HHS Public Access

Author manuscript

Neuroscience. Author manuscript; available in PMC 2020 June 15.

Published in final edited form as:

Neuroscience. 2019 June 15; 409: 195–203. doi:10.1016/j.neuroscience.2019.04.045.

A sexually dimorphic distribution of corticotropin-releasing factor receptor 1 in the paraventricular hypothalamus

Zachary J Rosinger[1], **Jason S Jacobskind**[1], **Rose M De Guzman**[1], **Nicholas J Justice**[2], **Damian G Zuloaga**[1]

[1]University at Albany, Department of Psychology, Albany, NY 12222.

[2]Center for Metabolic and Degenerative Diseases, Institute of Molecular Medicine, University of Texas Health Sciences Center, Houston, TX, USA.

Abstract

Sex differences in neural structures are generally believed to underlie sex differences reported in anxiety, depression, and the hypothalamic-pituitary-adrenal axis, although the specific circuitry involved is largely unclear. Using a corticotropin-releasing factor receptor 1 (CRFR1) reporter line, we report a sexually dimorphic distribution of CRFR1 expressing cells within the paraventricular hypothalamus (PVN; males > females). Relative to adult levels, PVN CRFR1-expressing cells are sparse and not sexually dimorphic at postnatal days 0, 4, or 21. This suggests that PVN cells might recruit CRFR1 during puberty or early adulthood in a sex-specific manner. The adult sex difference in PVN CRFR1 persists in old mice (20–24 months). Adult gonadectomy (6 weeks) resulted in a significant decrease in CRFRI-immunoreactive cells in the male but not female PVN. CRFR1 cells show moderate co-expression with estrogen receptor alpha (ERα) and high co-expression with androgen receptor, indicating potential mechanisms through which circulating gonadal hormones might regulate CRFR1 expression and function. Finally, we demonstrate that a psychological stressor, restraint stress, induces a sexually dimorphic pattern of neural activation in PVN CRFR1 cells (males >females) as assessed by co-localization with the transcription/neural activation marker phosphorylated CREB. Given the known role of CRFR1 in regulating stress-associated behaviors and hormonal responses, this CRFR1 PVN sex difference might contribute to sex differences in these functions.

Introduction

In numerous species, including rodents and humans, sex differences in anxiety, depression, and hypothalamic-pituitary-adrenal (HPA) axis function have repeatedly been reported (Heck & Handa, 2019; Kessler et al., 1994; Kessler et al., 2012; Nolen-Hoeksema, 1987), while the neurobiological underpinnings of these sex differences are largely unknown. Sex differences in brain corticotropin-releasing factor (CRF) and its receptors CRFR1 and CRFR2 represent intriguing potential mechanisms through which these behavioral and hormonal sex differences might be regulated, based on the known role of the CRF system in

Corresponding Author: Damian Zuloaga, Ph.D, Dept. of Psychology, University at Albany, 1400 Washington Ave Albany, NY 12222, dzuloaga@albany.edu, Phone: 518-442-4361, FAX: 518-442-4867h.

controlling depressive- and anxiety-related behaviors as well as the HPA axis (Britton et al., 1986; Deak et al., 1999; Muller et al., 2003; Timpl et al., 1998).

Region specific sex differences in CRF and CRF receptors have previously been reported. In the rat brain, CRF-expressing cells are more numerous in the male central amygdala, while females show greater CRF cell number in the preoptic area and bed nucleus of the stria terminalis (Funabashi et al., 2004; Karanikas et al., 2013; McDonald et al., 1994). Sex differences in CRFR1 have also been found in the rat anterior cingulate, piriform cortex, nucleus accumbens, and olfactory tubercle with females showing higher levels of expression (Wealthington et al. 2014). In voles, a sex difference in CRFR2 has been found in the bed nucleus of the stria terminalis (males > females; Lim et al., 2005). Also, our laboratory recently discovered a sexually dimorphic cluster of CRFR1 cells in the rostral anteroventral periventricular nucleus (AVPV) of the mouse which is prominent in females but virtually absent in males (Rosinger et al., 2017). Together, these and other sex differences in the CRF system likely contribute to sex differences in depression, anxiety, arousal, and HPA axis regulation (Bangasser et al., 2019; Bangasser & Wiersielis, 2018). Accordingly, sex differences have been reported in CRF regulation of stress-related behaviors. In mice deficient for both CRF receptors, changes in anxiety-like behaviors were sex-dependent (Bale et al., 2002), further supporting the idea that CRF and its receptors might regulate sex differences in stress-related behaviors.

The paraventricular nucleus of the hypothalamus (PVN) is a region critical to the regulation of the HPA axis, and controls several behavioral and autonomic functions as well (Abel & Majzoub, 2005; Eaton et al., 1996; Makino et al., 1995). In particular, CRF containing cells in the PVN play a central role in regulating HPA axis function by stimulating the release of adrenocorticotropic hormone from the pituitary (Hodges, 1970). CRFR1 cells in the PVN have also recently been revealed as key in regulating HPA axis tone as well as behavioral adaptations to chronic stress (Jiang et al., 2018; Ramot et al., 2017). PVN CRFR1 cells are demonstrated to control a local negative feedback loop that inhibits CRF cells to decrease HPA axis activation (Jiang et al., 2018). PVN CRFR1 cells are largely distinct from other primary cell phenotypes in the PVN, showing little to no co-localization with oxytocin and vasopressin and only about 8% co-localization with CRF-expressing cells (Ramot et al., 2017). Given reported sex differences in CRFR1 in other brain regions, we sought to investigate whether CRFR1 in the PVN was also sexually dimorphic and might therefore contribute to sex differences in HPA axis regulation and stress adaptation (Hodes et al., 2015; Oyola & Handa, 2017; Zuloaga et al., 2011).

