saline (PBS), followed by 40 ml 4 % paraformaldehyde. Brains were removed, stored in 4 % paraformaldehyde overnight, and then transferred to 30 % sucrose until sectioning.

**Single-Label Chromagen-Based IHC:** To visualize the developmental distribution of CRFR1-GFP-ir, sections were rinsed in phosphate-buffered saline (PBS; pH 7.6), incubated in 1% hydrogen peroxide and 0.4% Triton- X in PBS (PBS-TX) for 10 minutes. Next, tissue was re-rinsed in PBS and incubated in 4% normal goat serum (NGS) in PBS-TX for 1 hour. Tissue was then incubated overnight in primary antisera for GFP (rabbit, Life Technologies, A6455; 1:7500). The following day, tissue rinsed in PBS then incubated in biotinylated goat anti-rabbit antisera in PBS-TX (Vector Laboratories; 1:500) for 1 hour. After, tissue rinsed in PBS and was then placed in avidin-biotin complex (ABC Elite kit, Vector Laboratories; 1:1000). Tissue rinsed again in tris-buffered saline (TBS) and was placed in diaminobenzidine for 10 minutes to visualize CRFR1-GFP+ cells. Sections from wild type brains were utilized a negative control to assess CRFR1-GFP expression; this tissue displayed no labeling.
**Dual-Label Fluorescent IHC:** For phenotypic characterization of CRFR1 cells, we preformed several dual-label fluorescence IHC studies for CRFR1 including phosphorylated CREB (Cell Signaling; rabbit; 1:500), tyrosine hydroxylase (Millipore; rabbit; 1:500), kisspeptin (Millipore; rabbit; 1:500), estrogen receptor alpha (Santa Cruz; rabbit; 1:250), and glucocorticoid receptor (Santa Cruz; anti-rabbit; 1:250). Sections thoroughly rinsed in phosphate-buffered saline (PBS; pH 7.6), then incubated in 4% normal donkey serum (4% NDS) and 0.4% Triton-X in PBS (PBS-TX) for 1 hour. Immediately following incubation, tissue was placed into the primary antisera (chicken, GFP; abcam; 1:1000) and incubated at room temperature overnight. The following day, tissue was thoroughly rinsed in PBS (80 minutes) and then placed directly into secondary antisera (donkey anti-chicken, 488; 1:1000) in 4% NDS and PBS-TX for 2.5 hours. After, the tissue was transferred to the second primary antisera in 4% NDS and PBS-TX at room temperature overnight. On the third day, tissue was rinsed in PBS, then transferred to the second secondary antisera (anti-rabbit, 594; 1:250) for 2.5 hours. After which, tissue was rinsed again in PBS. Immediately following the final rinse, tissue was mounted and coverslipped with Santa Cruz hard set mounting media with DAPI when dry.

**Microscopic analysis:** Analysis of CRFR1 distribution was conducted on a Nikon 80i microscope equipped with a digital camera. The Allen institute mouse brain atlas (Lein et al., 2007) was used to identify P21 and P60 brain areas and an atlas of the developing mouse brain was used for P0 and P4 brains (Paxinos et al., 2007). Images were collected at a 20X objective. Following image collection, cells were bilaterally quantified within two atlas-matched sections using appropriated shaped ROIs placed around desired forebrain structures. CRFR1+ cells were visualized using the chromagen Diaminobenzidine (DAB) for development and maintenance studies, and were quantified using Image J software (NIH). Dual-labeled cells were quantified using the same Image J software, and considered co-localized when green
(CRFR1) and red labeled cells (secondary labels) overlapped, appearing yellow on the image overlay.

Statistical Analyses: Analysis of CRFR1-ir within the AVPV/PeN was performed using 2-way ANOVA for statistical analyses with age by sex, and sex by treatment, where listed. 1-way ANOVA was used for treatment by CRFR1-ir, where listed. Significant main effects/interactions were further analyzed using Bonferroni-corrected T-tests; significance level was set to \( p \leq 0.05 \) and data are displayed as arithmetic means ± SEM.

Results
2.1 Developmental Distribution of CRFR1 at P0, P4, and P21
2.1.1 Hypothalamus/Preoptic Area: A striking sex difference in CRFR1-ir was found in mice spanning all of the investigated developmental time points in the AVPV/PeN (P0, P4, P21) with females showing a dense cluster of CRFR1-ir cells along the 3rd ventricle (Figure 2.1; Table 2.1). This cluster was largely absent in males. The rostral MPOA, ventral to the anterior commissure was stable between P0 and P21, with moderate clustering of CRFR1-ir (Table 2.1). In the PVN CRFR1-ir was relatively low at all time-points assessed although there was an apparent increase between P4 and P21 (Table 2.1). The region directly dorsal to the PVN (peri-PVN) showed moderate levels of labeling throughout postnatal development (Table 2.1). The PeN/Int, which lies ventral to the caudal PVN contained moderate labeling at P0 and P4, but gradually became sparse by P21. Unlike the AVPV/PeN, no sex differences were present. Within the lateral hypothalamus, modest labeling was present throughout development with no apparent changes between P0 and P21 (Table 2.1). Within the caudal portion of the hypothalamus CRFR1-ir was dense in the dorsomedial hypothalamus of both sexes at P0, P4, and P21 (Table 2.1). Ventromedial hypothalamus immunolabeling was minimal and confined to the ventrolateral division (Table 2.1). The P0 and P4 arcuate nucleus contained sparse CRFR1-ir, though labeling became extensive at P21. Males at P0 and P21 appeared to have slightly
more CRFR1-ir within the anterior hypothalamus (Table 2.1). Ventral to the arcuate, little to no CRFR1-ir was localized in the median eminence (ME) at any age. Just lateral to the arcuate, the tuberal nucleus exhibited modest labeling with a small increase from P0 to P4, which returned to approximately P0 levels by P21 (Table 2.1). In the suprachiasmatic nucleus (Table 2.1), CRFR1-ir cells were moderate and relatively stable across development. In contrast, however, there was limited to no CRFR1-ir in the supraoptic nucleus (Table 2.1). For a full list of intensity ratings from this study, refer to Table 2.1.

2.1.2. CRFR1 quantification within selected brain regions: CRFR1-ir cells were quantified with the AVPV/PeN, PeN/Int, and arcuate nucleus. Two-way ANOVA in the AVPV/PeN of indicated a significant main effect of age (F(2, 24) = 8.71, p≤0.001) and sex (F(1, 24) = 21.08, p≤0.0001) (Figure 2.2A). Subsequent post hoc tests revealed a greater number of CRFR1 cells in females than males at P0 (p≤0.01), P4 (p≤0.001), and P21 (p≤0.01) (Figure 2.2A). Two-way ANOVA of PeN/Int CRFR1 cells revealed a significant age effect (F(2, 24) = 9.20, p≤0.001), with a greater number at P0 and P4 compared to P21 (Figure 2.2B). However, there was no sex effect or interaction within the PeN/Int. Two-way ANOVA of arcuate CRFR1 cells revealed a significant age effect (F(2, 24) = 24.11, p≤0.0001), with greater CRFR1-ir cells at P21 compared to earlier ages (Figure 2.2C). No sex effect or interaction was found in the arcuate nucleus. All sampled distributions met the criteria for normal distribution and equal variances.

2.2 AVPV/PeN CRFR1 Phenotypic Profiling
2.2.1. Tyrosine Hydroxylase (TH): Numbers of CRFR1, TH, and CRFR1/TH cells were assessed in male and female mice at P0 and P60. As previously reported we found the same sex difference in CRFR1-ir cells at P0 (females > males; t(8)=2.329, p≤0.05; Figure 2.1). No sex difference was found in the number of TH cells at P0 (Figure 2.3). Neither the percentage of CRFR1 cells that co-express TH (12%) nor the percentage of TH cells that co-express CRFR1 (17%) differed by sex at P0 (Figure 2.3). At P60, CRFR1 (t(8)=2.787, p≤0.05), TH (t(8)=3.755,
p≤0.01), and CRFR1/TH (t(8)=4.165, p≤0.01) cells were more abundant in females than males (Figure 2.4). The percentage of CRFR1 cells that co-express TH (t(8)=5.210, p≤0.001) and the percentage of TH cells that co-express CRFR1 (t(8)=5.802, p≤0.001) were also greater in females than males (Figure 2.4).

2.2.2. Kisspeptin (KISS): Numbers of CRFR1, KISS, and CRFR1/KISS cells were assessed at P60 in male and female mice. CRFR1 (t(8)=5.048, p≤.01), KISS (t(8)=3.285, p≤0.05), and CRFR1/KISS (t(8)=4.435, p≤0.001) cells were more abundant in females than males (Figure 2.5). There was a trend toward a greater percentage of CRFR1 cells that co-express KISS in females compared to males (t(8)=2.168, p=0.07). The percentage of KISS cells that co-express CRFR1 was significantly greater in females than males (t(8)=6.458, p≤0.001; Figure 2.5).

2.2.3. Estrogen Receptor Alpha (ERα): Quantification of dual label CRFR1/ERα co-localization showed that females had significantly more CRFR1/ERα co-localized cells than males (Age P60-70; Figure 2.6A). In addition, the male AVPV/PeN had significantly fewer ERα expressing cells (t(8)=2.691, p≤0.05), few of which co-express CRFR1, as indicated by a significantly higher percentage of CRFR1 cells that co-localize ERα in the female AVPV/PeN (t(8)=5.775, p≤0.001; Figure 2.6B). Co-localization of CRFR1 and ERα is also found in the AVPV/PeN of P0 female mice (Figure 2.6F).

2.2.4. AVPV/PeN CRFR1 co-localization with fluorogold: Quantification of dual label CRFR1/fluorogold co-localization for adult (P60-65) mice indicated that none of the AVPV/PeN CRFR1-expressing cells are neurosecretory (Figure 2.7). Little fluorogold was found near the sexually dimorphic CRFR1 AVPV/PeN cluster (Figure 2.7A-D) regardless of co-expression, although abundant fluorogold and CRFR1/fluorogold co-labeling was found in more caudal periventricular sections (Bregma -0.40; Figure 2.7E-H).
2.3 Neonatal (P0) Hormone Supplement

2.3.1. Neonatal (P0) Hormone Supplement: One-way ANOVA of CRFR1-expressing cells within the female AVPV/PeN revealed a main effect of hormone supplementation on CRFR1 expression ((F(2,14)=188, p≤0.001; Figure 2.8) where both TP and EB injection significantly reduced CRFR1 expression compared with female vehicle-injected animals (p≤0.001) (Figure 2.8 B, D). Post-hoc analysis further revealed a significant difference between treatments, where EB reduced CRFR1 cell number to a greater extent than TP (p≤0.05). One-way ANOVA of CRFR1 cells within the male AVPV/PeN revealed no significant effect of treatment on CRFR1 cell number (Figure 2.8C, E).

2.4 Adult (P60) Gonadectomy

2.4.1. Adult (P60) Gonadectomy (GDX) on CRFR1 Expression in the AVPV/PeN: A two-way ANOVA revealed a significant sex difference in AVPV/PeN CRFR1 cells (F(1,26)=75.50, p≤0.001 reproducing our previous data. However, adult (P60) GDX had no effect on CRFR1 expression in either sex (Figure 2.9).

2.5 AVPV/PeN CRFR1 post-Restraint, GR/CRFR1 co-expression

2.5.1. AVPV/PeN CRFR1 activation (pCREB co-expression) following a 30-minute restraint stress and CRFR1 co-localization with glucocorticoid receptor (GR): To determine whether acute psychogenic stress (30min restraint) would elicit activation of CRFR1 cells in the AVPV/PeN, we assessed the co-expression of CRFR1 and the transcription/neural activation marker phosphorylated (p) CREB. This analysis revealed a greater number of CRFR1/pCREB co-localized cells in females compared to males (t(10)=5.994, p≤0.001; Figure 2.10). The percentage of AVPV/PeN CRFR1 cells co-expressing pCREB was also greater in females (t(10)=2.241, p≤0.05; Figure 2.10). Quantification of dual-label CRFR1-GFP/GR indicated 75% co-localization in the female AVPV/PeN (Figure 2.11).
Discussion

Overall, the phenotypic profiling of the AVPV/PeN CRFR1 cells suggest a role for sexually dimorphic responsivity to circulating glucocorticoids and gonadal steroid hormones. We see that CRFR1 expression in the AVPV/PeN is sexually dimorphic by P0 (Figure 2.1), and is maintained throughout adulthood, independent of circulating gonadal hormones (Figure 2.9). This cell group also co-expresses many hallmark receptors implicated in stress responsivity, including ERα, GR, and shows activation following acute restraint stress (CRFR1/pCREB co-expression).

2.1 Developmental CRFR1 distribution. We investigated developmental distributions in the CRFR1 system between the female and male mouse forebrain, because there is little data on the development in current literature. Amplifying this problem is the fact that no antibodies to CRFR1 exist that are valid indicators of true CRFR1 expression. Therefore, we used an animal model (C57BL BAC CRFR1-GFP) as a validated CRFR1 reporter line (validated against an in situ hybridization for CRFR1, Justice et al., 2008). We report several changes over development, but the most intriguing finding was a stark dimorphic expression of CRFR1 in the female rostral anteroventral periventricular nucleus (AVPV/PeN), absent in the male from P0 onward (Fig 2.1; Rosinger et al., 2017). CRFR1 has been continually implicated in etiology of stress-related mood disorders such as anxiety, depression, and addiction (Chrousos 2009; Garcia-Carmona et al., 2015; da Silva et al., 2016; Holly et al., 2016; Schussler et al., 2016), which all are more prevalent in women than men. Therefore, it is possible that the CRFR1 of the female AVPV/PeN is influenced by stress and involved in stress-related adaptations, and the primary focus of the following studies was to gain a better understanding of the role of these CRFR1 cells for sex differences in the stress response. Regardless, the development of CRFR1 distribution was analyzed and compared across the rest of the mouse brain.
We report additional changes that take place across development, largely independent of sex. Generally speaking, CRFR1 increased with age in several regions including the medial amygdala, arcuate nucleus, paraventricular hypothalamus, medial septum, CA1 hippocampal area, and the lateral habenula. On the other hand, CRFR1 decreased with age in several other regions including the intermediate portion of the periventricular hypothalamic nucleus, and CA3 of the hippocampal formation.

The arcuate nucleus increased expression of CRFR1 as animals, male and female, significantly increased with age. The arcuate is responsive to psychological stress and the administration of CRF. Also, arcuate kisspeptin neurons are inhibited by local CRF administration (Li et al., 2010). In comparison, PVN CRFR1 levels were generally low (close to non-existent) until closer to P21, at which point there was a slight increase. However, we have later shown elsewhere that a sex difference in PVN CRFR1 does in fact appear (M > F; Rosinger et al., 2019b). This change is not present until after puberty (apparent by P60, but not at P21), illustrating that the CRFR1 system is still immature until a time point post-puberty. Others have shown that PVN CRF neurons project directly to PVN CRFR1 neurons (Ramot et al., 2017), and thereby modulate the stress response over time. Further information regarding the appearance of a male-biased sex difference in PVN CRFR1 will be addressed in chapter 4, investigating the influence of chronic variable stress on CRFR1 populations.

The MPOA was stable across the developmental time-points that were investigated (P0-P21), there were no sex differences in the expression of CRFR1, either. In general, there are anatomical sex differences in the MPOA (Morris et al., 2004), and the MPOA is known to influence sex-related behavior in rodents (Nutsch et al., 2016). Though we found no differences in CRFR1 expression, others have shown a sex difference in MPOA CRF (F>M) (McDonald et al., 1994; Funabashi et al., 2004). The MPOA is a site at which the HPA and HPG axes converge, and researchers have shown that androgens or estrogens binding cognate receptors
in the MPOA can modulate CRF and AVP release through projections to the PVN (Williamson and Viau, 2007; Williamson et al., 2010). Moreover, there are several sex differences in the MPOA distribution of both ERs and AR, and it is therefore possible that there are sex differences in the co-localization of MPOA CRFR1 and steroid hormone receptors, though further investigation is required.

When looking at CRFR1 within the bed nucleus of the stria terminalis, we found a moderate decline from P0 to P21, but the levels remained high compared with other regions throughout the entire developmental period. We did not find any sex differences in CRFR1 labeling, but others have shown in the rat that CRF is sexually dimorphic in expression, where females have more than males (Funabashi et al., 2004). Others have investigated CRFR2 as opposed to CRFR1, and in the vole there is a sex difference in BST CRFR2 binding, which was significantly higher in males than females (Lim et al., 2005), a finding that was replicated in the rat (Wealthington et al., 2014). CRFR2 is anxiolytic, and a global knockout of CRFR2 leads to increased anxiety-like behavior, and is likely involved in toning down HPA activity and behavioral response to stressors (Kishimoto et al., 2000; Reul and Holsboer, 2002).

The hippocampus mainly regulates the HPA axis through glucocorticoids binding GR. CA1 of the hippocampus displayed the greatest density of CRFR1 labeling of all regions we investigated. Within CA3 of the hippocampus, we noted some developmental changes in expression patterns with high levels at P0 and P4, and a decrease by P21. Contrasting our data, the adult DG has been shown to express CRFR1 in the granule cell layer (Rissman et al., 2012). CRF signaling in the DG is involved in learning and consolidation of fear memories (Blank, et al., 2002), therefore, an absence of CRFR1 in the granule cell layer may impact development of fear associated memories early in life (Akers et al., 2012). The hippocampus is sensitive to effects of stress exposure (Kleen et al., 2006; Czeh et al., 2015) and is involved in modulating anxiety- and depression-like behaviors in rodents (Fanselow & Dong, 2010), and
with humans, after the onset of major depression (Frodl et al., 2002). Further, CRF levels fluctuate during postnatal development, with an expression peak between P11-18, after which they begin to drop to approximate adult levels (Chen et al., 2001). Chronic CRF exposure to a developing hippocampus leads to adult hippocampal dysfunction, including cognitive impairment in both mice and rats (Ivy et al., 2010; Liao et al., 2014). In vitro administration of CRF reduces dendritic arborization, which is prevented by CRFR1 inhibition (Ivy et al., 2010).

Finally, investigation of amygdala sub-regions showed changes in CRFR1 during early postnatal development. The medial (MeA) and lateral amygdala (LA) showed increased CRFR1-ir at each developmental time point. The LA relays to the basolateral amygdala (BLA) via excitatory efferent pathways, from which the BLA projects to the CeA (Shekhar et al., 2005) and behavioral/emotional responses are then initiated. Therefore, increased CRFR1-ir within the LA indicates that CRF signaling within this region suggests potential changes in emotional regulation throughout development. The olfactory bulb projects to the amygdala and the MeA receives many of these inputs and controls both social and sexual behavior (Cushing et al., 2008; Sano et al., 2016). Within the CeA, however, CRFR1 is stable between P0 and P21, largely located within the medial subdivision of the CeA. CRFR1 in the CeA has been linked with different behaviors and biological functions including anxiety, alcohol dependence, and nociception, of which all have known sex differences (Haramati et al., 2011; Rouwette et al., 2012). The reported sex differences in CeA-mediated changes is likely not related to CRFR1 signaling alone, although it is possible that between puberty and adulthood, this changes.

Overall, developmental expression of CRFR1 is dynamic in the mouse, with sex and developmental differences in discrete areas; the data from our CRFR1 development investigation prompted a more detailed analysis of the sexually dimorphic expression of the female AVPV/PeN CRFR1. We thus performed several studies to hone in on the likelihood of these cells being involved in the female stress response.
2.2 Phenotypic/Chemical Characterization of AVPV/PeN CRFR1. After exploring the development of the CRFR1 system and finding sexually dimorphic CRFR1 expression within the AVPV/PeN, we aimed to understand the chemical composition of these cells. Therefore, we conducted a series of dual-label fluorescent immunohistochemical staining. There are other AVPV/PeN cell phenotypes that have been established as dimorphic (i.e., TH, KISS) and have been shown in the same pattern as what we report with CRFR1 (F>M) (Semaan et al., 2010; Poling & Kaufman, 2013; Scott et al., 2015), further suggesting this region of the brain may be more strongly implicated in the female neuroendocrine responses than in the male.

Our findings indicate that the CRFR1-expressing cells of the female AVPV/PeN are largely distinct from these other dimorphic cell groups. Specifically, the majority of CRFR1 cells of the AVPV/PeN do not co-express TH (only ~22%). Further, the majority of TH-expressing cells also do not co-express CRFR1 (~18% co-expression); importantly, the CRFR1 sex difference is present at P0, showing a relatively stable lack of co-expression pattern from the perinatal period into adulthood (P60). Other dimorphic expression such as the TH and KISS do not appear until later in postnatal development (Waters and Simerly, 2009; Poling and Kauffman, 2013), which could influence the development of the other sex differences in the AVPV and adjacent regions such as the MPOA. CRFR1 has already been shown to influence development of the olfactory bulb (Garcia et al., 2016), and it is possible that CRFR1 of the AVPV/PeN is serving a similar purpose, driving other developmental changes. The preoptic area has been implicated in maternal behavior for decades (Numan et al., 1977), and TH has been highlighted as a vital transmitter for the proper display of maternal behavior (Champagne et al., 2004; Stolzenberg et al., 2007). Numan and colleagues (1984) showed that it is preoptic projections to dopaminergic pathways like the ventral tegmental area, that allow for the display of maternal behavior. The expression of TH is dynamic, and changes on the basis of maternal experience (Scott et al., 2015). This collectively suggests that CRF signaling within the
AVPV/PeN could influence dopamine transmission, and thereby influence associated functions such as normal display of maternal or reproductive behaviors.

KISS+ cells showed a greater percentage of co-expression with CRFR1 cells (~35%) than TH/CRFR1 (~22%), showing that CRF can influence KISS function in the AVPV/PeN, including potentially disrupting KISS signaling, however most CRFR1 cells do not co-express KISS. This could theoretically contribute to stress-related changes in reproductive function. This theory is attractive because others have shown that CRF signaling in both the preoptic area and the arcuate nucleus block KISS signaling to GnRH neurons (Kinsey-Jones et al., 2009), which are vital for pulsatile LH releases, needed for normal ovulation, and typical ovarian hormone surges daily and across the estrus cycle. Research by Kinsey-Jones and colleagues (2010) shows that intracerebroventricular CRF infusion can significantly delay the onset of puberty in the female rat. Therefore, CRF binding CRFR1 in the female AVPV/PeN cells may be a key part of this pathway by which CRF inhibits female pubertal onset, though more information is needed with the mouse model. However, the majority of CRFR1 cells of the AVPV/PeN do not co-express KISS, and therefore likely serve multiple functions.

Rodent AVPV cells project to several fore- and hindbrain structures involved with reproductive and stress behaviors, as well as hormone release (medial preoptic area, ventral lateral septum, arcuate nucleus, paraventricular nucleus, and periaqueductal grey) (Gu & Simerly, 1997; Scott et al., 2015). These previous findings, in conjunction with our more recent data (Rosinger et al., 2019a), suggest the possibility that the CRFR1 cells of the female AVPV/PeN are involved in these aspects of behavior and control over hormone release. We investigated the anterograde projection patterns for the CRFR1 cells of the AVPV/PeN (see chapter 3) to further address this question. We do know that these cells are not neurosecretory (fluorogold, Figure 2.7A-D), instead suggesting their role in central signaling.
2.3 Neonatal (P0) Hormone Supplement on Development of Dimorphic AVPV/PeN CRFR1-GFP. The sex difference in CRFR1 is present from P0, suggesting a role for early life hormones in the organization of the nucleus. Previous research dating back decades, has consistently shown a role for perinatal androgen surges are responsible for masculinizing many hypothalamic and limbic structures (Raisman & Field, 1971; Morris et al., 2004). Our investigation therefore sought to determine the role for perinatal androgens in the masculinization of AVPV/PeN CRFR1 expression. We suspected that neonatal treatment with either testosterone, or the aromatized metabolite 17β-estradiol would masculinize the female, and not the male AVPV/PeN, because males would already be exposed to the perinatal androgen surge, regardless. This is in fact just what we found. As seen in Figure 2.8, neonatal treatment with either testosterone or estradiol successfully masculinized the female AVPV/PeN CRFR1 cell cluster. Further, we found that treatment with estradiol was more efficient in masculinizing the female AVPV/PeN than testosterone. We believe that, while estradiol can bind to either ERα or –β to exert its effects, it is likely that ERα is the receptor at which estradiol acted to masculinize this region. This is likely to be the case because we have demonstrated that there is a high level of ERα/CRFR1 co-expression at the time of birth in females (Figure 2.6), and previous work has shown that the AVPV/PeN is largely devoid of ERβ at the P0 time point (Zuloaga et al., 2014). It is most likely not regulated by the androgen receptor either; others have shown limited expression of AR anywhere around the AVPV/PeN at P0 (Juntti et al., 2010; Kanaya et al., 2014), and in addition, estradiol masculinized the nucleus to a greater extent than testosterone, further reinforcing that it is not a result of binding AR.

Apoptosis may be responsible for the change in expression following early life androgen exposure, because others have shown perinatal androgen surges masculinize the AVPV through apoptosis (Forger et al., 2004; Varnum et al., 2013). In our previous investigations, CRFR1 expression gradually declines in the female and male between P0 and P60 (Figure 2.1)
remaining stable into later life; this may reflect further/ongoing apoptosis of AVPV/PeN CRFR1 beyond early postnatal development. However, it is equally possible that androgen surges masculinize the expression of CRFR1 within neurons of the AVPV/PeN. The cells we see in the female may still exist in the male, just not expressing CRFR1. To test the hypothesis that apoptosis is responsible for the AVPV/PeN, future studies could be performed to label for markers of apoptosis in female and male perinatal mice. In addition, we noted that the sex difference in CRFR1 appears before other known sex differences (i.e., TH, KISS; Figure 2.3; Poling and Kauffman, 2013; Waters and Simerly, 2009). Therefore, the early expression of CRFR1 may influence subsequent development of other dimorphic cell phenotypes. Others have shown that CRF/CRFR1 signaling is vital for the development of synapses and dendritic contacts in the olfactory bulb (Garcia et al., 2014). Currently we have no direct information supporting the role of AVPV/PeN CRFR1 expression guiding development of other regions or cell phenotypes, but more research is certainly warranted in the future.

2.4 Adult (P60) Gonadectomy (GDX) on Maintenance of Sexually Dimorphic AVPV/PeN CRFR1. We have established that perinatal androgen exposure masculinizes AVPV/PeN CRFR1 expression. We then investigated the role of circulating gonadal hormones on the maintenance of the dimorphic CRFR1 expression in adulthood. If the AVPV/PeN CRFR1 required circulating ovarian hormones to maintain expression, then adult female GDX would result with less or no cells expressing CRFR1, mirroring the AVPV/PeN of male mice. We hypothesized that adult GDX would not change the male AVPV CRFR1 expression, because these animals have limited expression of these cells at all times leading up to GDX. Six weeks post-GDX, adult female and male BAC CRFR1-GFP mice were sacrificed to investigate if AVPV/PeN CRFR1 distribution was impacted by GDX in either sex. The 6-week recovery time point was picked based on other work in mice that showed a change in AVPV KISS-expression following the same 6-week recovery period (Brock and Bakker, 2013). Six weeks post-GDX, we
saw no changes in the female or male AVPV/PeN CRFR1, suggesting that the nucleus is organized during the perinatal period, and resilient to changes in adulthood circulating gonadal hormones. However, we reported in the perinatal data, that P0 supplement with androgens, such as testosterone, are capable of masculinizing the nucleus likely through actions at ERα.

Another factor is that we did not monitor estrus phase in this portion of the investigation. It is theoretically possible that animals could show a difference if visualized during proestrus, in which estrogens are increased, compared with GDX females. However, we doubt this is a factor, because the previous study mentioned (Brock and Bakker, 2013) did not monitor estrus cycle either, but showed reduced KISS expression in GDX female mice compared to gonad-intact female counterparts. In addition, our female GDX data show almost identical expression as gonadal intact females, with low levels of variation, further suggesting that phase-specific changes are not confounding the reported data. It is possible that other hormones such as prolactin or glucocorticoids could alter CRFR1 levels, and there is a portion of our lab that is investigating the impact of post-partum experience on AVPV/PeN CRFR1 expression. It is possible that there were transient changes in CRFR1 expression at an early time-point following the GDX, and other factors may compensate over time. However, this is outside of the scope of the current series of investigations, and will not be covered directly. Going forward, it would be interesting to perform estrus phase-wide monitoring of AVPV/PeN CRFR1, because others have shown altered glucocorticoid receptor levels based on the estrus phase in rats (Ramos-Ortolaza et al., 2017), and ovarian hormone levels in macaques (Sanchez, Reddy, and Bathea, 2010). In mice, some have reported that proestrus females have increased membrane density of CRFR1 compared with males in pyramidal cell dendrites (Williams et al., 2011). Regardless, the current data suggest that adult GDX on either sex has no influence on the expression pattern of CRFR1 in the AVPV/PeN.
2.5 AVPV/PeN GR/CRFR1 and CRFR1 activation following stress. We aimed to determine the GR/CRFR1 co-expression in the female and male mouse AVPV/PeN, and pCREB/CRFR1 following a single 30-minute restraint stress. Following histochemical analyses, we report the female AVPV/PeN co-expressed GR on ~75% of CRFR1-expressing cells (Rosinger et al., 2019a). However, these studies did not inform us of the definitive role; while we can be sure that about 75% of these cells are capable of binding corticosterone, we still didn’t know if they play an active role in driving sex-specific stress-related adaptations. We also reported a high level of pCREB/CRFR1 co-expression in the female after a single 30-minute restraint; this was not observed in stressed males or non-stressed female controls (Rosinger et al., 2019a). Due to the extended expression of pCREB in AVPV/PeN of stressed females (still present 60 minutes post-stress), we believe that these cells are involved in the stress response, thereby implicating a female-specific site at which circulating gonadal hormones and glucocorticoids can bind and potentially alter cellular function.

**Conclusion:** The investigations that comprise this chapter converge on furthering our understanding of a novel sex difference in CRFR1 expression within the POA. These cells are organized during the perinatal period, and maintain the dimorphic expression throughout development, adulthood, and into old age, regardless of adult circulating gonadal hormones. These factors were directly addressed through the neonatal hormone supplement and adult GDX studies. We report that the majority of the CRFR1-expressing cells in the female AVPV/PeN are distinct from previously known sex differences (e.g., TH, KISS) (Semaan et al., 2010; Poling & Kaufman, 2013; Scott et al., 2015). Moreover, we know these cells are involved in central signaling, due to the lack of fluorogold co-expression (Figure 2.7; Rosinger et al., 2019a). The female AVPV/PeN CRFR1 have a high level of GR co-expression and show pCREB-immunoreactivity following an acute stressor, indicating a female-specific site that responds to acute psychological stress. The prolonged pCREB expression following a single 30-
minute restraint stress points to the importance of investigating other alterations following stress. In addition, understanding the afferent and efferent connectivity of these CRFR1 cells will help us to establish if there is direct communication with known stress-responsive brain regions.
Figure 2.1. CRFR1 expression within the rostral region of the anteroventral periventricular nucleus (AVPV/PeN). (A) Representative images from female and male AVPV/PeN during developmental periods P0, P4, and P21. (B) High magnification images illustrating the AVPV/PeN for female and male subjects during the listed developmental periods. The dense clustering of CRFR1-ir can be seen clearly near the 3rd ventricle of females but not males (right). N = 3-4 per age/sex.
Table 2.1. Distribution of CRFR1 cells in the developing mouse forebrain. Density and intensity of CRFR1-ir was rated visually during developmental periods P0, P4, and P21, as having −, No label; +/−, minimal but still noticeable labeling intensity/density of cells; +, low labeling intensity/density of cells; ++, moderate labeling intensity/cell density; ++++, high labeling intensity/cell density; ++++, very high labeling intensity/cell density.

<table>
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<th>P21</th>
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<tr>
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<tr>
<td>inner cortical layers</td>
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Table 2.1. Distribution of CRFR1 cells in the developing mouse forebrain. Density and intensity of CRFR1-ir was rated visually during developmental periods P0, P4, and P21, as having −, No label; +/−, minimal but still noticeable labeling intensity/density of cells; +, low labeling intensity/density of cells; ++, moderate labeling intensity/cell density; ++++, high labeling intensity/cell density; ++++, very high labeling intensity/cell density.
Figure 2.2. Quantification of CRFR1 within selected brain regions. CRFR1-ir cells were counted in selected brain regions of P0, P4, and P21 mice. (A) In the AVPV/PeN, there are significant sex differences at all age groups, with females having more CRFR1-ir than males. (B) Within the intermediate division of the periventricular nucleus, there were no sex differences, but there were significantly fewer CRFR1-ir cells, regardless of sex, by P21, in comparison to P0 and P4. Finally, within the arcuate, there was a significant increase in immunoreactivity at P21 in comparison to P0 and 4. * Indicates sex difference, p < 0.01. # Indicates significant difference between P21 and other age groups, p<0.01. Data are shown as mean ± SEM. AVPV/PeN; ateroventral periventricular nucleus of the hypothalamus, periventricular nucleus (Int); periventricular nucleus of the hypothalamus, intermediate division.
Figure 2.3. Dual-label fluorescent immunohistochemistry comparing P0 male and female tyrosine hydroxylase/CRFR1. Female (A-D) and male (E-H) AVPV/PeN CRFR1, tyrosine hydroxylase (TH), and co-localization of CRFR1/TH on the day of birth (D, H; high-magnification display of images C, G respectively). (I) Comparison of male versus female CRFR1- and TH-expressing cells (green, red, respectively), and co-expression of TH/CRFR1 (yellow) illustrating a significant sex difference in P0 CRFR1 (F>M), but no observed differences in TH-ir. (J) Female versus male comparison of the percentage of CRFR1 cells that co-localize TH, and percentage of TH cells that co-express CRFR1. * Indicates statistical significance (p≤0.05), data reported as mean ± SEM. Inset boxes (C, G) indicate regions that were further magnified (D, H). White arrows indicate examples of co-localized cells. N=5 per sex.
Figure 2.4. Dual-label fluorescent immunohistochemistry comparing adult (P60) male and female tyrosine hydroxylase/CRFR1. Female (A-D) and male (E-H) AVPV/PeN CRFR1, tyrosine hydroxylase (TH), and co-localization of CRFR1/TH (D, H; high-magnification display of images C, G respectively). (I) Comparison of female versus male CRFR1- (green), TH-expressing cells (red), and co-expression of TH in CRFR1-GFP cells (yellow) illustrating significant sex differences in P60 CRFR1-ir, TH-ir, and co-labeled cells (all F>M). (J) Female versus male comparison of the percentage of CRFR1 cells that co-localize TH, and percentage of TH cells that co-express CRFR1 (both F>M). * Indicates statistical significance (p≤0.05), data are reported as mean ± SEM. Inset boxes (C, G) indicate regions that were further magnified (D, H). White arrows indicate examples of co-localized cells. N=5 per sex.
Figure 2.5. Dual-label fluorescent immunohistochemistry comparing adult (P60) male and female kisspeptin/CRFR1. Female (A-D) and male (E-H) AVPV/PeN CRFR1 (A, E), kisspeptin (KISS) (B, F), and co-expression (C, G). (D, H) high magnification display of images c, g are displayed respectively. (I) Comparison of female and male CRFR1-ir and KISS-ir, with significant differences in the number of CRFR1-ir (F>M), KISS-ir (F>M), and co-localized cells (F>M). (J) Female versus male comparison of the percentage of CRFR1 cells that co-localize KISS, and percentage of KISS cells that co-express CRFR1 (both F>M). * Indicates statistical significance (p≤0.05), data are reported as mean ± SEM. Inset boxes (C, G) indicate regions that were further magnified (D, H). White arrows indicate examples of co-localized cells. N=5 per sex.
Figure 2.6. Dual-label fluorescent immunohistochemistry comparing female and male CRFR1/ERα co-expression. (A) Co-localization of CRFR1 and ERα, and (B) illustrates this sex difference in ERα is also present in the percentage of CRFR1 cells that express ERα. Females therefore have more CRFR1 in general, and the few CRFR1 cells in the male do not co-express ERα. Dual-label fluorescent immunohistochemistry showing that females have significantly more CRFR1, ERα, CRFR1/ERα co-localized, and a higher percentage of CRFR1 cells that co-express ERα compared to males. (C-F) Representative images from a female AVPV/PeN showing a low magnification image of co-localized cells (C), CRFR1 (D; green), ERα (E; red), and co-expression of CRFR1/ERα (F; yellow). (G) Image showing co-expression of CRFR1 and ERα on the day of birth (P0) in the female BAC-CRFR1-GFP mouse AVPV/PeN. Arrows indicate examples of co-localized cells. * Indicates statistical significance (p≤0.05), all data are reported as mean ± SEM. N=5 per sex. ERα, estrogen receptor alpha.
Figure 2.7. Peripheral fluorogold injections do not back-fill cells of the sexually dimorphic region of AVPV/PeN CRFR1. Images showing fluorogold and CRFR1 labeling. (A-D) Few neurosecretory (fluorogold) were found near the sexually dimorphic CRFR1 AVPV/PeN nucleus and no CRFR1/fluorogold co-labeled cells were found. (E-H) However, high levels of fluorogold and CRFR1/fluorogold co-labeling was found in further caudal periventricular sections, where both females and males express CRFR1. Arrows indicate examples of co-localized cells. All images are from a female mouse (male not shown).
Figure 2.8. Neonatal hormone treatment with EB or TP masculinizes female AVPV/PeN CRFR1-ir. Neonatal (P0) hormone supplement influence on CRFR1 cell number in (A) female versus male mice treated with sesame oil (VEH), testosterone propionate (TP), or estradiol benzoate (EB). P0 treatment with either TP or EB significantly reduced the cell number in female mice, while having no impact on the male AVPV/PeN CRFR1 cells. Female treatment with EB reduced CRFR1 to a greater extent than TP. Representative images from females treated with (B) VEH, (C) TP, (D) EB, and a male treated with (E) VEH. * Indicates statistical significance (p≤0.001), + indicates p≤.05. Data are reported as mean ± SEM. N=6-7 per sex/treatment.
Figure 2.9. Adult (P60) gonadectomy has no effect on CRFR1 cells in females or males. (A) Gonadectomy did not alter CRFR1 cell number in either females or males. Representative images from female (B) and male (D) sham surgery compared with female (C) and male (E) gonadectomy, showing that removal of adult gonadal hormones does not change the expression pattern. * Indicates significant sex difference (p≤0.001), data are reported as mean ± SEM. N=8 per sex/treatment.
Figure 2.10. Dual-label fluorescent immunohistochemistry showing AVPV/PeN CRFR1 sex- and stress-specific cellular activation following acute restraint stress. (A) The female versus male expression levels of CRFR1 (F>M), and pCREB/CRFR1 co-localization (F>M). (B) Comparison between female and male co-expression, showing a sex difference in the percentage of activated CRFR1 cells (pCREB co-expression) following a 30-minute restraint stress (F>M). Representative images of a female AVPV/PeN from a (C) s; stressed and (D) ns; non-stressed mouse. * Indicates statistical significance (p≤0.05), data are reported as mean ± SEM. Arrows indicate examples of co-localized cells. N= 5 male, 7 female mice (males not shown).
Figure 2.11. Dual-label fluorescent immunohistochemistry showing CRFR1/GR co-expression in the female AVPV/PeN. The number of (A) CRFR1 and CRFR1/GR co-expressing cells, and (B) the percentage of female AVPV/PeN CRFR1 cells that express GR (~75%). (C) Representative image of CRFR1 (green) and GR (red) co-expression (yellow), and (D) high magnification image of co-localized cells in the AVPV/PeN. Inset box in (C) indicates region further magnified in (D). White arrows indicate examples of co-localized cells. N= 4 female mice.
Chapter 3: Anterograde Projection Sites of AVPV/PeN CRFR1