Materials and Methods

Animals:

Experiments were performed using a CRFR1 reporter mouse line (bacterial artificial chromosome identified by green fluorescent protein (BAC CRFR1-GFP; Justice et al., 2008) validated by comparing distributions of CRFR1-GFP to *in situ* CRFR1 mRNA expression. This comparison revealed similar anatomical localization of CRFR1 (Justice et al., 2008; Van Pett et al., 2000). Ages of mice ranged from neonatal (P0, P4, P21), adult (60–120 days) and old adult (20–24 months of age). The 20–24 month old mice were retired breeder males

and females. Nucleotide sequences used for genotyping by RT-PCR were: CCT ACG GCG TGC AGT GCT TCA GC forward and CGG CGA GCT GCA CGC TGC GTC CTC reverse EGFP350 primers. Actin 470 was used as an internal positive control validating the presence of DNA in each sample. Mice were maintained under a 12/12 L/D cycle (lights on at 0700), with food and water available *ad libitum.* All procedures have been approved by the University at Albany Institutional Animal Care and Use Committee (IACUC) and are in accord with National Institutes of Health guidelines.

Gonadectomy (GDX):

This procedure was performed to determine the role of adult gonadal hormones on maintenance of sexually dimorphic CRFR1 cell number, P60–70 male and female CRFR1-GFP mice were gonadectomized or sham operated (N= 32, 8 per treatment/sex). Orchidectomies and ovariectomies were performed as previously described in our published protocols (Jacobskind et al., 2017; Rosinger et al., 2018). All mice were administered postsurgery sc injections of the analgesic carprofen. Sham surgery involved anesthesization and incisions in the skin or scrotum (females and males respectively). At 6 weeks after surgery, brains were collected to determine effects on PVN CRFR1-GFP. Estrus stage was not assessed in intact females. A previous study reported a significant decrease in periventricular kisspeptin at 6 weeks post-ovariectomy compared to intact freely cycling mice (Brock & Bakker, 2013), thus supporting our selection of time point.

Restraint Stress:

Restraint stress was used to determine whether PVN CRFR1 cells are activated during psychogenic stress. Sixty-80 day old mice (N= 5 male, 7 female) were restrained in a tube (L: 6–4/5”, W: 3–9/10”, H: 2–3/5”) for 30 minutes inside a plastic cage with bedding cage. At 30 minutes mice were removed from the restraint tube and left in their home cage until they were sacrificed 120 minutes after the onset of restraint. This protocol was chosen based on a previous report which indicated a peak phosphorylated cAMP response element-binding protein (pCREB) response in the mouse PVN 120 minutes after restraint stress onset (Kwon et al., 2006). We have also reported extensive induction of pCREB within CRFR1 cells in the anteroventral periventricular nucleus at 2 hours after restraint stress (Rosinger et al., 2018).

Tissue collection:

Adult mice were sacrificed via cervical dislocation followed by rapid decapitation. Neonatal mice (P0, P4, and P21) were perfused with 4% paraformaldehyde. For all ages, brains were removed from the skull and placed in 4% paraformaldehyde for 24 hours at 4° C. Brains were then transferred to a 30% sucrose solution containing sodium azide. Brains were sectioned at 40 μm or 50 μm (P0 only) on a cryostat (Microm HM505E, MICROM international GmbH, Walldorf, Germany) in the coronal plane into 3 series (adult and P21 brains) or 2 series (P0 and P4 brains). P0 brains were sectioned at 50 microns due to the greater fragility of neonatal tissue. Sectioned brain tissue was stored at 4° C in cryopreservative solution until immunohistochemistry was performed.

Chromogen Immunohistochemistry:

CRFR1-GFP-ir was visualized to compare levels in male and female brains. Tissue sections were rinsed in phosphate-buffered saline (PBS; pH 7.6), and incubated in 1% hydrogen peroxide with 0.3% Triton-X in PBS (PBS-TX) for 10 minutes. Tissue was then rinsed in PBS and placed in 4% normal goat serum (NGS) with PBS-TX for 60 minutes. Tissue was transferred to incubation overnight in primary GFP antisera (rabbit, Life Technologies, RRID: AB221570; 1:7500). Tissue was rinsed in PBS the following day, which was followed by incubation in biotinylated goat anti-rabbit antisera with PBS-TX (Vector Laboratories; 1:500) for 60 minutes. Following rinses in PBS, brain tissue was placed in avidin-biotin complex (ABC Elite kit, Vector Laboratories; 1:1000). Brain tissue was then washed in tris-buffered saline (TBS), followed by placement in diaminobenzidine (chromogen) for 10 minutes to label CRFR1-GFP. A negative control (wild type brain sections) was used to assess CRFR1-GFP label. No signal was found in these wild type sections.

For assessment of CRFR1 co-localization with androgen receptor (AR), tissue was first processed for detection of CRFR1-GFP using an identical protocol with the exception of the primary antibody (chicken, GFP; Abcam; (RRID: AB300798; 1:10,000) and secondary antibody (biotinylated goat anti-chicken antisera with PBS-TX (Vector Laboratories; 1:500) used. Following labeling with DAB, tissue was rinsed in PBS 4× 30 minutes after which antigen retrieval was performed by placing free-floating sections (stored in floating Netwell inserts (Corning, USA)) into a 98° C citrate buffer solution for 15 minutes). After PBS rinses tissue was placed in a 4% NGS solution for 1 hour then placed into AR primary antibody (rabbit; Abcam; (RRID: AB11156085; 1:500). The next day, tissue was placed in biotinylated goat anti-rabbit antisera with PBS-TX (Vector Laboratories; 1:500), and after PBS rinses, into avidin-biotin complex (ABC Elite kit, Vector Laboratories; 1:1000). Tissue was then rinsed in sodium acetate buffer and placed into a Nickel/DAB solution followed by 2 rinses in sodium acetate buffer and 2 rinses in PBS prior to mounting. This protocol produced brown labeling for CRFR1 throughout the cell and a black nuclear label for AR. AR labeling in the present study showed a similar distribution as previously reported for AR protein and mRNA with high levels throughout the hypothalamus and particularly robust levels in the preoptic area, bed nucleus of the stria terminalis, medial amygdala, and CA1 (Chen et al., 2016; Allen Institute ISH database).

Fluorescent Immunohistochemistry:

Dual-label fluorescence immunohistochemistry was used to assess CRFR1-GFP-ir co-localization patterns with ERα and pCREB. Brain tissue was heavily rinsed in PBS (pH 7.6) to remove any excess cryopreservative, followed by incubation in normal donkey serum (4% NDS) and 0.4% Triton-X in PBS (PBS-TX) for 1 hour. Next, sections were placed into primary antisera ((pCREB); Cell Signaling; rabbit; (RRID: AB2561044; 1:500), or estrogen receptor alpha (Santa Cruz; rabbit; (RRID: AB631470; 1:250)) and incubated overnight at room temperature. The next day, sections were rinsed in PBS followed by incubation in secondary antisera (anti-rabbit, 594; 1:500) in 4% NDS and PBS-TX for 2.5 hours. After rinsing, sections were incubated in second primary antisera (chicken, GFP; Abcam; (RRID: AB300798; 1:1000 in 4% NDS and PBS-TX at room temperature overnight. On day three,

tissue was again rinsed in PBS, followed by incubation in another secondary antisera (donkey antichicken, 488; 1:1000) for 2.5 hours. Immediately following final rinses in PBS, and when dry, brain sections were mounted and coverslipped using mounting media (Santa Cruz hard set with DAPI). Validation for all antibodies used has previously been performed in our laboratory and others (Omoto et al., 2005; Quesada et al., 2007; Rosinger et al., 2017; 2018; Uribe-Marino et al., 2016).

Microscopic analysis:

Images of CRFR1-GFP and co-localization patterns were collected on a Nikon 80i microscope equipped with a digital camera. An adult mouse brain atlas (Allen Institute reference atlas) and an atlas of the developing mouse brain (Paxinos et al, 2007) was used to identify sections including the rostral (~Bregma −0.70) and middle PVN (~Bregma −0.82), where CRFR1 cells are primarily located. All images were collected using a 20× objective. For quantification, cells were counted bilaterally using ImageJ within two atlas-matched sections using triangular ROIs placed around the PVN (P0, 65620 μm^2; P4, 99011 μm^2; P21/adult, 117425 μm^2). Quantification of fluorescent dual-labeled cells was also performed using ImageJ, and cells were counted as co-localized when red nuclear proteins (ERα, pCREB) appeared within the nucleus of CRFR1-GFP cells, visualized as yellow nuclear label. CRFR1/AR dual-labeled cells were also quantified using ImageJ and identified as black nuclear AR label present within brown CRFR1-GFP cells. All cell counts are reported as immunoreactive cells per mm^2.

Statistical Analyses:

Student's t-tests were used to analyze CRFR1-GFP-ir and co-localization of CRFR1 with ERα and pCREB. Two-Way ANOVA was utilized to compare CRFR1-GFP cells in gonadectomy studies using sex (male, female) and treatment (gonadectomy, sham) as independent variables. Post hoc analyses were performed using Bonferroni corrected t-tests. Analysis was performed using GraphPad Prism v.5. Significance level was set at $p \leq 0.05$ and data are shown as means ± SEM. Standard parametric tests were utilized as sampled distributions for all analyses met the criteria for equal variances and normal distribution.

Results

Characterization of the sex difference in PVN CRFR1-GFP-ir cell number.

In P60 mice (N=7 male, 9 female), males had a greater number of PVN CRFR1-GFP labeled cells than females ($t(14)= 3.295$, $p \leq .01$, Figure 1). At P0, P4, and P21 no sex differences were observed in CRFR1-GFP labeled cells (Figure 2a). Notably, few cells CRFR1-GFP immunoreactive cells were found relative to adult levels (Figure 2b). In old mice (20–24 months old; N=6 male, 9 female) males continue to show a greater number of CRFR1-GFP-ir cells in the PVN compared to females ($t(13)= 2.505$, $p \leq .05$, Figure 3).

Gonadectomy effects on CRFR1-GFP cell number.

A 2-Way ANOVA revealed a significant effect of gonadectomy ($F(1,28)=4.97$, $p \leq 0.05$), sex (($F(1,28)=5.79$, $p \leq 0.05$) and an interaction between the two (($F(1,28)=5.20$, $p \leq 0.05$). Post

hoc tests revealed a significant decrease in CRFR1-GFP following gonadectomy in males but not females ($p \leq 0.05$; N=8 per sex/treatment; Figure 4).

CRFR1-GFP co-localization with Estrogen Receptor Alpha (ERα).

Analysis of dual-label CRFR1-GFP/ERα co-localization did not reveal sex differences in ERα expressing cells, CRFR1-GFP-ir/ERα co-localized cells, or the percentage of CRFR1-GFP-ir cells that co-localize ERα (N=5 male, 5 female; Figure 5a–b). Importantly, approximately 29% of PVN CRFR1 cells co-express ERα (Figure 5b). Similar percentages of co-labeled neurons were found in the rostral and middle sections of the PVN (Figure 5b). Co-labeled cells were distributed throughout both the dorsal and ventral subdivisions of the PVN.

CRFR1-GFP co-localization with Androgen Receptor (AR).

Two-Way ANOVA of CRFR1-GFP/AR in the PVN did not indicate sex differences in CRFR1-GFP-ir/AR co-localized cells, or the percentage of CRFR1-GFP-ir cells that co-localize AR (N=5 male, 4 female; Figure 6). However, there was a greater number of AR-positive cells in males ($t(7) = 2.572$, $p \leq .05$, Figure 6). Overall, approximately 65% of CRFR1 cells co-express AR with similar percentages of co-localization found in the rostral and middle PVN. Similar to ERα, AR/CRFR1 co-expressing cells were present in the dorsal and ventral aspects of the PVN.