Introduction

Our lab investigated the chemical composition of CRFR1 cells expressed in the female AVPV/PeN, and have shown that they are largely distinct from other known sexually dimorphic cell phenotypes, are sensitive to perinatal androgen surges, not dependent upon adult circulating ovarian hormones, and respond to acute psychological stress (Rosinger et al., 2017, 2019a). The data our lab has collected continue to point to female AVPV/PeN CRFR1 cells as a female-specific cell group that contributes to mediating observed sex differences in HPA axis activity and anxiety-like behaviors. Moreover, the AVPV as a structure is volumetrically larger in females, and early lesion studies point to its importance in spontaneous ovulation, and without an intact AVPV, LH surges are eliminated (Wiegand & Terasawa, 1982). In addition, the preoptic area in general is involved in changes following various types of stressor modalities (Scammell et al., 1993; Dobson et al., 2003; Jankord and Herman, 2008; Kinsey-Jones et al., 2009), including stress-induced changes in maternal care (Klampfl et al., 2018).

According to previous tract tracing research for the AVPV, we know that the cells in the female rat AVPV project to many hypothalamic and limbic areas all associated with both stress and reproductive behaviors, such as the PVN, PAG, MPOA, ARC, and ventral-lateral septal nuclei (Gu and Simerly, 1997). However, these data are limited to anterograde projections from non-specific AVPV cells in the rat, with no information for the mouse, or AVPV CRFR1 projection patterns in any species. We therefore aimed to establish the specific anterograde projection patterns of the CRFR1-expressing cells in the female AVPV/PeN to identify potential sex-specific circuits that mediate stress responses. We hypothesized that the female CRFR1 cell cluster in the AVPV/PeN would have efferent projections with hypothalamic, preoptic, and
limbic systems. Data along these lines would further implicate the AVPV/PeN involvement with observed sex differences in HPA activity and stress-induced behavioral changes.

In the present series of studies, we performed anterograde (N=6 females, 3 “hit”, 3 “miss”) tracer infusions into the female AVPV/PeN to determine projection patterns of the CRFR1 cells, through the use of two anterograde single-synapse rAAV tracer that binds and fills CRE-expressing cells in a mouse line bred to express CRE on CRFR1 cells. Our tracing data show that the female CRFR1 nucleus within the AVPV/PeN sends dense projections to many regions implicated in both sex and stress behavior.

Based on our current data, these cells send dense anterograde projections to the PVN, BST, PAG, among other regions. Coupling these data with the acute restraint-stress induced cell activation in the female AVPV/PeN CRFR1 cells, our present tracing studies provide more evidence that female AVPV/PeN CRFR1 cells are involved in the stress response, communicate with other regions known to regulate stress and reproduction, and therefore may contribute toward regulation of sexually divergent stress responses through these projections.

**Methods**

**Animals:** Heterozygous CRFR1-CRE+/− male mice on a C57BL background were bred with C57BL/6J wild-type female mice, and offspring were genotyped for expression of iCRE (1100bp) using the primers: R1-flipdown-5’ [CCAGTGCCCACCAGCCTTGTCCTAATAA] and R1-flipdown 3’ [TGTCCACCCCGCAGCCCAGAT], and actin364 used as the positive control assuring DNA presence in each sample. For DNA amplification: 64°C annealing temperature, 15 seconds for 30 cycles. Female mice that were CRE+ were used for unilateral anterograde AAV tracer infusions. Mice were assigned a “hit” or “miss” category following perfusion/histochemical staining through atlas matched identification. For animals to be assigned in the “hit” category, there needed to be cell body labeling within the range from rostral-most portion (bregma +0.30mm, DV 5.25-5.40mm), through the mid-point (bregma
+0.02mm, DV 4.80-5.45mm), and no labeling past the caudal-most portion (bregma: -0.22mm, DV 4.85-5.45mm). Animals that showed immunolabeling at any points outside of these ranges were then assigned into the “miss” category, and used as control infusions. In our animals, there is only CRFR1+ cell labeling within the PeN, and lateral to the AVPV/PeN CRFR1, there are no cells labeled. Therefore, as long as the infusion took place within the AVPV/PeN and we saw no CRFR1+ cells in regions adjacent, such as the MPOA or BST, we considered the infusion successful in AVPV/PeN-specific CRFR1-expressing cells.

**Stereotaxic Surgery:** Female mice were anaesthetized and maintained under an inhaled oxygen/isoﬂurane mixture (~2.5%–3%). After the head was secured in position, the skull was exposed with an incision along the midline scalp. Using the stereotactic coordinates (Bregma; AP: +0.25—0.15, ML: +0.10, DV: 5.47—5.43), to target the AVPV/PeN, I drilled a small hole into the skull, followed by a unilateral injection of virus into the left hemisphere of the AVPV/PeN. The needle was a Hamilton syringe (Hamilton Co; 10µL syringe, 30gauge replaceable needle), and was inserted with tracer delivered over a 1-minute timed pressure injection; the needle was then left in place for an additional minute before withdrawal) using a computer-controlled stereotaxic apparatus (Neurostar Robot Stereotaxic; SD326). Following surgery, animals were given 5 weeks to recover before intracardial perfusion and tissue collection.

**rAAV5/Ef1a-DIO-EYFP; single-synapse anterograde tracer:** To visualize anterograde projection paths from AVPV/PeN CRFR1 expressing cells, we used a previously validated viral tracer that binds CRE and fills CRE-expressing cell soma and axons, ﬂuorescing, and therefore showing the afferent fiber projections (Scott et al., 2015) (UNC GTC Vector Core, rAAV5/Ef1a-DIO-EYFP tracer). We injected 0.1µL over a 1-minute timed pressure injection into the stereotaxic coordinates for the left hemisphere of the AVPV/PeN (coordinates listed above) of female CRFR1CRE/+ mice (N=6 females; 3 hit, 3 miss). Following surgical injection, animals were given 5 weeks to recover, based on previous studies using the same tracer (Scott et al., 2015). Prior to
euthanasia, animals were placed into restraint for 30 minutes, and perfused one hour after restraint was completed. Animals were perfused immediately before tissue was collected for processing.

**Perfusion, Tissue Collection and Processing for Immunohistochemistry:** All mice for the tracing studies were injected with an overdose of ketamine (100 mg/kg)/xylazine (10 mg/kg)/acepromazine (3 mg/kg) (KXA) and, once anesthetized, intracardially perfused with 20 ml phosphate buffered saline (PBS), followed by 40 ml 4 % paraformaldehyde. Brains were then removed, stored in 4 % paraformaldehyde overnight, and then transferred to 30 % sucrose until fully dehydrated for sectioning. Brains were sectioned using a cryostat (Microm, HM 505E, Waldorf, Germany) into three alternate series at 40\(\mu\)m thickness.

**Immunohistochemistry:** To visualize the tracers, we performed a fluorescent staining to enhance GFP (EGFP). Briefly, sections rinsed in PBS, then incubated in 4% NDS and 0.3% PBS-TX (1 hour). Immediately following incubation, tissue went into the primary antisera (chicken, GFP; abcam; 1:1000), and incubated at room temperature overnight. The following day, tissue rinsed in PBS and then was placed into secondary antisera (donkey anti-chicken, 488; 1:1000) in 4% NDS and PBS-TX for 2.5 hours. After, tissue was thoroughly rinsed in PBS, and mounted on microscope slides. The tissue was coverslipped with Santa Cruz hard set mounting media as soon as the slides were dry.

**Microscopic analysis:** Analysis of CRFR1 distribution was conducted on a Nikon 80i microscope equipped with a digital camera. The AVPV/PeN and regions that showed labeled fibers were identified using the Allen institute mouse brain reference atlas (Lein et al., 2007). Images were collected at a 20x objective unless stated otherwise. Following staining, successful AVPV/PeN infusion sites were identified by comparison with atlas-matched sections (Lein et al., 2007), through overlaying images taken with DAPI from our mounting media (wavelength 358nm) to confirm region specificity. The brain was scanned at 10X to search for projection...
labeling for the anterograde tracer. When a region showed GFP+ fibers (anterograde-labeled), the fiber density was rated based on the following scale: --, no label; -/+ , minimal labeling; + moderate density; ++ dense labeling; +++ very dense labeling, similar to previous density rating methods published from our lab (Rosinger et al., 2017). An average for all successful tracer infusions was taken for each brain region density score to give an overall projection density rating (N=3 females; Table 3.1).

Results
Infusion sites: We report successful infusions in 3 females, and 3 missed hits. Additionally, there were several infusions that showed no labeling in the brain at all, suggesting that the needle tip was occluded while being lowered to the proper DV coordinate. The successful infusions, or “hits” were based on cell body labeling within the coordinate range described above in the methods. The first animal (shown in Fig 3.2A) showed densest infusion labeling at the mid-point range, defined above. The second animal showed densest infusion labeling at the rostral-point, and had primarily labeling toward the ventral end of the DV coordinates (~5.40mm; not shown). The third successful infusion showed densest labeling at the mid-point range, with primary labeling toward the ventral portion of the DV range (~5.40mm; not shown). All three animals with successful infusions showed labeling throughout the rostro-caudal extent of the AVPV/PeN, and no cell labeling lateral to AVPV/PeN CRFR1 cells (i.e., ML: ~+0.10-0.125mm).

Projections: Table 3.1 indicates the full list of relative density for projections for anterograde tracer-infused female C57BL6 CRFR1CRE+ mice. Following successful AAV tracer infusion into the female AVPV/PeN, we report that this cell cluster contains dense efferent projections to several portions of the hypothalamus, preoptic area, and hindbrain.

Several subcortical structures showed anterograde labeling. Regions included the PVN (very dense; Table 3.1, Figure 3.3A), surrounding AVPV (dense; Table 3.1), MPOA (very dense
labeling; Table 3.1, Figure 3.4), DMH (very dense; Figure 3.5A), and the paraventricular thalamus (PVT, moderate; Table 3.1).

The ventral lateral septum, BSTpr, and BSTdl all had dense labeling (Table 3.1, Figure 3.6). The medial amygdala and ventral tegmental area showed moderate anterograde labeling (Table 3.1). Finally, toward the hindbrain, there were additional regions that showed labeling, including the locus coeruleus (LC; moderate, Table 3.1), dorsal PAG (moderate; Figure 3.7A), dorsal raphe (light; Figure 3.7B), Barrington’s (moderate; Figure 3.7C), and parabrachial nuclei (moderate; Figure 3.7D).
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<th>Region</th>
<th>Anterograde Density</th>
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<tr>
<td>Lateral Septum (ventral)</td>
<td>++</td>
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<tr>
<td>Rostral Septum</td>
<td>--</td>
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<tr>
<td>MPOA</td>
<td>+++</td>
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<tr>
<td>AVPV (incl AVPV/PeN)</td>
<td>++</td>
</tr>
<tr>
<td>PVN</td>
<td>+++</td>
</tr>
<tr>
<td>DMH</td>
<td>+++</td>
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<tr>
<td>VMH</td>
<td>--</td>
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<tr>
<td>Anterior Hypothalamus</td>
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<td>Lateral Hypothalamus</td>
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<tr>
<td>Tuberal Nucleus</td>
<td>++</td>
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<td>PVT</td>
<td>+</td>
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<td>Arcuate Nucleus</td>
<td>++</td>
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<tr>
<td>MeA</td>
<td>-/+</td>
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<td>Basal Amygdala</td>
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<td>Cortical Amygdala</td>
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<tr>
<td>BSTdl</td>
<td>++</td>
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<tr>
<td>BSTav</td>
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<tr>
<td>BSTpr</td>
<td>++</td>
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<tr>
<td>Ventral Tegmental Area</td>
<td>+</td>
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<tr>
<td>Locus Coeruleus</td>
<td>+</td>
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<tr>
<td>Dorsal Tegmental</td>
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<tr>
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<tr>
<td>Lateral Ventral Parabrachial</td>
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<tr>
<td>PAG Dorsal</td>
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<tr>
<td>PAG Ventral</td>
<td>--</td>
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<tr>
<td>Dorsal Raphe</td>
<td>-/+</td>
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<tr>
<td>Median Raphe</td>
<td>--</td>
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<tr>
<td>Supraoptic Nucleus</td>
<td>+</td>
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Table 3.1. Anterograde projection sites with relative density. Regions were assigned the following scores to determine relative anterograde fiber label densities: -- no label; -/+ minimal labeling; + moderate density; ++ dense labeling; +++ very dense labeling. MPOA, medial preoptic area; AVPV, anteroventral periventricular nucleus; PVN, paraventricular nucleus of the hypothalamus; DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; PVT, paraventricular thalamus; MeA, medial amygdala; BSTdl, bed nucleus of the stria terminalis dorsolateral; BSTav, anteroventral BST; BSTpr, principle nucleus of the BST; PAG, periaqueductal grey.
Discussion

Based on previous work in the female rat, we know the AVPV in general sends projections to many limbic and hypothalamic regions (Gu and Simerly, 1997). However, the specific projection patterns of CRFR1+ cells of the female AVPV/PeN were unknown in any species. We therefore investigated both anterograde (N= 6, 3 “hit”, 3 “miss”) projection patterns for the CRFR1-expressing cells of the female AVPV/PeN. We know that the female AVPV/PeN is responsive to circulating gonadal hormones, glucocorticoids, and CRF, all of which have direct implications on HPA axis function, and mediating behavioral responses to stress. Therefore, establishing efferent projection sites will provide more evidence for AVPV/PeN CRFR1 as a novel female-oriented nucleus in the stress-response system.

The data from our tracer infusions show that AVPV/PeN CRFR1 sends efferent projections to many stress-associated and limbic regions including, but not limited to, the PVN, BST, amygdala, portions of the preoptic area, and even hindbrain regions, such as the parabrachial nucleus, Barrington’s nucleus, the locus coeruleus, and dorsal raphe. The majority of regions to which the AVPV/PeN CRFR1 cells project collectively have multifaceted influences on various portions of the stress response and associated behavior. For example, several of the anterograde projection sites terminate in limbic structures that modulate emotional valence during stress, and others are to hypothalamic brain regions that have direct evidence in HPA axis regulation, and driving behavioral responses to stress.

PVN: The PVN showed very dense fiber labeling following the anterograde infusion (Figure 3.3A), suggesting that CRFR1 within the female AVPV/PeN sends information to a key region for orchestrating the stress response. Understanding the role of the AVPV/PeN in modifying PVN activity will shed light on the role of the AVPV/PeN in the female-specific stress adaptation. It is possible that CRFR1 cells of the female AVPV/PeN synapse onto CRF-expressing cells in the PVN, and could influence PVN activation; others have shown local PVN
CRFR1 feeds back and inhibits PVN CRF during stress (Jiang et al., 2018), protecting the PVN from stress-induced dysregulation. Based on these previous findings (Jiang et al., 2018), it is possible that AVPV/PeN CRFR1 is also contributing to reducing PVN CRF activity through a similar mechanism in the female mouse. Communication from the AVPV/PeN to the PVN may also alter the synthesis and release of either oxytocin or vasopressin. This would provide a region- and cell-specific pathway for stress-induced alterations to endocrine function in the female mouse; oxytocin, vasopressin and CRF all play key roles in HPA axis regulation (Herman et al., 1997; Nishioka et al., 1998; Jankord & Herman, 2008). In addition, it is important for future studies to determine if female AVPV/PeN CRFR1 cells are GABA- or Glutamatergic, because this will explain the influence of cellular activation in AVPV/PeN CRFR1 on downstream targets, such as the PVN. If the activated AVPV/PeN CRFR1 cells are GABA-ergic, then the activity occurring in the female AVPV/PeN CRFR1 may be protective against chronic stress-induced PVN activity.

**DMH and septum:** The dorsomedial hypothalamus (DMH) inhibits the stress response (Herman & Cullinan, 1997; DiMicco et al., 2002; Ebner et al., 2012), and lesions to the DMH increase HPA axis activation (Ebner et al., 2012). Others have shown that the DMH is a part of an integrative pathway that converges at the PVN to inhibit PVN activation (Myers et al., 2014). The DMH has GABAergic inhibitory projections to PVN CRF-expressing cells (Roland and Sawchenko, 1993, Boudaba et al., 1996, Radley et al., 2009), showing that the DMH can reduce HPA axis activation, and possibly reduce anxiety- or depression-behavior by preventing chronic CRF release. We have already shown that AVPV/PeN CRFR1 are active during acute stress in only females (Figure 2.10), and now we show that the cluster of CRFR1 sends dense efferent projections to the DMH, showing that stress-induced cellular activity in the AVPV/PeN likely has a direct influence on DMH signaling. Because the DMH has an important role for inhibiting the stress response, it is possible that AVPV/PeN activation of DMH cells helps to
enhance DMH inhibitory signaling to the PVN. However, the female AVPV/PeN CRFR1 could also be sending inhibitory signals to DMH cells. Therefore, it may increase HPA axis activation by disinhibiting the PVN through DMH inhibition.