CRFRI-GFP-ir activation (pCREB co-expression) in the PVN following a 30-minute restraint stress.

To determine whether PVN CRFR1-GFP cells respond to stress in a sexually dimorphic manner, co-localization of CRFR1-GFP cells and a marker for transcription/neural activation (pCREB) was assessed. Males showed a greater number of CRFR1-GFP/pCREB co-expressing cells than females following exposure to psychogenic stress ($t(11) = 3.369$, $p \leq .01$; N=6 male, 7 female; Figure 7a). The percentage of CRFR1-GFP/pCREB co-expressing cells did not significantly differ between males and females (Figure 7b). High levels of co-localized cells were found throughout the dorsal and ventral aspects of the PVN. A subset of brains were collected from mice that were not exposed to restraint stress. These brains showed little to no pCREB in the PVN (See example image in Figure 7d).

Discussion

This study revealed a novel sexually dimorphic cluster of CRFR1 expressing cells in the PVN that is present throughout adult life. Interestingly, this sex difference is not present in the neonatal period through postnatal day 21. Independent of sex, CRFR1 expression was sparse at P0-P21 postnatal time points and levels in females and males likely do not reach full expression until puberty or early adulthood. However, once the sex difference develops it remains present throughout the lifespan as evidenced by the greater number of CRFR1 cells present in 20–24 month old mice.

By comparison, a recently discovered sexually dimorphic cluster of CRFR1 cells in the AVPV (greater cell number in females) is sexually dimorphic during the early neonatal

period and the sex difference persists into adulthood (Rosinger et al., 2017). This AVPV sex difference is controlled by perinatal exposure to androgens, and their subsequent conversion to estrogens, which decreases CRFR1 cell number in males (Rosinger et al., 2017). However, in adulthood neither gonadectomy of males or females affects CRFR1 levels in the AVPV, indicating that the AVPV CRFR1 sex difference is mediated by the organizational, and not activational, effects of androgens and estrogens (Rosinger et al., 2018). On the contrary, the male biased PVN CRFR1 population appears to be maintained by androgens in adulthood, since gonadectomy decreases CRFR1 cell number in males. This suggests the PVN CRFR1 sex difference is potentially less influenced by early life gonadal hormone exposure since the sex difference does not appear until after postnatal day 21. However, the possibility remains that early life gonadal hormone exposure organizes PVN cells in such a way that pubertal or adult hormones can act on these cells to differentially induce CRFR1 expression. Since estrus phase was not assessed, it is possible that a difference may have been detected when estrogen levels are peaking (proestrus), although the almost identical CRFR1-GFP cell numbers reported in these groups and minimal variability suggest that circulating adult estrogens in females produce little effect on this cell phenotype. It also remains possible that CRFR1 may have been altered in the female PVN in the early stage following gonadectomy but that compensatory changes in gene expression may have eliminated any effect by 6 weeks post-castration. For example, long term gonadectomy is known to induce changes in estrogen, androgen, and glucocorticoid receptor expression, all of which can potentially modify CRFR1 levels (Handa et al., 1996; Sarvari et al., 2017; Patchev and Almeida, 1996).

The decrease in PVN CRFR1 following gonadectomy in males is likely regulated by androgen activity at either androgen or estrogen receptors. Testosterone and its metabolite 5α-dihydrotestosterone can act directly via androgen receptors. Alternatively, testosterone can be converted to estradiol by the enzyme aromatase. Subsequently, estradiol binds estrogen receptors including ERα and ERβ. Here we report a high and moderate co-localization of CRFR1 cells with AR and ERα respectively, indicating potential receptor mechanisms through which androgens might regulate the sex difference in PVN CRFR1. It also remains possible that gonadal hormone regulation of PVN CRFR1 occurs through binding of ERβ, which is also highly expressed in the mouse PVN (Zuloaga et al., 2014; Oyola et al., 2017). Treatments with selective gonadal steroid hormone receptor agonists could be utilized in future studies to assess specific receptor sites of action. Interestingly, the presence of ERα in the PVN is species specific, even within rodents. In rats ERα is sparse, while in mice ERα is moderately distributed, in the PVN (Shugrue et al., 1997; Mitra et al., 2003; McClellan et al., 2010). This suggests that gonadal hormone regulation of stress responses in the PVN may also be species specific. The sustained sex difference in old (20–24 month) mice likely reflects testosterone levels that only modestly decline with age and remain relatively high throughout the lifespan in male mice (Hamrick et al., 2006).

PVN CRFR1 neurons have been demonstrated to play a critical role in behavioral and endocrine stress responses (Jiang et al., 2018; Ramot et al., 2017). In males, genetic deletion of CRFR1 from the PVN disrupts changes in anxiety-like behavior that occur after chronic social defeat stress (Ramot et al., 2017). CRFR1 cells have been shown to project to both canonical PVN brainstem target nuclei such as the NTS and PAG, as well as and several

forebrain nuclei not previously recognized as PVN targets including the lateral septum and bed nucleus of the stria terminalis (Jiang et al., 2018). Forebrain CRFR1 projections might regulate behavioral responses such as those associated with anxiety- and depressive-like behaviors while brainstem projections likely mediate autonomic responses. PVN CRFR1 neurons also synapse on neighboring CRF neurons within the PVN (Jiang et al., 2018). This local microcircuit has been proposed to provide feedback to the HPA axis by modulating CRF neuron activity and subsequent CRF release (Jiang et al., 2018).

In this study, we demonstrated that the number of PVN CRFR1 cells activated (CRFR1/pCREB positive) by acute stress is greater in male than in female mice. Increased number and excitability of PVN CRFR1 neurons in males might be a key mechanism that causes sexually dimorphic HPA axis activity and behavioral adaptations in response to stress. Short periods of variable stress exposure have been demonstrated to induce depression-like behavior in female but not male mice (Hodes et al., 2015). Furthermore, stress-induced release of ACTH and corticosterone are consistently higher in female rodents (Handa & Weiser, 2014; Zuloaga et al., 2011). The sexually dimorphic distribution and activation of PVN CRFR1 cells may contribute to differences in these behavioral and hormonal responses given the known role of this cell type in modulating distinct features of the stress responses (Ramot et al., 2017; Jiang et al., 2018).

Adult circulating gonadal hormones have also been repeatedly demonstrated to regulate anxiety- and depressive-like behaviors as well as HPA axis function. Testosterone suppresses stress-induced corticosterone release and decreases anxiety-related behaviors (Carrier et al., 2015; Fernandez-Guasti et al., 2005; Handa et al., 1994; Oyola & Handa, 2017; Zuloaga et al., 2008) although the specific anatomical sites involved in these effects are largely unclear (Handa & Weiser, 2014). The known role of the PVN as regulator of HPA axis and stress-related behavioral responses, along with the high levels of gonadal hormone receptors and aromatase in the PVN, indicate this an intriguing central site for gonadal steroid hormone regulation of these functions (Chen et al., 2016; Foidart et al., 1995; Handa & Weiser, 2014; Mitra et al., 2003; Zuloaga et al., 2014). Interestingly, the decrease in PVN CRFR1 following gonadectomy reported in the current study is opposite to the finding that gonadectomy increases PVN *CRF* in the rat (Bingaman et al., 1994). The opposing androgen regulation of CRF and CRFR1 might be factors that underlie androgen suppression of the HPA axis. Specifically, androgen induced decreases in CRF are predicted to increase HPA axis drive while increases in CRFR1 are expected to decrease drive (Jiang et al., 2018).

Sex differences in CRFR1 expression within select brain regions of the rodent brain, including the anterior cingulate, piriform cortex, nucleus accumbens, and AVPV, have also been previously reported (Rosinger et al., 2017; Wealthington et al., 2014). In four vole species, a sex difference in *CRFR2* is found in the bed nucleus of the stria terminalis with males showing greater expression (Lim et al., 2005). Beyond differences in expression, sex differences in CRFR1 dendritic morphology and trafficking are present in the locus coeruleus, which might result in greater sensitivity to CRF in females (Bangasser et al., 2010; 2011; Bangasser, 2013). Gonadal hormones have also been shown to regulate CRFR1 in the AVPV and CRFR2 in the lateral septum (Rosinger et al., 2017; Weiser et al., 2008). Together, these findings suggest that discrete distributions of CRF and its receptors,

including the present finding in the PVN, might be critical to sex differences in and gonadal hormone regulation of stress-associated behaviors and HPA axis function.

Results of this study revealed a gonadal hormone- and stress-responsive sexually dimorphic group of CRFR1 neurons in the PVN. This cell cluster represents an intriguing site for sex differences in regulation of the HPA axis as well as anxiety- and depressive-like behaviors. In the human population there are dramatic sex differences in anxiety and depression, with women twice as likely to be diagnosed compared to men (Kessler et al., 1994; Kessler et al., 2012; Nolen-Hoeksema, 1987). Understanding the neural mechanisms that underlie these striking sex differences in anxiety and mood is necessary in order to develop effective, and possibly sex-specific, treatments for these debilitating disorders.

Acknowledgments

Danielle Fico, Nicole Bulanchuk, and Shannon Park are acknowledged for their role in data collection. Support was provided by University at Albany Research Initiation Funds (DZ), NIH-MH119631 (DZ) and NIH-MH112768 (NJ).

References

Abel KB, Majzoub JA (2005), Molecular biology of the HPA axis. Ed. Steckler T, Kalin NH, and Reul JMHM. Techniques in Behavioral and Neural Sciences 15(1):79–94.

Bale TL, Picetti R, Contarino A, Koob GF, Vale WW, Lee KF (2002), Mice deficient for both corticotropin-releasing factor receptor 1 (CRFR1) and CRFR2 have an impaired stress response and display sexually dichotomous anxiety-like behavior. J Neurosci 22(1):193–199. [PubMed: 11756502]

Bangasser DA (2013), Sex differences in stress-related receptors: “micro” differences with “macro” implications for mood and anxiety disorders. Biol Sex Differ 4(1):2. [PubMed: 23336736]

Bangasser DA, Curtis A, Reyes BA, Bethea TT, Parastatidis I, Ischiropoulos H, Van Bockstaele EJ, Valentino RJ (2010), Sex differences in corticotropin-releasing factor receptor signaling and trafficking: potential role in female vulnerability to stress-related psychopathology. Mol Psychiatry 15(9):877, 896–904.

Bangasser DA, Eck SR, Ordones Sanchez E (2019), Sex differences in stress reactivity in arousal and attention systems. Neuropsychopharmacology 44(1):129–139. [PubMed: 30022063]

Bangasser DA, Zhang X, Garachh V, Hanhauser E, Valentino RJ (2011), Sexual dimorphism in locus coeruleus dendritic morphology: a structural basis for sex differences in emotional arousal. Physiol Behav 103(3-4):342–51. [PubMed: 21362438]

Bangasser DA, Wiersielis KR (2018), Sex differences in stress responses: a critical role for corticotropin-releasing factor. Hormones (Athens) 17(1):5–13. [PubMed: 29858858]

Bingaman EW, Magnuson DJ, Gray TS, Handa RJ (1994), Androgen inhibits the increases in hypothalamic corticotropin-releasing hormone (CRH) and CRH-immunoreactivity following gonadectomy. Neuroendocrinology 59(3):228–34. [PubMed: 8159272]