The lateral septum (LS) is important for inhibiting ACTH and CORT synthesis; lesions to the LS increase ACTH and CORT, and activating the LS reduces struggle behaviors on the forced swim test, which illustrates the role of the LS in active coping strategies (Singewald et al., 2011). In the current study, the lateral ventral septum (LSv) showed dense anterograde fibers, demonstrating that cellular activation in AVPV/PeN CRFR1 can influence LSv function, potentially affecting stress coping. Based on the current data we do not know if CRFR1 signaling from the AVPV/PeN to the LSv is protective, though previous studies show that LS activation is protective. Going forward, it will be important to understand the role that signaling from the AVPV/PeN to the LSv has for cellular activity in the LSv. It may be that AVPV/PeN CRFR1 serves to amplify the LSv function in coping, though the opposite could also be true.

**BST and Amydala:** In our study, we show dense labeling in the dorsolateral and principal nucleus of the BST, and very dense labeling in the anteroventral nucleus of the BST. The BST projects to the PVN (Cullinan et al., 1993; Jankord & Herman, 2008) influencing the stress response by incorporating amygdala activity into a relay to the PVN (Dong et al., 2001a). Lesions to the anterior BST reduce serum ACTH, and c-Fos RNA in the PVN (Choi et al., 2007). In the present study, BST sub-nuclei had labeling following the anterograde tracer infusion, including the dorsolateral (dl), anteroventral (av), and principle nucleus (pr), showing female AVPV/PeN CRFR1 cells can relay information to the BST, a vital structure in organizing the stress response. AVPV/PeN CRFR1 projections to the BSTav and −dl may involve opposing circuits, depending on the activated cells. For example, different signaling from specific CRFR1 cells of the AVPV/PeN may activate the BSTav to increase ACTH as part of HPA feedback, while during different contexts, the BSTdl may be activated to reduce production of ACTH.
Female AVPV/PeN CRFR1 also send dense anterograde projections to the BSTpr, which expresses high levels of gonadal hormone receptors and is known to influence the stress response (Choi et al., 2007). The BSTpr has GABA-ergic cells that innervate the PVN, and lesions to the BSTpr result with increased plasma ACTH and impaired serum CORT recovery following a restraint challenge (Choi et al., 2007). Because the BSTpr has direct inhibitory projections to the PVN, it is possible that AVPV/PeN CRFR1 activity influences the relay between the BSTpr and PVN. It is difficult currently to know if AVPV/PeN activity increases or reduces BSTpr inhibitory signaling to the PVN, but understanding this would help to better establish the role of the AVPV/PeN in the female stress response. Future investigations could establish if the same AVPV/PeN CRFR1 cells are projecting to all of the BST nuclei, or if specific sub-groups of CRFR1 in the AVPV/PeN are projecting to specific regions.

Like the BST, the amygdala modulates the HPA axis and emotional valence in the face of stressors (Herman et al., 2005). The MeA shows substantial c-Fos activation following acute restraint stress (Cullinan et al., 1995), and when the MeA is stimulated CORT is secreted (Dunn & Whitener, 1986). In our tracing study, the MeA showed fibers in the anterograde infusion. Therefore, it is possible that AVPV/PeN signaling to the MeA could modify the HPA axis response to stress. It will be important to understand the phenotype of the anterograde-projecting CRFR1 cells; depending on if they are primarily GABA- or Glutamatergic, would help build an understanding of how these cells modulate MeA activity. If these cells were GABA-ergic, this would imply that stress-induced activation of the AVPV/PeN CRFR1 would be involved in blocking stress-induced MeA activation.

Despite the reported labeling, it is difficult to ensure that the fiber densities we reported are representative of terminal projection sites, as opposed to labeled axons passing through a region. Therefore, additional work is needed to confirm the terminal projection sites based on the labeling we report above. To confirm these projection sites, we could perform another series
of stereotax infusions targeting the same coordinates, using a synaptophysin-cre tracer (Jiang et al., 2018). Synaptophysin is expressed on axon terminals, therefore this tracer would show only terminal fibers instead of the entire axon, avoiding any potential confusion about projection sites. Following imaging, it would become clear if the fibers we report are projecting to the given regions of interest, or are axons passing through.

**General Conclusion:** In our tracing studies, we show that the female-specific AVPV/PeN CRFR1 is densely connected with the hypothalamus, limbic system, and hindbrain. Interestingly, some of the densest anterograde projections went to the PVN which furthers the implications of the AVPV/PeN in the stress response. We believe that the evidence overall points to AVPV/PeN CRFR1 involvement in the female-specific stress response.
Figure 3.1. Stereotaxic Experimental timeline; AAV anterograde tracers. The image above illustrates the anterograde infusion experimental timeline. Animals had infusions into the AVPV/PeN and allowed to recover for 5 weeks before a restraint and perfusion. Brain tissue was collected immediately following intracardial perfusion. The tracer was injected to the left hemisphere of the female AVPV/PeN.
Figure 3.2. Anterograde tracer infusion into AVPV/PeN target and missed-hit control. (A) Successful female AAV anterograde infusion site at the AVPV/PeN (hit). The green cells are CRFR1CRE/+ soma that picked up the anterograde viral tracer. (B) Female injected with the anterograde AAV that missed the target with infusion spanning parts of the dorsal POA and ventral BST, which are in close proximity, but unique from the AVPV/PeN CRFR1 population. 3V, third ventricle; AC, anterior commissure; POA, preoptic area; dashed oval on the left frame delineates missed infusion site; 3V, third ventricle; dashed rectangle delineates the AVPV/PeN.
Figure 3.3. Dense anterograde projections from the female AVPV/PeN CRFR1 to the PVN. Of all the regions that received projections from female AVPV/PeN CRFR1 cells, the PVN had the highest density. (A) The anterograde innervation of the PVN from a successfully infused AVPV/PeN. (B) Labeling seen from the “miss” infusion site listed above showing CRFR1 cells from the MOPA/BST project to regions surrounding the PVN, together with (A) further suggests that activity in the female AVPV/PeN CRFR1 cells specifically may modulate the PVN. 3V, third ventricle. The red dashed lines denote the PVN in the various images. 3V, third ventricle; MPOA, medial preoptic area; PVN, paraventricular nucleus of the hypothalamus.
Figure 3.4. MPOA receives afferent input from the female AVPV/PeN CRFR1 cell cluster. Note the dense fiber volume in the medial preoptic area (blue dashed circle). Photograph taken showing AAV+ fibers in the MPOA from the anterograde infusion. MPOA, medial preoptic area.
Figure 3.5. Anterograde projections to the DMH and tuberal nucleus. (A) Anterograde projection from a successful female AVPV/PeN infusion which shows labeling within the DMH (blue outline) and tuberal nucleus (white outline), while avoiding the VMH (orange outline) altogether. (B) Anterograde projection pattern from the female “miss” infusion, projecting to the VMH, distinct from the AVPV/PeN infusion. DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; Tuberal, tuberal nucleus; 3V, third ventricle.
Figure 3.6. AVPV/PeN projects to the lateral septum, and several BST nuclei. Fibers from the anterograde tracer infusion can be seen in various regions associated with the HPA axis including the (A) ventral lateral septum, (B) BST principal nucleus, and (C) dorsolateral BST. Note the dense labeling in the principal and dorsolateral BST. AC, anterior commissure; LV, lateral ventricle; LSv ventral lateral septum; BSTpr, bed nucleus of the stria terminalis, principle nucleus; BSTdl, dorsolateral bed nucleus of the stria terminalis.
Figure 3.7. Female AVPV/PeN anterograde projections reach hindbrain structures. The female AVPV/PeN CRFR1 nucleus projects far-caudal despite its rostral positioning. Additionally, the majority of projections remain medial, periventricular, and largely remain unilateral, with the exception being the PAG, where there are fiber densities on both sides of the cerebral aqueduct. (A) Fiber densities are seen around the PAG, (B) the dorsal raphe, (C) Barrington’s nucleus, and (D) the parabrachial nucleus. PAG, periaqueductal grey; CA, cerebral aqueduct.
Chapter 4: Influence of Chronic Variable Stress on CRFR1 Expression

Introduction

The acute stress response is healthy and adaptive for animals, recruiting resources that enable successful escape or defense behaviors. Essentially, the acute stress response is important for allowing the animal to make attempts to re-establish homeostatic balance following stress exposure (Sapolsky, 2002). However, chronic activation of the stress response is generally detrimental and can lead to stress-related mood disorders like anxiety, depression, or addiction. Chronic stress leads to chronic CRF release, and chronic CRFR1 activity has been shown to precipitate the onset of stress-related mood disorders such as anxiety and depression (Chrousos 2009; Garcia-Carmona et al., 2015; da Silva et al., 2016; Holly et al., 2016; Schussler et al., 2016). Considering the sex difference in lifetime prevalence (F>M; Kornstein et al., 2000; Kessler et al., 2005), it is important to investigate any sex differences in the underlying neural circuitry. Therefore, the interplay between corticotropin-releasing factor (CRF) and its receptor 1 subtype (CRFR1) may have an influence on the sex differences seen in stress-related mood disorder.

Chronic stress involves prolonged CRF release and CRFR1 activation, which can lead to altered baseline levels of ACTH, CORT, and alterations in response to subsequent stress exposure (Aguilera, 1994). Using genetic models that over-express CRF, researchers are able to investigate the impact of chronic CRF/CRFR1 signaling in an attempt to model chronic stress-induced activity. These data show chronic CRFR1 activity causes elevated basal levels of CORT, in addition to impaired HPA axis response to subsequent stress (Coste, Murray and Stenzel-Poore, 2001). Others have shown that chronic CRF/CRFR1 activity amplifies depression-like behaviors (Chen et al., 2018), and that blocking CRFR1 prevents these same behaviors. Chen and colleagues (2018) further demonstrate that chronic stress sensitizes the
CRF/CRFR1 system in the basolateral amygdala, thereby altering it for future experiences, both at baseline and in response to future stressors. Earlier research in the rat has shown that CRF-expressing cells of the PVN downregulate c-Fos expression following chronic stress (Bonaz and Rivest, 1998), suggesting that these cells are altered by ongoing activation. Like CRF, CRFR1 activation (via c-Fos) in the PVN has been shown to change following chronic social defeat stress (Ramot et al., 2017) in males, with recruited c-Fos/CRFR1 co-expression. Interestingly, these researchers also showed that while CRF in the PVN is negatively regulated by glucocorticoid exposure, PVN CRFR1 is positively regulated in male mice following exposure to the synthetic glucocorticoid dexamethasone (Ramot et al., 2017). Collectively, these findings indicate that CRF and CRFR1 are dynamic in expression and sensitive to the impacts of chronic stress. The alterations in CRF/CRFR1 may be a mechanism by which behaviors are altered following chronic stress.

Female and male rodents exhibit differences in adaptations to chronic stress. These sex differences involve physical changes within the brain, as well as altered behavior. A study investigating rats after chronic stress showed sex differences in brain-derived neurotrophic factor and phosphorylated CREB (pCREB), with levels changing in a sex- and stress-dependent manner in regions implicated in stress regulation (Lin et al., 2008). Following variable stress exposure, females but not males show increased corticosterone (CORT) levels and increased indices of anxiety- and depression-like behavior (Hodes et al., 2015). Sex differences in the CRF/CRFR1 system are believed to contribute to the observed sex differences in the stress response.

Using a validated CRFR1 reporter mouse line (bacterial artificial chromosome identified by green fluorescent protein (BAC CRFR1-GFP); Justice et al., 2008) we have previously identified a two clusters of CRFR1-ir cells; one is increased within the female AVPV/PeN (Rosinger et al., 2017), and the second shows higher levels of CRFR1-ir in the male PVN
(Rosinger et al., 2019b). We recently reported a high level of co-expression of AVPV/PeN CRFR1 cells with glucocorticoid receptor (Rosinger et al., 2019a), which suggests that high levels of glucocorticoids due to chronic stress may alter this cell cluster. Furthermore, AVPV/PeN CRFR1 cells respond to a single psychogenic stress in a sex-specific manner, where only female CRFR1 cells in the AVPV/PeN express pCREB two hours after the onset of a 30-minute restraint (Rosinger et al., 2019a). As with the female AVPV/PeN, the PVN in male mice shows extended expression of pCREB following restraint. Both of these CRFR1 nuclei are stress-responsive, and sexually dimorphic, suggesting that these cell groups may be mediating sex-specific adaptations to chronic stress.

The present investigation therefore aimed to explore chronic stress-induced changes in CRFR1 expression and neural activation in AVPV/PeN. We also investigated the role of chronic stress-induced changes in PVN CRFR1, based on the known importance in the stress response, in addition to the sex difference in CRFR1 expression (Rosinger et al., 2019b). We exposed female and male BAC CRFR1-GFP reporter mice (Justice et al., 2008; Rosinger et al., 2017) to chronic variable stress. Furthermore, we investigated the effect of CVS in other brain regions that are known to regulate stress including the BST and amygdala. Following 9 days of chronic variable stress (CVS), we show increased anxiety-like behavior independent of sex, and report that CRFR1 expression and activity is dynamic; the expression levels change based on the brain region, prior stress exposure, and sex of the mouse. These findings suggest that there are sex-specific neural circuits that mediate biological adaptations and behavioral expression of anxiety-like behavior.

Methods

Animals: Subjects were female and male mice (N=24, 6/sex per treatment) on a C57BL/6J background, using a validated bacterial artificial chromosome (BAC) identified green fluorescent protein specific to CRF receptor 1 (CRFR1) (BAC GFP-CRFR1). Animals were maintained
under a 12/12 L/D cycle (lights on at 0700), with food and water available *ad libitum*, aside from when portions of the CVS paradigm required food deprivation overnight. All procedures in this study were approved by the University at Albany Institutional Animal Care and Use Committee and are in accordance with the National Institutes of Health guidelines.

**Chronic Variable Stress (CVS):** Adult male and female BAC GFP-CRFR1 mice were randomly divided into chronic stress or control conditions. CVS animals were housed in a separate vivarium. Control animals were not handled until the open field assessment. Stressed animals went through a 9-day variable stress paradigm (Table 4.1). Briefly, animals were exposed to one or two variable stressors, daily. On days in which two stressors were present, there was a minimum of a 2-hour period between each stressor. The same is said for conditions in which animals were food-deprived the night before, also having a 2-hour period between reinstated access to food, and subsequent stressors. The stressors used are from a previously validated paradigm (Johnson et al., 2015) that has shown to change HPA axis tone, and influence behavioral indices of anxiety. Animals were exposed to the following battery of variable stressors: Wet cage (3hr), food deprivation overnight (12 hours), 45° cage tilt (3hr), tail suspension (10min), cold water swim (5min, or 10min), naphthalene exposure (1hr), restraint stress (30min).

**Open Field:** All animals from both treatment groups were exposed to a final 10-minute video-recorded open field assessment. This was performed with to assess the anxiety-like behavioral phenotype in female and male mice following chronic stress exposure. The apparatus consisted of a clear Plexiglas cube (16” x 16” x 16”) with an open top. The test was illuminated from above, and a camera was mounted on the ceiling for behavior recording. AnyMaze software (Stoelting Co.) was used to superimpose inner and outer areas over the apparatus, from which latency to first center entry, number of center entries, total center time, and the total locomotion were all calculated. Additionally, rearing, and defecation were hand-scored by an observer that
blinded to treatment conditions as added measurements for anxiety-like behavior. Mice were individually placed into one corner, and allowed to roam freely for 10 minutes. After 10 minutes, animals were removed and returned to their home cages. The apparatus was cleaned with 70% ethanol between each test. We analyzed the first 5 minutes of the OFT exposure for each animal, because others have shown that this time frame in the OFT successfully identifies anxiety-like behavior in the mouse (Zuloaga et al., 2008).

**Tissue Collection for immunohistochemistry:** All animals underwent cervical dislocation followed by rapid decapitation ninety minutes after the onset of open field exposure. The brains were removed and placed in 4% paraformaldehyde (PFA), then stored overnight at 4°C. The next day, brains were transferred into a 30% sucrose cryoprotectant solution, where they remained at 4°C until sectioned. For immunohistochemistry, brains were coronally sectioned at 40µm, into 3 series sectioned using a cryostat (Microm HM505E, MICROM international GmbH, Walldorf, Germany). Tissues were placed into cryopreservative and stored at 4°C until immunohistochemistry was performed.

**Immunohistochemistry (IHC):** We performed dual-label fluorescent IHC to determine co-expression of CRFR1+ cells with c-Fos or pCREB following open field exposure on day 10. Similar methods were taken as in previous chapters. Briefly, sections were rinsed in PBS, then incubated in 4% NDS and 0.3% PBS-TX for one hour. Following incubation, tissue was placed into either primary antisera c-Fos (Santa Cruz; rabbit; 1:250) or pCREB (Cell Signaling; rabbit; 1:500) and incubated overnight (at room temperature). The next day, tissue was again rinsed in PBS and then placed into secondary antisera (anti-rabbit, 594; 1:1000) diluted into 4% NDS and PBS-TX, for a total of 2.5 hours. After secondary exposure, the tissue was transferred to the second primary antisera, GFP (Abcam; chicken; 1:2000) in 4% NDS and PBS-TX at room temperature, overnight. On the final day, tissue was rinsed (PBS), and transferred to the second secondary antisera (anti-chicken, 488; 1:1000) for 2.5 hours. After the tissue came out of
secondary, it was immediately rinsed in PBS, mounted and coverslipped with Santa Cruz hard set mounting media, containing DAPI, when dry.

**Microscopy and Statistical Analyses:** Assessment of CRFR1, c-Fos, and pCREB labeling was performed on a Nikon 80i microscope using a digital camera, at 20X magnification. The Allen institute mouse brain atlas (Lein et al., 2007) was used to identify the desired brain areas. Specifically, quantitative assessment of absolute CRFR1, c-Fos, pCREB, and activated CRFR1 (via co-expression of GFP with either c-Fos or pCREB immunoreactivity) was performed after fixing proper regions of interest on representative images of the AVPV/PeN (rectangle), arcuate nucleus (triangle), paraventricular nucleus of the hypothalamus (triangle), medial preoptic nucleus (circle), dorsolateral (circle) and anteroventral (oval) bed nucleus of the stria terminalis, and the central (circle) and ventral basolateral amygdalae (circle). Cells were counted using ImageJ software to estimate various cell densities within the selected regions. Two bilateral regions were used for quantification within each brain region. These analyses were utilized to estimate cell density within these selected regions. 2-way ANOVA was used for statistical analyses with sex and stress as factors. Significant main effects/interactions were further analyzed using Bonferroni corrected T-tests. Significance level was set at p≤0.05 and data are shown as means ± SEM.