Britton KT, Lee G, Dana R, Risch SC, Koob GF (1986), Activating and ‘anxiogenic’ effects of corticotropin releasing factor are not inhibited by blockade of the pituitary-adrenal system with dexamethasone. Life Sci. 39(14):1281–6. [PubMed: 3489874]

Brock O, Bakker J (2013), The two kisspeptin neuronal populations are differentially organized and activated by estradiol in mice. Endocrinology 154(8):2739–2749. [PubMed: 23744640]

Carrier N, Saland SK, Duclot F, He H, Mercer R (2015), The anxiolytic and antidepressant-like effects of testosterone and estrogen in gonadectomized male rats. Biol Psychiatry 78:259–69. [PubMed: 25683735]

Chen CV, Brummet JL, Jordan CL, Breedlove SM (2016), Down, But Not Out: Partial Elimination of Androgen Receptors in the Male Mouse Brain Does Not Affect Androgenic Regulation of Anxiety or HPA Activity. Endocrinology 157(2):764–73. [PubMed: 26562258]

Deak T, Nguyen KT, Ehrlich AL, Watkins LR, Spencer RL, Maier SF, Licinio J, Wong ML, Chrousos GP, Webster E, Gold PW (1999), The impact of the nonpeptide corticotropin-releasing hormone antagonist antalarmin on behavioral and endocrine responses to stress. Endocrinology. 140(1):79–86. [PubMed: 9886810]

Eaton MJ, Cheung S, Moore KE, Lookingland KJ (1996), Dopamine receptor-mediated regulation of corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus.

Fernandez-Guasti A, Martinez-Mota L (2005), Anxiolytic-like actions of testosterone in the burying behavior test: Role of androgen and GABA-benzodiazepine receptors. Psychoneuroendocrinology 30:762–70. [PubMed: 15919582]

Foidart A, Harada N, Balthazart J (1995), Aromatase-immunoreactive cells are present in mouse brain areas that are known to express high levels of aromatase activity. Cell Tissue Res 280(3):561–74. [PubMed: 7606769]

Funabashi T, Kawaguchi M, Furuta M, Fukushima A, Kimura F (2004), Exposure to bisphenol A during gestation and lactation causes loss of sex difference in corticotropin-releasing hormone-immunoreactive neurons in the bed nucleus of the stria terminalis of rats. Psychoneuroendocrinology 29:475–485. [PubMed: 14749093]

Hamrick MW, Ding KH, Pennington C, Chao YJ, Wu YD, Howard B, Immel D, Borlongan C, McNeil PL, Bollag WB, Curl WW, Yu J, Isales CM (2006), Age-related loss of muscle mass and bone strength in mice is associated with a decline in physical activity and serum leptin. Bone 39(4):845–53. [PubMed: 16750436]

Handa RJ, Kerr JE, DonCarlos LL, McGivern RF, Hejna G (1996), Hormonal regulation of androgen receptor messenger RNA in the medial preoptic area of the male rat. Brain Res Mol Brain Res. (1–2):57–67.

Handa RJ, Nunley KM, Lorens SA, Louie JP, McGivern RF, Bollnow MR (1994), Androgen regulation of adrenocorticotropin and corticosterone secretion in the male rat following novelty and foot shock stressors. Physiol Behav 55(1):117–24. [PubMed: 8140154]

Handa RJ, Weiser MJ (2014), Gonadal steroid hormones and the hypothalamo-pituitary-adrenal axis. Front Neuroendocrinol. 35(2):197–220. [PubMed: 24246855]

Heck AL, Handa RJ (2019), Sex differences in the hypothalamic-pituitary-adrenal axis' response to stress: an important role for gonadal hormones. Neuropsychopharmacology. 44(1):45–58. [PubMed: 30111811]

Hodes GE, Pfau ML, Purushothaman I, Ahn HF, Golden SA, Christoffel DJ, Magida J, Brancato A, Takahashi A, Flanigan ME, Menard C, Aleyasin H, Koo JW, Lorsch ZS, Feng J, Heshmati M, Wang M, Turecki G, Neve R, Zhang B, Shen L, Nestler EJ, Russo SJ (2015), Sex Differences in Nucleus Accumbens Transcriptome Profiles Associated with Susceptibility versus Resilience to Subchronic Variable Stress. J Neurosci 16;35(50):16362–76.

Hodges JR (1970), The hypothalamus and pituitary ACTH release. Prog Brain Res. 32:12–20. [PubMed: 4321514]

Jacobskind JS, Rosinger ZJ, Zuloaga DG (2017), Hypothalamic-pituitary-adrenal axis responsiveness to methamphetamine is modulated by gonadectomy in males. Brain Res 15;1677:74–85.

Jiang Z, Rajamanickam S, Justice NJ (2018), Local Corticotropin-Releasing Factor Signaling in the Hypothalamic Paraventricular Nucleus. J Neurosci 21;38(8):1874–1890.

Justice NJ, Yuan ZF, Sawchenko PE, Vale W (2008), Type 1 corticotropin-releasing factor receptor expression reported in BAC transgenic mice: implications for reconciling ligand-receptor mismatch in the central corticotropin-releasing factor system.J Comp Neurol 511(4):479–96. [PubMed: 18853426]

Karanikas CA, Lu YL, Richardson HN (2013), Adolescent drinking targets corticotropin-releasing factor (CRF) peptide labeled cells in the central amygdala of male and female rats. Neuroscience 249:98–105. [PubMed: 23628776]

Kessler RC, Petukhova M, Sampson NA, Zaslavsky AM, Wittchen HU (2012), Twelve-month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the United States. Int J Methods Psychiatr Res 21:169–184 [PubMed: 22865617]

Kessler RC, McGonagle KA, Zhao S, Nelson CB, Hughes M, Eshleman S, Wittchen HU,Kendler KS (1994), Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. Arch Gen Psychiatry 51(1):8–19. [PubMed: 8279933]

Kwon MS, Seo YJ, Shim EJ, Choi SS, Lee JY, Suh HW (2006), The effect of single or repeated restraint stress on several signal molecules in paraventricular nucleus, arcuate nucleus and locus coeruleus. Neuroscience. 142(4):1281–92. [PubMed: 16938401]

Lim MM, Nair HP, Young LJ (2005), Species and sex differences in brain distribution of corticotropin-releasing factor receptor subtypes 1 and 2 in monogamous and promiscuous vole species. J Comp Neurol 487:75–92. [PubMed: 15861459]

Makino S, Smith MA, Gold PW (1995), Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus. Endocrinol. 136(8):3299–3309.

McClellan KM, Stratton MS, Tobet SA (2010), Roles for gamma-aminobutyric acid in the development of the paraventricular nucleus of the hypothalamus. J Comp Neurol. 518(14):2710–28. [PubMed: 20506472]

McDonald AJ, Mascagni F, Wilson MA (1994), A sexually dimorphic population of CRF neurons in the medial preoptic area. Neuroendocrinology 5:653–656.

Mitra SW, Hoskin E, Yudkovitz J, Pear L, Wilkinson HA, Hayashi S, Pfaff DW, Ogawa S, Rohrer SP, Schaeffer JM, McEwen BS, Alves SE (2003), Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. Endocrinology. 144(5):2055–67. [PubMed: 12697714]

Müller MB, Zimmermann S, Sillaber I, Hagemeyer TP, Deussing JM, Timpl P,Kormann MS, Droste SK, Kühn R, Reul JM, Holsboer F, Wurst W (2003), Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. Nat Neurosci. 6(10):1100–7. [PubMed: 12973355]

Nolen-Hoeksema S (1987), Sex differences in unipolar depression: evidence and theory. Psychol Bull. 101(2):259–82. [PubMed: 3562707]

Omoto Y, Imamov O, Warner M, Gustafsson JA (2005), Estrogen receptor alpha and imprinting of the neonatal mouse ventral prostate by estrogen. Proceedings of the National Academy of Sciences 102:1484–1489.

Oyola MG, Handa RJ (2017), Hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axes: sex differences in regulation of stress responsivity. Stress 20(5):476–494. [PubMed: 28859530]

Oyola MG, Thompson MK, Handa AZ, Handa RJ (2017), Distribution and chemical composition of estrogen receptor β neurons in the paraventricular nucleus of the female and male mouse hypothalamus. J Comp Neurol 525(17):3666–3682. [PubMed: 28758220]

Patchev VK, Almeida OF (1996), Gonadal steroids exert facilitating and “buffering” effects on glucocorticoid-mediated transcriptional regulation of corticotropin-releasing hormone and corticosteroid receptor genes in rat brain. J Neurosci 16(21):7077–84. [PubMed: 8824343]

Paxinos G, Halliday G, Watson C, Koutcherov Y, Wang H (2007), Atlas of the developing mouse brain at E17.5, P0, and P6, vol. 1 New York: Elsevier.

Quesada A, Romeo HE, Micevych P (2007), Distribution and localization patterns of estrogen receptor-beta and insulin-like growth factor-1 receptors in neurons and glial cells of the female rat substantia nigra: localization of ERbeta and IGF-1R in substantia nigra. J Comp Neurol 503(1): 198–208. [PubMed: 17480015]

Ramot A, Jiang Z, Tian JB, Nahum T, Kuperman Y, Justice N, Chen A (2017), Hypothalamic CRFR1 is essential for HPA axis regulation following chronic stress. Nature Neuroscience 20(3):385–388. [PubMed: 28135239]

Rosinger ZJ, Jacobskind JS, Park SG, Justice NJ, Zuloaga DG (2017), Distribution of corticotropin-releasing factor receptor 1 in the developing mouse forebrain: a novel sex difference revealed in the rostral periventricular hypothalamus. Neuroscience. 361:167–178. [PubMed: 28823817]

Rosinger ZJ, Jacobskind JS, Bulanchuk N, Malone M, Fico D, Justice NJ, Zuloaga DG (2017), Characterization and gonadal hormone regulation of a sexually dimorphic corticotropin releasing factor receptor 1 cell group. J Comp Neurol doi: 10.1002/cne.24588.

Sárvári M, Kalló I, Hrabovszky E, Solymosi N, Liposits Z (2017), Ovariectomy Alters Gene Expression of the Hippocampal Formation in Middle-Aged Rats. Endocrinol. 158(1):69–83.

Shughrue PJ, Lane MV, Merchenthaler I (1997), Comparative distribution of estrogen receptor-alpha and –beta in the rat central nervous system. J Comp Neurol. 388(4):507–25. [PubMed: 9388012]

Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK, Blanquet V, Steckler T, Holsboer F, Wurst W (1998), Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat Genet. 19(2):162–6. [PubMed: 9620773]

Uribe-Mariño A, Gassen NC, Wiesbeck MF, Balsevich G, Santarelli S, Solfrank B, ... Schmidt MV (2016), Prefrontal Cortex Corticotropin-Releasing Factor Receptor 1 Conveys Acute Stress-Induced Executive Dysfunction. Biological Psychiatry 80(10):743–753. [PubMed: 27318500]

Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C, Prins GS, Perrin M, Vale W, Sawchenko PE (2000), Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. J Comp Neurol 428(2):191–212. [PubMed: 11064361]

Wealthington JM, Hamki A, Cooke BM (2014), Sex- and region-specific pubertal maturation of the corticotropin-releasing factor receptor system in the rat. J Comp Neurol 522:1284–1298. [PubMed: 24115088]

Weiser MJ, Goel N, Sandau US, Bale TL, Handa RJ (2008), Androgen regulation of corticotropin-releasing hormone receptor 2 (CRHR2) mRNA expression and receptor binding in the rat brain. Exp Neurol 214(1):62–8. [PubMed: 18706413]