**Results**

4.1 Anxiety-Like Behavior (Open Field) and Blood Corticosterone

*Latency to First Center Entry:* A 2-way ANOVA revealed a significant effect of CVS treatment on the latency to first center entry (F(1,20) = 9.89) p < 0.01, where CVS-treated animals independent of sex took significantly longer before their first center entry (Figure 4.2A).

*Total Center Entries:* A 2-way ANOVA revealed a significant effect of treatment on the number of center entries in the open field (F(1,20) = 7.66) p<0.02. Further analysis revealed that the
chronic stressed males made significantly less center entries compared with non-stressed males (p<0.05), a pattern not seen in the female counterparts (Figure 4.2B).

Time in Center: A 2-way ANOVA revealed a significant main effect of treatment on the amount of total time in the center of the open field (F(1,20) = 10.50) p<0.005, regardless of sex, with chronic stressed animals spending significantly less time in the center compared with all non-stressed animals (Figure 4.2C).

Distance Traveled: A 2-way ANOVA revealed no significant difference in total distance traveled regardless of sex or treatment, indicating no effect of CVS on general locomotor activity (Figure 4.2D).

Rearing and defecations: A 2-way ANOVA on rearing revealed no differences on the number of rears (Supplemental S4A) regardless of sex or treatment condition. Finally, a 2-way ANOVA revealed no effect of treatment or sex on the amount of defecations in the open field (Supplemental S4B).

Radioimmunoassay for blood Corticosterone: A 2-way ANOVA revealed a main effect of sex on the amount of serum CORT collected at 90 minutes after open field onset, showing that all males had significantly less serum CORT than females, regardless of stress condition (F(1,19) = 4.685) p=0.0434, [NS Female=84.959850ng/mL ± 24.749010, CVS Female=60.540550ng/mL ± 8.362371; NS Male=45.669270ng/mL ± 10.105340, CVS Male=44.386550 ± 3.643398]. CORT data are reported as standardized mean serum ng/mL ± SEM (Supplemental S5).

4.2 Immunohistochemistry (IHC)

AVPV/PeN: A 2-way ANOVA of CRFR1-ir within the AVPV/PeN revealed significant main effects of sex (F>M; (F(1,20) = 121.37) p<0.0001), an interaction with treatment (F(1,20) =
12.19) p<0.01), further post-hoc analysis showed a significant difference for stress treatment on female CRFR1 expression (CVS females > NS females; t(10) = 3.81, p<0.004) (Figure 4.3A). The number of c-Fos/CRFR1+ cells also showed a significant main effect of sex (F>M; (F(1,20) = 12.52) p<0.01) (Figure 4.3B). Additionally, pCREB/CRFR1 labeling showed main effects of sex (F(1,20) = 54.99) p<0.001), stress (F(1,20) = 11.32) p<0.01), and an interaction (F(1,20) = 10.65) p<0.01), all of which showed the same directionality (Female > Male) (Figure 4.3C).

Another 2-way ANOVA comparing c-Fos levels showed a main effect of sex (F(1,20) = 12.52) p<0.01), where females had more overall c-Fos expression than males (Table 4.1). Comparison within females further revealed that stressed females had significantly more pCREB/CRFR1 cells in the AVPV/PeN than non-stressed females (p<0.001). Total AVPV/PeN pCREB-ir was not different for any of the treatment groups (Table 4.1). There was no impact of treatment on any measurements for the male animals.

*Paraventricular Nucleus of the Hypothalamus (PVN):* A 2-way ANOVA on PVN CRFR1 cells/mm² revealed a main effect of sex on CRFR1-ir, (F(1,20) = 21.12) p<0.001, where males of both conditions had significantly more CRFR1-ir than females (Figure 4.4A). In addition, there was a main effect of treatment on c-Fos/CRFR1 (F(1,20) = 11.23) p<0.01 (Figure 4.4B). Based on previous data from our lab (Rosinger et al., 2019b), we planned to compare CVS males versus non-stressed males in PVN c-Fos/CRFR1, predicting that there would be treatment-dependent impact on the male PVN. A Student’s t-test demonstrated that indeed the CVS males had significantly fewer c-Fos/CRFR1 cells/mm² than non-stressed males (t(10)=3.54, p<0.01; Figure 4.4B). Another 2-way ANOVA of total c-Fos-ir within the PVN revealed a significant main effect of treatment, where CVS-exposed males had significantly reduced c-Fos-ir within the PVN compared with non-stressed males (F(1,20) = 32.70) p<0.0001; Table 4.1. Finally, there was a main effect of sex on the total number of pCREB/CRFR1 co-expressing cells, where males regardless of stress treatment had significantly more pCREB/CRFR1 co-expression than
females (F(1,20) = 17.40) p<0.001 (Figure 4.4C). The total amount of pCREB in the PVN did not differ regardless of sex or treatment (Table 4.1).

**Arcuate Nucleus of the Hypothalamus (ARC):** A 2-way ANOVA of total c-Fos-ir within the ARC revealed a significant main effect of treatment (F(1,20) = 13.27) p<0.002, where stressed animals had significantly less c-Fos-ir than non-stressed, regardless of sex (Table 4.1). Results show no effect of sex or treatment on the total amount of CRFR1-ir within the ARC (Figure 4.5). In addition, there were no impacts of treatment or sex on the number of c-Fos/CRFR1 or pCREB/CRFR1 within the ARC (Figure 4.5). However, there was a trend toward a main effect of CVS to reducing the amount of c-Fos/CRFR1-GFP (p<0.06; Figure 4.5).

**BSTav:** A 2-way ANOVA of total c-Fos expression within the BSTav revealed a significant main effect of treatment (F(1,19) = 11.04) p<0.01, where CVS animals showed significantly less total c-Fos than non-stressed counterparts (Figure 4.5). Upon further examination, it was shown that CVS females specifically showed a significant decrease in total c-Fos+ cells compared with non-stressed females (t(10)=2.64 p<0.05; Table 4.1). Further, a 2-way ANOVA on the amount of c-Fos/CRFR1 co-expression revealed a significant main effect of stress condition, where CVS-exposed animals all had significantly less c-Fos/CRFR1 cells/mm² (F(1,20) = 7.39) p<0.05 (Figure 4.5). An additional comparison revealed and effect of treatment, showing that CVS females had a significant decrease in c-Fos/CRFR1 compared with non-stressed females (t(10)=3.35, p<0.01; Figure 4.5). There were no main effects of sex on the total c-Fos in the BSTav (Table 4.1) or on total CRFR1-ir (Figure 4.5). Additionally, a 2-way ANOVA of both the total pCREB+ cells/mm² (Table 4.1) and the total pCREB/CRFR1+ cells/mm² (Figure 4.5) revealed no significant differences regardless of sex or treatment.

**BSTdl:** A 2-way ANOVA of total c-Fos expression within the BSTdl revealed a significant main effect of treatment (F(1, 20) = 13.03) p<0.01, and a significant main effect of sex (F(1,20) = 4.56) p<0.05; CVS females had significantly less c-Fos within the BSTdl compared with non-
stressed females (t(10)=3.72) p<0.01; Table 4.1). Another 2-way ANOVA of c-Fos/CRFR1 co-expression revealed no significant differences (Figure 4.5). Comparisons for pCREB/CRFR1 co-expression also showed no differences regardless of sex or treatment (Figure 4.5).

**BLAv:** A 2-way ANOVA revealed a significant main effect of treatment on the expression of total c-Fos present (F(1, 20) = 14.66) p<0.01, further examination revealed that CVS males had significantly less total c-Fos in the BLAv than non-stressed males (t(10)=3.02, p<0.05; Table 4.1). Another 2-way ANOVA revealed a significant main effect of treatment in the number of c-Fos/CRFR1 cells/mm² (F(1, 20) = 15.66) p≤0.001; Figure 4.5), animals in the CVS treatment had significantly less c-Fos/CRFR1 than those in the non-stressed treatment, independent of sex (Figure 4.5). There was no impact of sex on the levels of CRFR1 cells/mm². Another 2-way ANOVA revealed no differences in pCREB expression regardless of sex or treatment (Table 4.1). Further, there were no differences in the number of pCREB/CRFR1 cells/mm², regardless of sex or treatment (Figure 4.5).

**CeA:** There were no significant differences between female and male total CRFR1-ir, or in c-Fos/CRFR1 co-expression, regardless of sex or treatment (Figure 4.5). Further, there were no differences regardless of sex or treatment in pCREB/CRFR1 co-expression (Figure 4.5). However, analysis of pCREB cells/mm² expression in the CeA revealed an interaction (F(1,20) = 9.38) p<0.05), indicating that CVS male mice had a significant reduction in total pCREB cells/mm² in the CeA compared with all other animals (p≤0.05) (Table 4.1).

**Discussion**

The current study investigated the effects of chronic variable stress on CRFR1 cells within select rodent forebrain structures, including a sexually dimorphic structure within the female AVPV/PeN that has recently been reported (Rosinger et al., 2017). We found that our 9-day CVS paradigm produces an anxiety-like behavioral phenotype, independent of sex, indicating that this variable stress paradigm is effective in eliciting behavioral changes (Figure
4.2). In addition, CVS significantly alters levels of CRFR1 and cell activation in a region- and sex-dependent manner.

**CVS induction of Anxiety-like Behavior and Serum CORT:** Following CVS exposure, both female and male mice exhibited increased markers of anxiety-like behavior in the final open field challenge, compared with stress-naïve mice. Specifically, CVS-exposed female and male mice had a greater latency to center entry, fewer center entries, and less time in the center compared with non-stressed mice (Figure 4.2A-C), independent of general locomotor activity (Figure 4.2D). The increased anxiety-like behavior in both female and male mice after CVS suggests that both female and male mice are behaviorally susceptible to our paradigm, which lines up with previous reports using similar methods (Mineur, Belzung and Crusio, 2006; Cotella et al., 2019). While both sexes displayed stress-induced anxiety-like behavior in the open field, there are likely different mechanisms and neural networks involved in female versus male adaptations to CVS. In addition, comparisons between male and female mice have shown that females may be more vulnerable to sub-chronic stress than males (Hodes et al., 2015), where sub-chronic stress induced behavioral and transcriptional changes in only female mice. While our current investigation reports no sex differences in open field behavior, it is possible that a milder CVS paradigm may better produce a sexually dimorphic behavioral response to the final open field stress (Hodes et al., 2015). CVS has been shown to cause chronic increases in CORT production following subsequent stress exposure in both sexes (Herman, Adams, and Prewitt, 1995; Cotella et al., 2019), and others have shown that chronic stressed females have higher CORT than stressed male counterparts (McCormick et al., 2005). Our data show no treatment effect within either female or male mice on serum CORT. However, the blood in our study was collected at 90 minutes after the open field (at the time of brain collection) and levels likely reflect recovery, not peak, serum CORT. Therefore, it is possible that peak or even basal CORT levels may have been altered by the current CVS paradigm. Regardless, our data show that
females still had significantly more serum CORT than males (Supplemental S5), and visually, the levels in males were elevated from normal baseline, which is ~20ng/mL in C57BL/6 males (Flint & Tinkle, 2001) as opposed to ~45ng/mL which we saw in all of our male C57BL/6J mice, suggesting that the open field exposure was able to elicit CORT activity in both sexes. Further, we show that while CORT may be elevated in the males, there was no difference at recovery between CVS and NS males (Supplemental S5).

**Rostral Anteroventral Periventricular Nucleus (AVPV/PeN):** In the present study, we build off of the previous findings which reported an increase in cell activation of CRFR1 within the female, but not male, AVPV/PeN (pCREB/CRFR1) following a single 30-minute restraint stress (Rosinger et al., 2019a). We expand this to a 9-day chronic variable stress, which significantly upregulates female CRFR1 expression to approximately double that of non-stressed female counterparts (no changes in males) (Figure 4.3). Others have shown that cells in the female rat AVPV project to stress-related regions such as the PVN and BST (Gu and Simerly, 1997), and data from our lab suggest that the AVPV CRFR1 specifically projects to the same regions in the female mouse (Table 3.1). These data suggest that the adaptations in AVPV/PeN CRFR1 are modifying downstream HPA axis-related brain regions after restraint stress. Research has also shown that the AVPV expresses sexually dimorphic levels of both tyrosine hydroxylase (TH) and kisspeptin (F>M; Simerly, 1989; Simerly et al., 1997; Semaan et al., 2010; Brock, et al. 2015; Scott et al., 2015). It is possible that CRFR1 could be up-regulated in neurons within the AVPV/PeN that co-express KISS or TH cells, thereby potentially influencing reproductive functions including LH surges and sexual behavior, considering that the AVPV plays a role in ovulation (Simerly et al., 1997). In addition, the AVPV/PeN displays experience-dependent plasticity following maternal experience (Scott et al., 2015), and the changes that we see following female CVS suggest that stress may modulate the functions. For example, some have shown that CRF administration into the MPOA can delay puberty in female
rats (Kinsey-Jones et al., 2010), by inhibiting the luteinizing hormone surge driven by kisspeptin signaling in the preoptic area (Kinsey-Jones et al., 2009). It is therefore likely that the changes we see in the female AVPV/PeN CRFR1 expression can alter reproductive functions, as well.

We predicted that AVPV/PeN CRFR1 would increase female c-Fos/CRFR1 co-expression after CVS. This was based on previous data showing a recruitment of cellular activity within CRFR1 cells of the male PVN following chronic social defeat stress (Ramot et al., 2017). In our study, the CRFR1/c-Fos co-labeling was relatively sparse, regardless of treatment in the AVPV/PeN. Keeping that in mind, the female mice of both treatments still had more AVPV/PeN c-Fos/CRFR1 than males. Regardless of previous stress exposure the female c-Fos/CRFR1 pattern confirms female-specific engagement of AVPV/PeN CRFR1 in the stress response regardless of previous stress experience. The present data suggest that chronic or prolonged activation of these cells promotes increased CRFR1 expression. This possibly occurs as a result of inducing the phosphorylation of CREB, another transcriptional marker of cellular activity. The elevated pCREB measured in the AVPV/PeN at two hours following the open field may be due to phosphorylation of calcium/calmodulin dependent protein kinase II, driving the upregulation (CaMKII; Kwon et al., 2006). CaMKII is one of the major downstream targets of calcium signaling (Lucic, Greif, and Kennedy, 2008), and leads to auto-phosphorylation, driving increased expression of pCREB long after calcium signaling has ended. In addition, pCREB influences transcription of c-Fos and many other genes involved in cell function and survival (Ahn et al., 1998; Mayr and Montminy, 2001; Lonze et al., 2002; Ortega-Martinez, 2015). The sustained pCREB expression in the female AVPV/PeN from the present study suggests that genes associated with phosphorylating CREB are influenced by stress. Though there are many possible consequences of prolonged pCREB expression, others have shown that prolonged pCREB-immunoreactivity is co-expressed in cells with upregulated CRFR1 mRNA production, in
the medial prefrontal cortex (Uribe-Marino et al., 2016), giving more evidence that pCREB may be driving the observed increased CRFR1 expression.

Although it is possibly pCREB driving the upregulation in CRFR1 expression, it is also possible that chronic GR activation in the CVS females is what caused these results, because chronic CORT activity at GR is known to lead to HPA axis dysregulation (Vial, 2002; Abel & Majzoub, 2005), and because we report a high level of CRFR1/GR co-expression in our animals. Regardless, future studies on GR-mediated CRFR1 expression in the AVPV/PeN would be interesting. Overall, the current data point toward the AVPV/PeN as a potential female-specific site at which circulating stress- and gonadal hormones can modulate cellular function and possibly influence behavior.

**Paraventricular Nucleus of the Hypothalamus (PVN):** In a previous study from our lab, we reported a male-biased sex difference in CRFR1-ir of the adult mouse paraventricular nucleus of the hypothalamus (PVN, M>F; Rosinger et al., 2019b). We also showed a high level of AR/CRFR1 co-expression, in addition to reporting that PVN CRFR1 cells co-express pCREB following an acute 30-minute restraint stress (Rosinger et al., 2019b). In the current investigation, we extend these findings to a significant attenuation of the total c-Fos+ cells/mm² in the PVN of all CVS-treated animals. Moreover, we show that in CVS males, significant c-Fos attenuation occurs within CRFR1-ir cells (not seen in females). This lines up with previous work; others have shown that chronic stress will attenuate PVN c-Fos; reduced PVN c-Fos has been shown for 30 days following CVS exposure in male rats (Ostrander et al., 2009), a finding that has also been shown by others at various time points (Bonaz and Rivest, 1998; Girotti et al., 2006). In addition, Ramot and colleagues (2017) showed that CRFR1 activation in the PVN is related to the animal’s previous stress exposure. Following chronic social defeat, for example, male mice showed increased cFos/CRFR1+ cells in the PVN (Ramot et al., 2017), which was shown in a separate portion of the study to be glucocorticoid-mediated (Ramot et al., 2017).
Jiang and colleagues (2018) showed increased c-Fos in PVN CRFR1 reduces HPA axis activation, therefore reduced c-Fos activation in PVN CRFR1 in the present investigation could be maladaptive. Future studies are necessary to determine if the increased pCREB expression was due to CRFR1 activation by CRF directly. However, CRFR1 antagonists have been shown to reduce similar responses within the PVN after restraint stress (Imaki et al., 1995). Therefore, while this is a likely possibility, direct comparisons are still needed.

Males also had significantly more PVN pCREB/CRFR1 cells/mm² than females (Figure 4.4C), regardless of treatment condition, which is in line with previous work showing that pCREB is elevated at 2-hours post stress in the male PVN regardless of acute or repeated restraint stress (Kwon et al., 2006). Extended production of pCREB is indicative of various ongoing transcriptional changes in the PVN (Legradi et al., 1997; Chocyk et al., 2000; Lee et al., 2003), and this suggests that the male PVN undergoes more pCREB-mediated changes than the female, independent of prior stress. Future work should aim to clarify any male-specific pCREB mediated transcriptional changes to PVN CRFR1, because we do not know if elevated pCREB/CRFR1 is conferring an advantage to males or not. Research has shown that there is a CRFR1—CRF circuit within the PVN where CRFR1 signals back to CRF expressing cells to reduce subsequent activation of CRF cells (Jiang et al., 2018). In addition, earlier work focusing on CRF, showed increased expression of both glutamate and norepinephrine on CRF-expressing soma, increasing excitatory signaling in the face of ongoing stress (Flak et al., 2009). It is therefore possible that CRF cells in the PVN are signaling locally to CRFR1 to drive the prolonged pCREB expression in all males.

Other Hypothalamic and Limbic Structures

*CVS impact on total CRFR1, pCREB/CRFR1*: Aside from the AVPV/PeN, no other regions we examined showed any changes in levels of CRFR1 following CVS. In addition, none of the regions we examined demonstrated any treatment or sex differences in pCREB/CRFR1
co-expression, aside from the AVPV/PeN and the PVN, mentioned above. Repeated stress did not alter pCREB expression in many brain regions. Specifically, we reported no effect of treatment on pCREB/CRFR1 co-expression within the arcuate, bed nucleus of the stria terminals (anteroventral and dorsolateral), central nor basolateral amygdala nuclei, although CVS did alter c-Fos.

CVS influence on c-Fos/CRFR1 expression: Following exposure to chronic stress, we discovered two nuclei with downregulated c-Fos/CRFR1 co-expression, the BSTav and BLAv. Within the female BSTav, there was a significant reduction for CVS-exposed females compared with NS female counterparts with no differences in males (Figure 4.5, Supplemental S6). In addition, c-Fos/CRFR1 co-expression was decreased by CVS in both sexes in the ventral basolateral amygdala (Figure 4.5, Supplemental S6). Finally, we report a trend toward reduced c-Fos/CRFR1 expression within the arcuate nucleus of CVS-exposed female and male mice (Figure 4.5).

The BST is involved with the stress response, serving as a relay from other limbic sites to the PVN (Cullinan et al., 1993), specifically through GABAergic signaling (Herman et al., 2002). Data from other studies suggest that CRF acts in the BST to increase anxiety-like behavior through acting at CRFR1 and not CRFR2 (Sahuque et al., 2006). Our current investigation reports a significant reduction in c-Fos/CRFR1-ir cells in the anteroventral BST (BSTav) in female CVS mice. Other work has shown cells expressing CRF within portions of the BST (dl, dm, and ventral) all show increased c-Fos co-expression following acute stress exposure (Butler et al., 2016). The reduction of c-Fos/CRFR1 is possibly reflective of longer-term alterations/adaptations in the female BST following CVS, including potential habituation of CRFR1 cells in the BST to the chronic stress-induced activation. In addition, we report a significant reduction of total c-Fos in the BSTdl, specific to CVS-exposed female mice (Table 4.1). The BSTdl sends projections to the CeA that increase anxiety and are distinct from
projections to the lateral hypothalamus and VTA (Yamauchi et al., 2018), which reduce anxiety. Therefore, the influence of the downregulated BST c-Fos on stress-induced alterations depends on the phenotype of BSTdl neurons that showed downregulated c-Fos. If reduced activation is taking place in CeA-projecting neurons, it may be protective, though if these cells project to the VTA, downregulation could increase anxiety (Yamauchi et al., 2018). Overall, our data suggest that in female mice, the BST is altered by CVS exposure, either by reduced CRFR1/c-Fos activation (BSTav), or global c-Fos reductions (BSTdl). The CVS-induced reductions in cell activation of discrete BST nuclei may predispose the female mouse to HPA axis dysregulation, and could be a sex-specific circuit that underlies development and expression of female anxiety-like behavior. Future work on the BST in the context of chronic stress adaptations is needed to better understand the potential role it plays in sex-specific behavioral stress responses and HPA axis activation.

The amygdala has been consistently implicated in fear and anxiety responses (Walker, Toufexis, and Davis, 2003; Carvalho-Netto et al., 2011). Previous work has shown that chronic restraint stress increases synaptic density in the BLA (Carvalho-Netto et al., 2011), which has been positively correlated with increased anxiety-like behavior in the elevated plus maze (Vyas et al., 2005). Moreover, others have suggested that CRFR1 activity in the BLA, but not the CeA, is implicated with stress-induced anorexia, (Jochman et al., 2005), another condition in which a sex difference exists for the prevalence (F>M; Pope et al., 1984). CRF/CRFR1 in the basolateral amygdala is also involved in the recall of a traumatic experience (Hollis et al., 2016), which is blocked by using a CRFR1 antagonist; showing that CRFR1 within the BLA is involved in consolidating fear memories. In the present study, we report a significant attenuation in c-Fos/CRFR1+ cells of the BLAv following CVS. Unlike the BSTav, the BLAv expressed attenuated c-Fos/CRFR1 in all CVS mice, indicating that it might be involved in more generalized habituation to chronic stress in both sexes. Reduced c-Fos/CRFR1 from the
present study lines up with other work in rats (Ostrander et al., 2009) which showed a reduction in total c-Fos expression following CVS (not specific to CRFR1-expressing cells). In addition, CRF/CRFR1 activity in the BLA has been shown to influence depressive-like behavior in male rats after exposure to chronic stress (via repeated forced swim) by sensitizing neuronal excitability within the BLA (Chen et al., 2018). Predictable, repeated foot shocks have been shown to cause a similar increased c-Fos expression in the BLA (Westenbroek et al., 2003). Overall, the data demonstrate that it depends on stressor modality; predictable stressors result in increased neuronal activation, while unpredictable stressors reduce BLA cellular activity. It may be that increased CRFR1 activity in the BLA during repeated predictable stress is protective and demonstrates an alternative form of learning, possibly helping the animal cope with the ongoing stress. If this is the case, our CVS data demonstrate an amygdala nucleus that has become dysregulated, thereby increasing the probability of the CVS animal displaying increased anxiety-like behavior, when stressors are unpredictable, which is what our behavior data show.

Within the arcuate nucleus of the hypothalamus, there was a trend toward a decrease in c-Fos/CRFR1+ cells in CVS mice (Figure 4.5). Others have shown that chronic stress leads to reduced c-Fos, but not pCREB, in the ARC (Kwon et al., 2006). Our data suggest that this reduction may be occurring specifically within CRFR1-expressing cells. The arcuate nucleus is a hot-spot for many hypothalamic functions, largely homeostatic. Specifically, the arcuate is known to be involved with glucose intake, cardiovascular regulation, and various hormone/transmitter release, all of which is regulated through neuroendocrine signaling to the pituitary, culminating with increased release of various pituitary-derived messengers (Palkovits, 2008; Sapru, 2014; Hussain et al., 2015). The arcuate is also responsive to psychological stress and to the administration of CRF. Further, GnRH neurons of the arcuate are inhibited by local CRF administration (Li et al., 2010). It is possible that the reduced expression of c-Fos in
arcuate CRFR1-expressing cells could impact any of these functions such as altering appetite, gonadal hormone surges, or overall homeostatic balance.

**General Conclusion:** Chronic stress precipitates the onset of stress-related mood disorders, which exhibit a sex bias in lifetime prevalence rates (F>M), and dysregulation/alteration in CRFR1 function has been implicated in the onset of these conditions (Chrousos 2009; Garcia-Carmona et al., 2015; da Silva et al., 2016; Holly et al., 2016; Schussler et al., 2016). In the present study, we report that CVS exposure selectively impacts discrete CRFR1-containing cell groups in the female and male mouse, and the neural alterations occurred in tangent with CVS-induced anxiety-like behavior in the open field test. The PVN CRFR1 population is protective in males, according to data from others, showing that CRFR1 signals back to PVN CRF neurons to inhibit further CRF activity (Jiang et al., 2018), implying that the downregulation of PVN CRFR1/c-Fos we report in our study may be maladaptive for CVS-exposed males. Of all of the regions we investigated, the AVPV/PeN was the only to upregulate expression of CRFR1, not just cell activation, showing this is a female-specific nucleus that is vulnerable to chronic stress induced alterations. In the female AVPV/PeN, dysregulated CRFR1 expression may contribute to complications associated with stress-related mood disorders, such as diminished sex-drive, fertility, and impaired maternal behavior, or CVS-induced increases in anxiety or depression-like behavior.
Figure 4.1. Chronic Variable Stress (CVS) Paradigm. Female and male animals were randomly assigned to the CVS or non-stressed (NS) treatment group. Animals in the CVS group had various unpredictable stressors, either once or twice daily, and on day 10 the animals were exposed to a final open field where behavior was analyzed. Animals in the NS group were left in their home cage until the final open field exposure. At 90 minutes after the onset of the open field, animals were restrained and euthanized via rapid cervical dislocation, and trunk blood was collected to measure blood corticosterone.
**Figure 4.2. CVS induces anxiety-like behavior in the open field test regardless of sex.** On day 10 following CVS onset, male and female CVS and control animals were exposed to a final open field, and anxiety-like behavior was assessed. (A) The latency to first entry, which was significantly longer for CVS animals (f and m) than non-stressed animals. (B) Total number of center entries, showing that CVS animals made significantly fewer center entries than non-stressed counterparts, regardless of sex. (C) The total amount of time spent in the center was significantly shorter for CVS-exposed than non-stressed animals, regardless of sex. (D) Total distance traveled in meters was not different for any groups. NS, non-stressed; CVS, chronic variable stress. (*) Main effect of stress, p < 0.05.
Figure 4.3. CVS impact on AVPV/PeN CRFR1, c-Fos/CRFR1, and pCREB/CRFR1 staining and co-expression cells/mm². (A) CVS females showed a significant increase in CRFR1-ir compared to all other treatment groups. (B) Representative images of CVS and NS female and male AVPV/PeN CRFR1. (C) c-Fos/CRFR1 co-expression was significantly higher in all female AVPV/PeN than male, regardless of treatment condition. (D) Representative images of CVS and NS female and male c-Fos/CRFR1 showing a female-specific reduction in c-Fos/CRFR1 cells. (E) All females, regardless of treatment had increased pCREB/CRFR1 in the AVPV/PeN than males, and CVS females specifically had higher pCREB/CRFR1 than any other groups. (F) Representative images of NS and CVS female and male pCREB/CRFR1 in the AVPV/PeN showing female specific increased pCREB. 3V, third ventricle; NS, non-stressed; CVS, chronic variable stress. * Indicates a main effect of sex, (F>M; p < 0.05). (+) indicates a significant effect of treatment, (CVS>NS; p > 0.05).
Figure 4.4. PVN CRFR1, c-Fos/CRFR1, and pCREB/CRFR1 co-expression. Following a 9-day CVS schedule, the PVN showed male-specific reduction in cellular activation via c-Fos/CRFR1. (A) The female PVN had significantly less CRFR1 than the male, regardless of treatment, with no treatment effect in CRFR1 expression for either sex. (B) Representative PVN CRFR1 images for female and male mice from NS and CVS conditions. (C) CVS males had significantly less c-Fos/CRFR1 co-localization than non-stressed male counterparts, which was not seen in females. (D) Representative c-Fos/CRFR1 expression in female and male mice from NS and CVS treatments. (E) Males had an overall greater amount of pCREB/CRFR1 than females, regardless of treatment condition, (F) with representative PVN pCREB/CRFR1 photographs from female and male mice in both NS and CVS conditions. NS, non-stressed; CVS, chronic variable stress. Data are presented as mean ± SEM, and significance threshold set to p<0.05. * Indicates statistical significance p<0.05.
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<td>pCREB</td>
<td>659.795 ± 62.369</td>
<td>1046.882 ± 93.050%</td>
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Table 4.1. Total c-FOS and pCREB immunoreactivity in associated limbic and hypothalamic sites. * Indicates main effect of sex (Female > Male). ^ Indicates main effect of chronic stress (NS > CVS). & Indicates effect of chronic stress on females only (CVS Female < all others). % Indicates interaction of chronic stress and sex, with further post-hoc analysis showing CVS males had significantly reduced pCREB+ cells/mm². NS, non-stressed; CVS, chronic variable stress; AVPV/PeN, rostral anteroventral periventricular nucleus; PVN, paraventricular nucleus of the hypothalamus; ARC, arcuate nucleus; BSTav, anteroventral portion of the bed nucleus of the stria terminalis; BSTdl, dorsolateral portion of the bed nucleus of the stria terminalis; BLAv, ventral basolateral amygdala; CeA, central amygdala. Data reported as mean cells/mm² ± SEM, with the significance threshold set at p≤0.05.
Figure 4.5. CRFR1, c-Fos/CRFR1, and pCREB/CRFR1 co-localization. There was a main effect of CVS treatment on the total c-Fos/CRFR1+ cells in the (K) BLAv showing that all CVS-exposed animals had less c-Fos/CRFR1 expression than non-stressed counterparts. There was also a main effect of CVS treatment for the c-Fos/CRFR1 in the BSTav, further comparison revealed that NS females had significantly more c-Fos/CRFR1 co-expression than CVS females (B). The columns, from left to right, depict CRFR1 cells/mm², c-Fos/CRFR1 cells/mm², and pCREB/CRFR1 cells/mm² respectively within the (A-C) anteroventral BST, (D-F) dorsolateral BST, (G-I) central amygdala, (J-L) ventral basolateral amygdala, and (M-O) arcuate nucleus. + Indicates main effect of stress on outcome, p≤0.05. * Indicates an effect of sex on outcome, p≤0.05. & Indicates a trend toward main effect of treatment, p = 0.056. NS, non-stressed; CVS, chronic variable stress.
Chapter 5: Delineating a role for the cell phenotype ERα/CRFR1 in anxiety and depressive-like behaviors and activation of the HPA axis

Introduction

Gonadal hormones are involved in modulating the HPA axis and regulating anxiety- and depressive-like behaviors through activity at cognate receptors (Burgess et al., 1992; Handa et al., 1994; Viau 2002; Weiser and Handa, 2009; Zuloaga et al., 2011; Klein and Romeo, 2013; Handa and Weiser, 2014; Ramos-Ortolaza et al., 2017). Activity at ERα is generally anxiogenic and increases activation of the HPA axis (Burgess and Handa, 1992; Weiser and Handa, 2009), while signaling at ERβ or AR has been shown as anxiolytic, and reduces HPA axis activation (Burgess et al., 1992; Handa et al., 1994; Handa et al., 1994; Lund et al., 2006; Zuloaga et al., 2011). However, we do not know how estrogens act to in the brain to impact observed effects.

Based on previous work in our lab, we show a sex difference in the percentage of ERα/CRFR1+ cells in the AVPV/PeN where the majority of CRFR1 within the female AVPV/PeN co-expresses ERα (~80%), and the few CRFR1+ cells in the male AVPV/PeN do not. Our lab has shown co-expression of CRFR1/ERα in other regions aside from the AVPV/PeN. Specifically, it should be noted that approximately 29% of CRFR1 neurons in the PVN, 65% of CRFR1 neurons in the medial amygdala, and 35% of the CRFR1 neurons in the medial preoptic area co-express ERα, further indicating it as a site through which gonadal hormones can bind and modulate CRFR1-associated functions. Preliminary studies in our lab show the female AVPV/PeN CRFR1-expressing cells are active (increased pCREB/CRFR1 co-expression) after an acute 30-minute restraint stress (not seen in male mice). This couples with evidence from previous research showing that ERα perpetuates HPA activation in response to stress (Burgess et al., 1992; Handa et al., 1994; Zuloaga et al., 2011). In addition, some have even argued that ERα is a “master regulator” of the stress response, based on modifications to downstream gene
expression (Lorsch et al., 2018). Overall, it is possible that estrogens binding ERα on CRFR1-expressing cells are a key factor in driving sex differences in the HPA axis and stress-related behaviors.

Because both CRFR1 and ERα are known to modify the HPA axis and stress-related behaviors, the aim of the present investigation was to test the hypothesis that the ERα/CRFR1 cell phenotype is key in modulating the HPA axis, and acute behavioral stress response. We generated a transgenic mouse line that had ERα globally removed from CRFR1-expressing cells (ERαlox/lox / CRFR1Cre/+ ) in female and male mice, and tested the anxiety- and depression-like behavioral responses to select acute stressors. We subjected female and male mice to a behavioral analysis that involved single exposure to the following behavioral assays: open field test, light/dark test, novelty suppressed feeding test, and the tail suspension test, and a final 30-minute restraint stress. During the 30-minute restraint, tail blood was collected at baseline (within 3 min of touching home cage), peak (at the time of removal from the restraint tube), and recovery (1 hour after removal from the restraint tube), to determine the role of the ERα/CRFR1 phenotype on the CORT response. We report no acute behavioral effects of the ERα/CRFR1 cell phenotype in any of the behavioral analyses used. Further, after collecting blood serum for CORT analysis at baseline, peak, and recovery, we report no differences within either female or male mice based on genetic background. We believe that the combined influence of many factors including a mixed strain background, and global deletion of ERα from CRFR1 from ontogeny may have prevented our ability to determine the genetic influence on an acute behavioral response to a stressor in adult female and male mice.

Methods

Animals: We generated a conditional knockout mouse line that had ERα removed from CRFR1-expressing cells (CRFR1Cre/+ / ERαlox/lox), to test the influence of the ERα/CRFR1 cell phenotype on adult regulation of HPA activity, and anxiety- / depression-like behaviors. First, we
bred a homozygous female and male \(\text{ER}\alpha^{\text{lox/lox}}\). These animals were crossed with \(\text{CRFR1}^{\text{CRE/+}}\) animals. The next cross involved mating subsequent offspring that were \([\text{CRFR1}^{\text{CRE/+}} / \text{ER}\alpha^{\text{lox/WT}}]\) with \([\text{ER}\alpha^{\text{lox/lox}}]\) mice to give us the conditional knockout \((N=21, 11\ \text{female}, \ 10\ \text{male}; \text{CRFR1}^{\text{Cre/+/ER}\alpha^{\text{lox/lox}}})\). The female and male \(\text{CRFR1}^{\text{CRE/+}}\) mice express a reporter on the CRE promoter (tdtomato), which allows for dual-label IHC against dsRed (labeling CRE+ cells) and ERα. For the study, offspring of the final cross were genotyped for the expression of iCRE (1100bp) using the same primers and annealing temperature as listed in Chapter 3. A second PCR was performed on the same animals to confirm the presence of homozygous floxed ERα (1280bp), using the following primers: forward [TGG GTT GCC CGA TAA CAA TAA C], and reverse [AAG AGA TGT AGG GCG GGA AAA G]. Finally, we used animals genotyped as \(\text{CRFR1}^{\text{CRE/+}}/\text{ER}\alpha^{\text{WT}}\) \((N=15; \ 6\ \text{female}, \ 9\ \text{male})\), \(\text{CRFR1}^{\text{CRE/-}}/\text{ER}\alpha^{\text{lox/lox}}\) \((N=18; \ 10\ \text{female} \ 8\ \text{male})\), and wild type mice \((N=23, \ 10\ \text{female}, \ 13\ \text{male})\) all as non-knockout control groups.

**Ovariectomy (OVX) (Day 1-3):** Adult female mice \((N=37)\) were ovariectomized (OVX). Briefly, female mice were anesthetized with isoflurane and the incision areas were cleaned with ethanol and betadine. Small bilateral incisions were made through the skin and muscle wall overlying the ovaries. The ovaries were retracted using forceps, ligated between the ovary and uterine horn, and removed using surgical scissors. Muscle walls were closed using dissolvable sutures and the skin was closed with surgical staples. The analgesic carprofen \((0.05\ \text{mg/kg})\) was injected sc for two days post operatively.

**Estradiol (E2) Replacement (Day 9):** Adult female mice \((N=37)\) were given a subcutaneous implant of estradiol dissolved into sesame oil to form a concentration of 25µg/mL within each pellet. Animals were given two days post-implant before behavior testing commenced. Based on previous studies, this approach has previously been shown to keep estradiol at levels approximating proestrus in the mouse and by two days post-implant, LH surges occur based on
previous studies that used this method (Stephens et al., 2015). All female mice had approximately two weeks of estradiol exposure (see experimental timeline).

**Open Field (Day 11):** All animals from both treatment groups were exposed to a 5-minute video-recorded open field assessment on day 11 (anxiety-like behavior). The apparatus used was a Plexiglas cube (16" x 16" x 16") with opaque sides and an open top. The test was illuminated from above, with a camera mounted on the ceiling for behavior recording. AnyMaze software (Stoelting Co.) was used to superimpose inner and outer areas over the apparatus, from which the number of inner arm entries and total time spent in the inner area were calculated. In addition, AnyMaze software was used to calculate general locomotion (meters traveled). Defecations were hand-scored after each mouse was removed from the apparatus. All mice were individually placed into one corner (bottom left-hand corner), and allowed to roam freely for 5 minutes. After 5 minutes, animals were removed and returned to their home cages. The apparatus was cleaned with 70% ethanol between each test.

**Light/Dark Box (Day 13):** All animals were placed into a light/dark box on day 13 (anxiety-like behavior). The apparatus is composed of two equal size chambers. One of the chambers was brightly illuminated, and the other was an opaque (“dark”) chamber with minimal light entering from a small doorway. The mice were placed into the dark side of the apparatus and allowed to move freely for 5 minutes, and were video monitored for an assessment of the following behaviors: number of peaks from dark chamber into light chamber, latency to first enter the light chamber, total number of entries into the light chamber, and time spent in the light chamber. Peaking was defined as any time that a mouse had its head visibly in the light chamber, but remaining inside the dark box. Latency to enter the light box was measured in seconds and defined by subtracting the time at which the mouse was placed into the dark chamber and when it first fully entered the light chamber. If a mouse failed to enter the light box, it was given a
score of 300 seconds (5 min). Mice with longer latency to enter the light chamber, less distance traveled, and fewer transitions, are considered to have increased baseline anxiety-like behavior. The apparatus was cleaned with 70% ethanol between each test.

**Novelty Suppressed Feeding (NSF) (Day 15)**: All animals were exposed to the novelty suppressed feeding test on day 15 (anxiety-like behavior). Animals had their food removed the morning before the NSF took place. The next day, animals were placed into a clear Plexiglas cube (16” x 16” x 16”) with transparent sides in a dimly-lit room. The cube had a small amount of bedding (one standard home cage worth) and a single piece of standard chow was placed on a petri glass in the center of the cube, for increased visibility. Animals were placed into the NSF and allowed to freely explore for the full 10 minutes. Animals were recorded from a front-facing Logitech web camera and later the latency to eat was hand-scored by an observer blinded to treatment conditions. Animals were assigned the maximum amount (10 minutes, or 600 seconds) if they did not eat the food pellet in the allotted time. If the animal displayed a hunched position, with forearms up and the mouth moving, they were considered eating, and latency to eat was recorded upon first display of this behavior. All animals were left in the NSF for the full 10 minutes after which they were returned to their home cage. The protocol was designed from a previously validated NSF model (Zhang et al., 2018).

**Tail Suspension Test (TST) (Day 17)**: All animals, with the exception of home cage controls were administered the tail suspension test on day 17 (depression-like behavior). Briefly, animals were suspended upside-down by the tip of their tail for 6 minutes, with video monitoring for the amount of time the animal spends struggling to orient right-side up. Animals that spend more time passively hanging upside-down are considered helpless and classified as having a more depressive behavioral phenotype. Passive hanging versus active struggling was scored using ButtonBox software by an observer blinded to treatment conditions.
**Restraint Stress (Days 19-21):** All animals with the exception of home cage controls, were given a final 30-minute restraint stress one week after the tail suspension test (days 19-21), and had tail blood drawn before restraint (0min, *baseline*), upon removal from restraint (30min, *peak*), and 90 minutes after restraint onset (*recovery*). For specific information regarding the restraint tube, see the methods from chapter 3, as the same tube was used. The restraint tube was wiped with 70% ethanol between each test.

**Blood Collection, CORT Radioimmunoassay:** At collection time points listed above, blood was collected in EDTA-coated tubes for a radioimmunoassay on CORT. The blood collected at the recovery time-point was trunk blood immediately following cervical dislocation. Blood was centrifuged at 5,500 g for 10 min and the supernatant was transferred to a new tube and stored at -80°C until assay. Plasma samples were analyzed using commercial I125 corticosterone radioimmunoassay following manufacturer instructions (MP Biochemicals, LLC, Orangeburg, NY, USA). Intra-assay coefficient of variation was 4.4%.

**Tissue Collection:** At 90 minutes after removal from restraint, each animal was given a rapid cervical dislocation at which point trunk blood was collected for the recovery time-point, after which the brain was removed. Following tissue extraction, each brain was placed into a 4% paraformaldehyde solution in phosphate buffered saline (4% PFA) overnight. The next day, tissue was transferred to a 30% sucrose solution at 4°C until sectioning. Tissue from select animals was sectioned on a cryostat at -22°C into three alternating 40µm series and placed in cryopreservative until immunohistochemistry used to validate the knockout (shown below).

**Immunohistochemistry:** To confirm that ERα was removed from CRFR1-expressing cells, we performed a dual-label fluorescent for dsRED and estrogen receptor alpha. This was possible because the CRFR1<sup>CRE/+</sup> animals were bred to express tdTomato (red fluorescent protein) on the CRE promoter; therefore, running a dual-label IHC for ERα and dsRED was sufficient for visualization of co-expression. Sections were thoroughly rinsed in phosphate-buffered saline.
(PBS; pH 7.6), then incubated in 4% normal donkey serum (4% NDS) and 0.4% Triton-X in PBS (PBS-TX) for 1 hour. Immediately following incubation, tissue was placed into the primary antisera (Santa Cruz rabbit SC542 rabbit, ERα; 1:250) and incubated at room temperature overnight. The following day, tissue was quickly rinsed (15min) in PBS and then placed into secondary antisera (Alexafluor anti-rabbit, 488; 1:200) in 4% NDS and PBS-TX for 2.5 hours. After secondary, the tissue was thoroughly rinsed (80 min) and then blocked in 4% NDS for one hour before being transferred into the second primary antisera (Rockland 200-101-379 goat anti-RFP, 1:250) in 4% NDS and PBS-TX at room temperature overnight. On the third day, tissue was rinsed in PBS, then transferred to the next secondary antisera (Alexafluor anti-goat, 594; 1:200) for 2.5 hours. After which, tissue was rinsed again in PBS (15min total). Immediately following the final rinse, tissue was mounted and coverslipped using Santa Cruz hard set mounting media when dry.

Microscopy and Statistical Analysis: Females and males were all exposed to behavioral tests that measure either anxiety- or depression-like behavior in mice. Animals were all compared by genotype within sex using a 1-way ANOVA, data are reported as the mean ± SEM, with the threshold for significance set at p≤0.05, and used Newman-Keuls multiple comparison test between genetic backgrounds within each sex, unless otherwise stated. Male and female mice were analyzed separately because males were not given a gonadectomy like the females, which doesn’t allow for direct statistical comparisons. Images for knockout validation were conducted on the same microscope and accompanying apparatus as in previous chapters. Briefly, the Nikon Eclipse microscope was used to capture co-localization images from atlas-matched sections within the AVPV/PeN and POA to show that ERα KO females showed limited to no co-expression of ERα/CRFR1.
Results

Validation of Knockout: We performed a dual-label immunohistochemistry to establish that our animal model was properly generated. We report that our knockout animals showed minimal to no co-localization of ERα/CRFR1 while animals that were CRFR1^{CRE/+} demonstrated ERα/CRFR1 co-expression in the AVPV/PeN and POA (Figure 5.2). The AVPV/PeN and POA were selected because these regions had high ERα/CRFR1 co-expression in WT females.

Open Field Test (OFT): Following exposure to the open field, we report no differences based on genetic background in either female or male mice in latency to first center entry, time spent immobile, total center entries, time in the center, or the total distance traveled (Figure 5.3).

Light/Dark Box (LD): Following exposure to the light/dark box, we report no significant differences regardless of genetic background in either sex for number of light peaks, latency to enter the light box, total light entries, or total time in the light box (Figure 5.4).

Novelty Suppressed Feeding (NSF): Upon analysis of the novelty-suppressed feeding, we report no significant differences regardless of genotype or sex on the latency to begin eating in a novel environment (Figure 5.5).

Tail Suspension Test (TST): There were no significant differences in the tail suspension test, regardless of genotype or sex for latency to passive hanging or struggle duration (Figure 5.6).

Serum corticosterone: We collected blood samples surrounding a final 30-minute restraint stress. Specifically, we collected tail blood at baseline (0 minutes), peak (30 minutes), and recovery (90 minutes) after the onset of restraint. Following a radioimmunoassay, we report no differences within either sex based on their genetic background. We do repeat a commonly reported sex difference overall on serum CORT ng/mL, where female mice, regardless of
genetic background had more serum CORT than males, when matched and qualitatively compared against each time-point. It is important to keep in mind that due to our experimental design, we could not make a direct comparison between female and male serum CORT (Figure 5.7).

Discussion

We investigated the role of the ERα/CRFR1 cell phenotype in anxiety- and depression-like behavior. Surprisingly, we found no behavioral differences in either female or male mice, regardless of genetic background. We confirmed these data were not from an ineffective knockout model (Figure 5.2). There are several factors that could be influencing the lack of behavioral effect. First, the animals generated had no ERα expressed on CRFR1 cells from ontogeny, which likely had other influences on the development of the HPA axis and associated brain systems. Second, both CRFR1 and ERα are widely expressed throughout the mouse brain, including regions aside from the AVPV/PeN such as the PVN, MPOA, hippocampus, prefrontal cortex, and amygdala, among other stress-responsive regions (Viau, 2002; Blank et al., 2003; Abel and Majzoub, 2005; Jankord & Herman, 2008; Klampfl et al., 2018; Chen et al., 2018; Ross et al., 2019), and are not always co-expressed. Therefore, cells expressing either ERα or CRFR1 were undisturbed and could have contributed toward compensation in regulating the HPA axis and stress-related behavior.

Recent work has shown that the effect of estrogens acting at ERα largely depends on the brain region in which increased ERα signaling occurs (Morgan et al., 2004). Depending upon the brain region, estrogenic activity at ERα can have opposing biological and behavioral influences. For example, a recent study showed that overexpression of ERα in the nucleus accumbens afforded both female and male mice resilience to depression-like behavior (Lorsch et al., 2018). Others have shown that ERα stimulation in the MPOA drives increased anxiety-like behavior (Spiteri et al., 2012); similar results were reported for ERα ex activation in the
Collectively this suggests that the lack of behavior or CORT data may be due to conflicting influences across the rodent brain.

Alongside ERα, the impact of CRFR1 activation on the HPA axis and stress behavior also varies by brain region. For example, a CRFR1-specific agonist acting in the medial prefrontal cortex attenuates c-Fos activity in the amygdala (Pentkowski et al., 2013), and CRFR1 activation in the globus pallidus also reduces HPA axis activity and anxiety-like behavior (Sztainberg et al., 2011). In addition, others have shown that PVN CRFR1 cells reduce HPA activation, (Jiang et al., 2018). However, activating CRFR1 in the BLA increases anxiety-like behavior (Cipriano et al., 2016). It is possible that the ERα we see on a subpopulation of CRFR1 cells in the PVN (~29%; Rosinger et al., 2019b) contributes to the “protective” effect seen in male mice. These data also highlight the importance of species-specific phenotypic explorations, because in the rat PVN, for example, there is no ERα expression, but high amounts of ERβ (Shughrue et al., 1997).

Our lack of behavior data may also be influenced by the mixed strain background (129/Sv and C57BL6) we used, possibly increasing behavioral variability. Studies have shown strain influences on behavioral analysis suggesting that the cross between two strains that we used may have influenced these assessments. C57/BL6 mice tend to have high levels of locomotion and lower levels of baseline anxiety when confronted with stressors such as the open field test, compared with mice of other backgrounds (Crawley et al., 1997). For the light/dark box, the same authors (Crawley et al., 1997) report that C57/BL6 mice have a significantly higher baseline number of light entries in general than a majority of other strains. When we designed and conducted this experiment, we did not anticipate mixed strain behavioral variability, because others have used the ERα flox line on the same mixed background and reported significant behavioral changes in knockout lines (Wu & Tollkuhn,
There now exists ERα/CRFR1 floxed mice on a C57 background, which would enable testing without the influence of a mixed strain background.

Research has consistently shown that both ERα and CRFR1 cause increased synthesis and release of CORT (Viau 2002; Seale et al., 2004; Klein and Romeo, 2013; Handa and Weiser, 2014). Therefore, we hypothesized that ERα/CRFR1 knockout animals would have an altered CORT response to acute stress from removing the estrogenic influence on CRFR1 cells. In the present investigation following a radioimmunoassay, we show no differences based on genetic background in either sex for serum CORT (Figure 5.7). It is possible that cells co-expressing ERα/CRFR1 are not important in mediating stress-related functions. There are still ERα- and CRFR1-expressing cells throughout the brain in our mouse model, and it may be that eliminating the co-expression is not influential enough to alter serum CORT levels. Had we seen altered CORT in response to acute stress, then it would be interesting to investigate the role of ERα/CRFR1 cells with CVS exposure. Differences in serum CORT based on genetic background would show that these mice would have chronic high levels of CORT, which over time can facilitate the onset of behavioral changes such as increased anxiety- or depression-like behavior. Support comes from research using chronic CORT injections in male rats that reported increased depression-like behavior (Gregus et al., 2005), and in mice, those bred naturally to have higher baseline CORT were also later shown to display increased depression-like behavior (Malisch et al., 2008). However, considering the lack of genetic influence on serum CORT in either sex, it seems unlikely that this cell phenotype would have any bearing on long-term alterations.

The data from the current investigation suggest that the cell phenotype ERα/CRFR1 is not vital in regulating either the biological response of the HPA axis or mediating the behavioral components of acute stress. However, because of the different region-specific impacts of both ERα and CRFR1 activity, it is also possible that regions specific knockout methods should be
employed to more deeply assess the role of the cell phenotype in driving any sex differences in HPA axis regulation or behavioral responses to stress. An AVPV/PeN-specific knockout of ERα from CRFR1, for example, would show whether estrogens mediate CRFR1 activation in response to stress. Other region-specific ERα knockouts from CRFR1, such as in the BST or amygdala, would test if estrogenic signaling at CRFR1 regulates CRFR1 activity, the HPA axis, or stress-related behaviors.
Figures

Figure 5.1. Experimental Timeline. Female mice underwent ovariectomy and were then given 6-9 days of recovery before receiving a subcutaneous implant of estradiol. Behavior testing commenced at two days post-supplement, at which point female mice have been previously shown to mirror normal proestrus continuously for the duration of the pellet life (up to 14 days post-implant). Males were left undisturbed until day 11. Female and Male mice were given two days between each behavior assay. On days 19-21 for the restraint stressor and CORT collection, the cohort was divided into three groups, to ensure that all samples could be collected before noon to avoid circadian CORT variation. Animals were separated into single-housed cages for 2 days before the final restraint. OVX, ovariectomy; OFT, open field test; LD, light/dark box; NSF, novelty-suppressed feeding test; TST, tail suspension test; CORT, corticosterone.
**Figure 5.2. Validation of ERα knockout from CRFR1-expressing cells.** Non-floxed, CRE+ controls (left column) and ERα/CRFR1 knockout (right column) animals were compared to ensure that ERα was removed from CRFR1<sup>CRE/+</sup> expressing cells. CRFR1<sup>CRE/+</sup> cells (red), ERα+ cells (green). Yellow indicates CRFR1/ERα co-expression in the (A, B) AVPV/PeN and the (C, D) POA. (E, F) High magnification of the AVPV/PeN and (G, H) the POA of CRE+ and KO animals respectively. White arrows indicate examples of animals with CRE+ showing wild type co-localization. (I, J) Representative graphs showing the percentage of co-localization in WT and KO animals within the AVPV/PeN and POA, respectively. AVPV/PeN, rostral anteroventral periventricular nucleus; POA, preoptic area; all images are from female mice. POA, preoptic area. Data are reported as arithmetic mean ± SEM, * p<0.01.
Figure 5.3. Influence of ERα/CRFR1 on open field behavior. No differences within either sex based on genotype for any of the measured behaviors. From left to right, latency to first center entry, total time immobile, total center entries, total time in center, and total distance traveled showed no differences based on genetic background. Top row (A-E) are the female and bottom row (F-J), male mice. A 1-way ANOVA was conducted using genetic background as the factor within each sex. Data are reported as the arithmetic mean ± SEM, the significance threshold set to p<0.05.
Figure 5.4. Light Dark Box Behavior. Though it may appear as if the CRFRsummary CRE+/+ males had a lower latency to enter the light box, there were no significant differences regardless of genotype on any of the light/dark box behaviors assessed (number of peaks into light box, latency to enter light box, number of entries into light box, total amount of time in the light box). Females are the left column, males the right. Data are reported as the arithmetic mean ± SEM, and the significance threshold was p<0.05.
Figure 5.5. **Novelty Suppressed Feeding Behavior.** In all animals (female and male), there were no differences in the latency to eat in the novelty-suppressed feeding test, regardless of genotype. (A) Female latency to eat, and (B) male latency to eat. Note how visually similar WT and KO latencies are for both female and male mice. Data are reported as average latency in seconds to eat, ± SEM and the threshold for significance was set to p<0.05.
**Figure 5.6. Tail Suspension Test.** There were no differences based on sex or genetic background for the latency to stop struggling, or total struggle duration during the tail suspension test. (A) Female latency to stop struggling and (B) female struggle duration by genetic background. (C) Male latency to stop struggling, and (D) male struggle duration by genetic background. Control groups: [ERα lx/lx = ERαlox/lopx/CRE1CRE/-]; [CRE+ = ERαWT/CRE1CRE/+]; [WT = wild type]; [KO = ERαlox/lopx/CRE1CRE/+ , knockout]. Data are reported as arithmetic mean ± SEM with the significance threshold set at p<0.05.
Figure 5.7. Serum CORT from female and male mice based on genotype. Blood was collected at three time points: baseline (0 minutes), peak (30 minutes from onset), and recovery (90 minutes from onset) following an acute 30-minute restraint stress. (A) Female serum CORT was not different for any animals regardless of genetic background. (B) Male serum CORT was not significantly different for any animals regardless of genetic background. However, a visual comparison shows that the females at each time point, respective to males, had higher serum CORT, regardless of genetic background in either sex. Bar colors: [Purple, ERα<sup>lx/lx</sup>/CRFR<sub>1</sub><sup>CRE/+</sup>]; [black, ERα<sup>WT</sup>/CRFR<sub>1</sub><sup>CRE/+</sup>]; [green, wild type]; [red, knockout line, ERα<sup>lx/lx</sup>/CRFR<sub>1</sub><sup>CRE/+</sup>].
Our laboratory initially sought to investigate the development of the CRFR1 system in the female and male mouse. Prior to our investigations, there had not been data available on the development of CRFR1 in the mouse, in large part due to a lack of effective antibodies targeting CRFR1. Our lab utilized an animal model with GFP attached to the promoter region of the CRFR1 gene, thereby allowing us to use histochemical methods to visualize GFP, and thereby visualize CRFR1. CRFR1 has been implicated in the etiology of stress-related mood disorders such as anxiety, depression, PTSD, and addiction; hyper-activation or dysregulation of CRFR1 can precipitate the onset of these conditions (Chrousos 2009; Garcia-Carmona et al., 2015; da Silva et al., 2016; Holly et al., 2016; Schussler et al., 2016). Stress-related mood disorders have sex differences in prevalence (Female > Male). We therefore believed that any potential sex differences in CRFR1 distribution or activation may contribute to an understanding of the different neural pathways by which female and male mice respond and adapt to stress.

During the initial investigations we reported many dynamic regions of CRFR1 expression that changed based on age. We did not have any a priori hypotheses at the outset regarding any particular brain regions; we did hypothesize there would be differences between female and male mice, along with differences across development. Following the developmental investigation, we found that the AVPV/PeN was sexually dimorphic from the day of birth, present in females and largely absent from males. The AVPV/PeN is located within the preoptic area and others have previously shown that the AVPV itself has implications with reproductive and maternal behavior (Simerly et al., 1997; Scott et al., 2015). Others have shown an overall volumetric difference, and in the expression of other markers such as tyrosine hydroxylase and kisspeptin (Simerly, 1989; Simerly et al., 1997; Semaan et al., 2010; Brock, et al. 2015; Scott et al., 2015), all higher in females than males.
The sexually dimorphic expression of CRFR1 in the AVPV/PeN prompted us to characterize the chemical composition, to help understanding the role these cells may play in the stress response. After a series of histological studies, we report that the CRFR1 within the female AVPV/PeN highly co-expresses both ERα and GR (Figures 2.6, 2.7; Rosinger et al., 2019a), and that these cells are only involved with central nervous system signaling (Figure 2.11). Further, we showed that these cells are mostly independent from the previously reported dimorphic TH and KISS cell groups. While some of the CRFR1 cells do co-express either TH or KISS, the majority do not. However, both TH and KISS cells had relatively higher percentages of CRFR1 here, suggesting that CRFR1 is co-expressed on several different cell types in the AVPV/PeN, and that different environmental experiences alter different cell types. For example, it is possible that CRFR1 signaling in the AVPV/PeN could interrupt KISS-ergic signaling, causing stress-related impairments in sex behavior or reproduction. Along similar lines, the TH+ cells in the female AVPV/PeN may become more specifically altered following parental-related stress, especially since TH is known to change following maternal experience. There are additional CRFR1 that largely do not co-localize with either KISS or TH, which suggests that these cells are involved in other systems. Overall, the phenotypic characterization of AVPV/PeN CRFR1 suggests that these cells are involved in diverse functions, all of which are subject to the influence of female stress exposure.

We had shown on the day of birth, the CRFR1 cell cluster was present in only females, which led us to hypothesize that it was the perinatal androgen surge in males driving the early masculinization of the AVPV/PeN CRFR1. We gave P0 infusions of either TP, EB, or vehicle, and at P21, we showed females that got either EB or TP had minimal expression of CRFR1 and therefore successful defeminization of the AVPV/PeN CRFR1 cluster, illustrating a mechanism by which this sexual dimorphism is established. We believe these effects are mediated through activity at ERα, because it is expressed on AVPV/PeN CRFR1 cells at P0 (Rosinger et al.,
2019a), while others have shown a lack of ERβ until later time-points (Zuloaga et al., 2014). Because others have shown limited expression of androgen receptor around the AVPV/PeN at P0 (Juntti et al., 2010; Kanaya et al., 2014), and further that estradiol, which does not bind AR, masculinized the nucleus, we can eliminate the possibility that AVPV/PeN CRFR1 is masculinized via AR activity. After the perinatal development study, we tested the influence of circulating gonadal hormones by performing gonadectomy on adult female and male mice (P60), showing that after 6 weeks post-GDX, there were no differences within either sex when comparing to intact adults (Rosinger et al., 2019a). However, performing a follow-up investigation using testosterone treatment in OVX females would test the role of androgens in adulthood masculinization of the AVPV/PeN. It is possible that there is not a sensitive period in the female AVPV/PeN CRFR1, and that sufficient androgen exposure in adulthood could eliminate female expression.

Following the neonatal and adult gonadal hormone investigations, the next step involved determining any relationship between these cells and the female stress response. After a single 30-minute restraint, we report a female-specific increase in both general AVPV/PeN, and in AVPV/PeN CRFR1-specific activation (Rosinger et al., 2019a). The sex- and stress-specific cellular activation suggests these cells are engaged during acute stress, and could likely be modifying other portions of the HPA axis or limbic system. It is possible that the AVPV/PeN CRFR1 is involved in female-specific negative feedback, because of the activation following stress, and the high level of co-expression with glucocorticoid receptor (Figure 2.11).

After we found that these cells were engaged during acute stress, we aimed to describe anterograde projections from female AVPV/PeN CRFR1-expressing cells. We infused a single-synapse anterograde tracer to describe the efferent connectivity of female AVPV/PeN CRFR1 with the rest of the mouse brain. Others have shown that non-specific cells in the female rat AVPV reach hypothalamic and limbic brain regions (Gu & Simerly, 1997), and there was no
information specific to CRFR1-expressing cells or the mouse. Following tracer infusion into the AVPV/PeN, we showed the CRFR1 cells send dense anterograde projections to the PVN, BST, DMH, and other hypothalamic sites implicated in the stress response, such as the septum and to a lesser degree, the amygdala. The data we report shows similar projections as those within the female rat, and moreover we are now able to show that stress-activated CRFR1 is reaching many of these stress-responsive zones in only female mice. When comparing our anterograde CRFR1 data with another recent study that traced PVN CRFR1 (Jiang et al., 2018), we have evidence suggesting that many of the female AVPV/PeN CRFR1 send efferent projections to regions that have been shown to relay directly to PVN CRFR1 neurons. For example, the lateral septum, BST, and DMH all project to PVN CRFR1 (Jiang et al., 2018), and all of these sites show anterograde fiber labeling from the AVPV/PeN CRFR1 in our study (Chapter 3). Since we know that the AVPV/PeN CRFR1 is activated during stress, it is possible this is a nucleus that modulates hypothalamic signaling to PVN CRFR1. In addition, the PVN received dense and direct innervation from the AVPV/PeN CRFR1. It is therefore possible that different stimuli stimulate different regions to impact and ultimately modulate the PVN tone, before a net output is produced (i.e., increased or reduced pituitary-targeted CRF/AVP, increased oxytocin signaling, etc.). Overall, the CRFR1 cells in the female AVPV/PeN are densely connected with a substantial number of other brain regions known to regulate stress, giving more support that this cluster may have a role in regulating or modifying stress-related adaptations in the female.

Chronic stress is known to precipitate HPA axis dysregulation, stress-, and anxiety-like behavior in rodents (Aguilera, 1994; Hodes et al., 2015), specifically through CRFR1 (Chen et al., 2018). Therefore, we tested the influence of CVS on female and male CRFR1 populations in CRFR1<sup>GFP/+</sup> mice. The most surprising discovery following our 9-day CVS was a female-specific upregulation of CRFR1 expression within the AVPV/PeN. Female mice that went through the CVS procedure showed approximately double the CRFR1-immunoreactivity than non-stress
female controls. The females from the CVS treatment also expressed significantly more CRFR1/pCREB than non-stressed controls. No effects were seen in the male AVPV/PeN, regardless of treatment condition. The maintained increase in AVPV/PeN CRFR1/pCREB-ir may have been driving the upregulation of CRFR1 in the female AVPV/PeN. However, it is also possible that chronic GR activation in the CVS females is what caused the upregulation of CRFR1 expression that we report, because chronic exposure to CORT and hyper-activation of GR is known to lead to HPA axis dysregulation. We currently do not know the mechanism by which this upregulation takes place, nor do we know the functional significance of upregulated CRFR1 in the female AVPV/PeN; future experiments will aim to assess these questions. Interestingly, both female and male mice that underwent CVS demonstrated anxiety-like behavior in the open field test, which suggests that the 9-day CVS paradigm we used was effective for both sexes. This also serves to highlight that different neural circuits are likely involved for females versus males that ultimately end with a similar anxiety-like behavioral phenotype, lending even more support for pursuing sex-specific treatments for stress-related mood disorders.

Following the CVS study, we hypothesized that the cell phenotype ERα/CRFR1 was critical in mediating either the acute biological or behavioral stress response in mice. To test this hypothesis, we created a transgenic line of mice that had ERα removed from CRFR1 using a common CRE/lox system. We were able to demonstrate a successful knockout of ERα from CRFR1, though we saw no behavioral differences within either sex based on genetic background. Further, we saw no differences in serum CORT within either sex when comparing genetic background at any time-points. It seems that a global knockout of ERα from CRFR1 does not cause alterations in acute behavioral or biological responses to stress, but further investigations are needed. It is possible that the opposing influences of ERα and CRFR1 depending upon the brain region and context play a role in the overall lack of effect based on
genetic background (Morgan et al., 2004; Spiteri et al., 2010; Sztainberg et al., 2011; Spiteri et al., 2012; Pentkowski et al., 2013; Cipriano et al., 2016; Jiang et al., 2018; Lorsch et al., 2018; Ramot et al., 2018). It is also likely that because these animals had lacked ERα expressed on CRFR1 since they were in utero, that other compensatory mechanisms took place, such as increased ERα on other cell types aside from CRFR1. At the moment, more research is needed to properly probe this question. Finally, it is also possible that the mixed-strain background contributed to the behavioral variability, though others using a very similar ERαlox/lox model on mixed strain mice showed genotype-dependent behavioral effects on aggression (Wu & Tollkuhn, 2017), suggesting mixed strain variability may not have been a factor.

Going into future studies, it will be important to investigate the role of CRF within the AVPV/PeN and associated regions, and communication with CRFR1 expressing cells, through the use of CRF-CRE animals, which are currently in our vivarium. We have already identified a sexually dimorphic group of CRF cells (F>M) that are proximal to the AVPV/PeN CRFR1 cell cluster (data not shown). These CRF cells may be part of a circuit through which CRFR1 in the female AVPV/PeN is regulated, especially since our tracing studies show bidirectional signaling between AVPV/PeN CRFR1, the surrounding AVPV, and MPOA. Even though we report a lack of acute behavioral data, it would be interesting to have region-specific knockouts of ERα from CRFR1, such as removing ERα from CRFR1 in the AVPV/PeN or PVN, alone. Doing so would give us spatiotemporal control over the knockout, thereby giving us more specific research questions moving forward, now that we have shown a global ERα/CRFR1 knockout did not influence acute stress behavior or HPA activation.

At this point, we believe that the AVPV/PeN CRFR1 cells in the female are involved in the acute stress response, and adaptations following chronic stress. Therefore, another important factor for the future would be selective ablation of CRFR1 cells in the AVPV/PeN to see if this group has any impact on CVS behavior, or on HPA axis activation following either
acute or chronic stress. The female-specific upregulation of CRFR1 we reported from the CVS study (chapter 4) implies a role for the AVPV/Pen CRFR1 in female CVS-induced changes. The increase may be protective, though increased CRFR1 activity in the MPOA (a region adjacent to AVPV/Pen) impairs a variety of behaviors hormone release in females (Dobson et al., 2003; Kinsey-Jones et al., 2009; Kinsey-Jones et al., 2010; Klampfl et al., 2018). Therefore, it is likely that the upregulation we report is maladaptive for females following chronic stress exposure. In addition, we believe that these cells are also important for various factors of maternal care. Investigations are currently underway regarding CRFR1 in the AVPV/Pen and postpartum anxiety-like behavior. Preliminary evidence suggests these cells are modified to some capacity following postpartum experience. Finally, optogenetic tools have become more widespread in their use, and employing such tools would be beneficial for understanding the impact of directly activating or inhibiting AVPV/Pen CRFR1. In doing so, we could assess if these cells directly mediate stress or maternal behavior. With stimulation of AVPV/Pen we could also investigate the influence on PVN, BST, and other regions to which we now know it projects. For example, after AVPV/Pen CRFR1 activation, we could look for changes in cell number, activation state of cells in these areas (i.e., altered levels of c-Fos or pCREB). Along with the cell influence, we could monitor any behavioral changes that may occur as a result of stimulating the AVPV/Pen CRFR1 cells. The use of optogenetics provides an alternative to CVS; optogenetics could be used for varying degrees of chronic AVPV/Pen CRFR1 stimulation over a given period of days and prescribed lengths of time, to compare behavioral or biological changes that may take place following varying levels of chronic stimulation. Performing this would enable us to directly test the role of AVPV/Pen CRFR1 cells in regulating female-specific stress adaptations.

Women have an increased risk of developing stress related mood disorders compared with men, but scientists still lack a clear understanding of why this is the case. It is important to understand any sex differences in the underlying neural circuitry that may explain the observed
sex differences in the prevalence rates for stress-related mood disorders. Based on our current understanding of the AVPV/PeN CRFR1 population, these cells exist primarily in the female mouse, starting at P0 continuing into old age, are stress responsive, and significantly upregulate expression following chronic variable stress. These CRFR1 cells also have dense afferent and efferent projection patterns with many hypothalamic, limbic, and hindbrain structures with known involvement in the HPA axis and behavioral responses to stress. These cells are largely absent from the male, and the few CRFR1 cells in the male AVPV/PeN are not otherwise of the same chemical composition (i.e., no activation following stress, no ERα co-expression). The evidence surrounding the AVPV/PeN CRFR1 from this series of investigations points to this cluster of cells as a female-specific site at which stress can modify HPA activation and behavioral response to stress.
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Supplemental S1. CRFR1-GFP expression in septal and hypothalamic regions (Chapter 2). (A-C) Representative images of the lateral septum, medial septum, and diagonal band for P0, P4, and P21. Note that distribution within the medial septum seems to increase slightly across development. The lateral septum appears to slightly decrease by P21, in comparison to earlier ages. The diagonal band increases slightly from P0 to P4, but it appears to level off at P4, with no visual differences from P4 to P21. (D-F) Representative images from the BST and the medial preoptic area at ages P0, P4, and P21. (G-I) Representative images for the nucleus just dorsal to the paraventricular hypothalamus (P-PV), the paraventricular hypothalamus, the anterior hypothalamus, and the lateral hypothalamus at ages P0, P4, and P21. Within the anterior hypothalamus, there appears to be a slight decrease in CRFR1-GFP-ir density between P4 and P21. MS; medial septum, LS; lateral septum, DB; diagonal band, BST; bed nucleus of the stria terminalis, MP; medial preoptic area, P-PV; nucleus dorsal to the paraventricular hypothalamus, PV; paraventricular hypothalamus, AN; anterior hypothalamus, LA; lateral hypothalamus.
Supplemental S2. CRFR1-GFP expression in the caudal hypothalamus (Chapter 2). (A-C) Representative images of the intermediate periventricular nucleus, dorsomedial hypothalamus, ventromedial hypothalamus and the arcuate nucleus at (A) P0, (B) P4, and (C) P21. Within the intermediate region of the paraventricular nucleus, there is a decrease in CRFR1-GFP-ir from P4 to P21. There are slight increases in CRFR1-GFP-ir for the dorsomedial hypothalamus across developmental windows measured. Little labeling was seen within the ventromedial hypothalamus across ages. Importantly, the arcuate nucleus displays a dramatic increase in labeling between P4 and P21 (D-F). Pe; intermediate periventricular nucleus, DMH; dorsomedial hypothalamus, VMH; ventromedial hypothalamus, ARC; arcuate nucleus of the hypothalamus.
Supplemental S3. CRFR1-GFP expression within the cerebral cortex, hippocampus, and amygdala (Chapter 2). (A-C) In the cerebral cortex CRFR1 immunoreactivity changes dramatically during postnatal development. At P0, labeling is greatest within middle cortical layers while at P4 there are dense and defined CRFR1 layers in both outer and deep cortical layers. By P21 density is greatest in outer and middle cortical layers. Overall, CRFR1-GFP-ir at P0 is somewhat lower than the other ages across regions of cortex. (D-F) Representative images of the hippocampus for P0, 4, and 21, respectively. There is a gradual increase in CA1 pyramidal layer density throughout developmental time points assessed, while there is a stark decrease in immunoreactivity within the CA3 region from P0 to P21. CRFR1-GFP-ir also decreases slightly by P21 within the dentate gyrus. (G) Representative high magnification image of the hilus, the most densely labeled portion of the dentate gyrus. (H-J) Representative pictures of the amygdala at P0, 4, and 21. There is a gradual increase in CRFR1-GFP-ir within the LA and MeA between P0 and P21. Labeling is generally higher in the basomedial amygdala than in the other regions of the amygdala, throughout development. DG, dentate gyrus; L, lateral amygdala; M, medial amygdala; Ce, central amygdala; BM, basomedial amygdala; BL, basolateral amygdala; CO, cortical amygdala.
Supplemental S4. Open field rearing and defecations (Chapter 4). (A) There were no differences in the total number of rearing behaviors in the open field test, regardless of sex or stress treatment. (B) There were no differences between sex or stress treatment on the total number of defecations during the open field test. Data are reported as the mean ± SEM, and the significance threshold was set to p<0.05. White bar, NS; non-stressed. Black bar, CVS; chronic variable stress exposure.
Supplemental S5. Serum CORT in ng/mL following 9-day CVS (Chapter 4). Blood was collected at 90 minutes after the onset of open field. Results show a main effect of sex, where females had significantly more serum CORT than males, regardless of treatment. Of note, compared with other literature, the males from both CVS and NS conditions show elevated CORT from baseline, showing that males are in recovery and have less serum CORT at this point compared with females of both treatment groups. Data are shown as average CORT present in ng/mL ± SEM; * indicates p≤0.05.
Supplemental S6. BLAv and BSTav show treatment- and sex-specific reductions in CRFR1/c-Fos activation (Chapter 4). (A) CVS-exposed mice showed a reduction in CRFR1 activation in the ventral BLA. (B) CVS females showed a significant reduction in CRFR1 activation in the BSTav, while there were no differences for the males, regardless of stress condition. NS, not stressed; CVS, chronic variable stress. (C) High-magnification images of BLAv and (D) BSTav. Arrows indicate CRFR1/c-Fos co-expression, white dashed boxes indicate location of high-magnification images.