Zuloaga DG, Poort JE, Jordan CL, Breedlove SM (2011), Male rats with the testicular feminization mutation of the androgen receptor display elevated anxiety-related behavior and corticosterone response to mild stress. Horm Behav 60(4):380–8. [PubMed: 21801726]

Zuloaga DG, Zuloaga KL, Hinds LR, Carbone DL, Handa RJ (2014), Estrogen receptor β expression in the mouse forebrain: age and sex differences. J Comp Neurol 522(2):358–71. [PubMed: 23818057]

Zuloaga DG, Morris JA, Jordan CL, Breedlove SM (2008), Mice with the testicular feminization mutation demonstrate a role for androgen receptors in the regulation of anxiety-related behaviors and the hypothalamic-pituitary-adrenal axis. Horm Behav 54(5):758–66. [PubMed: 18775430]

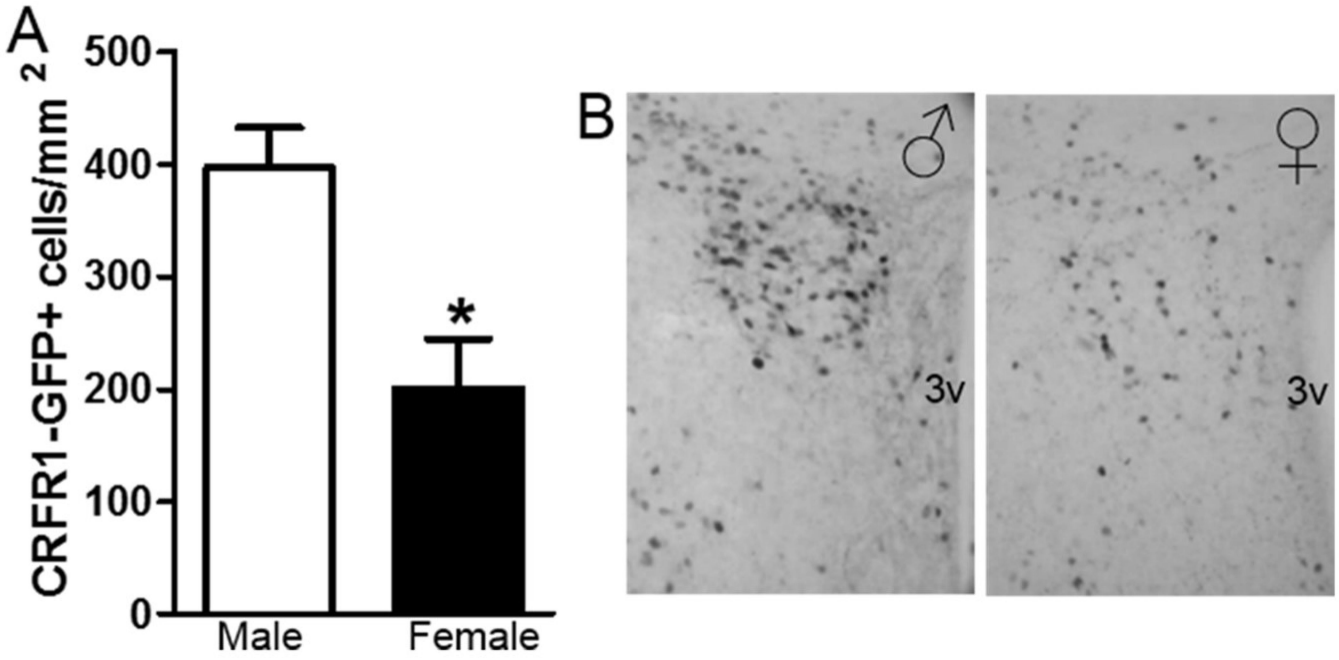

Figure 1. Sex difference in CRFR1-GFP cells in the PVN.

(A) Male mice have a greater number of CRFR1-GFP containing cells in the PVN compared to females. (B) Representative images of CRFR1-GFP cells in an adult (P60) male and female mouse brain. *p≤ 0.01. 3v; 3rd ventricle.

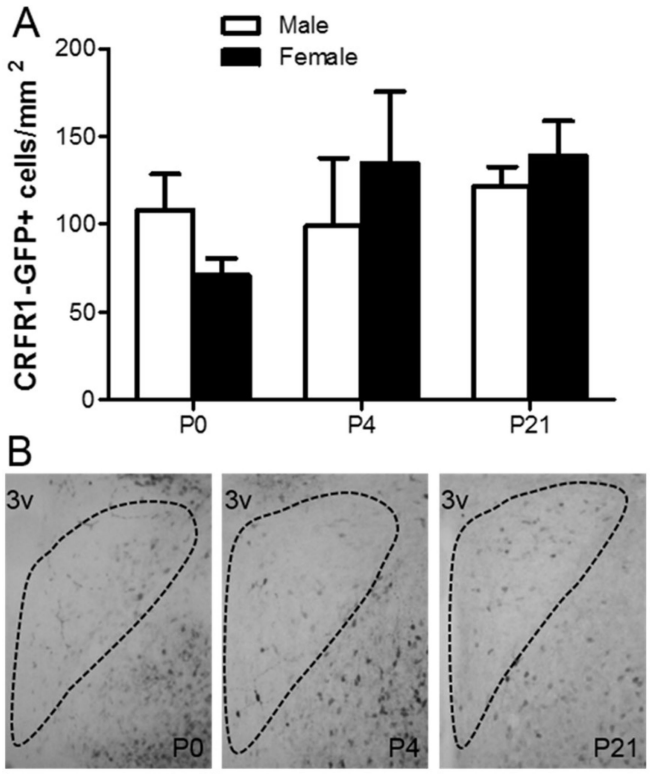

Figure 2. CRFR1-GFP cell number in the P0, P4, and P21 mouse PVN.

(A) No sex differences were found in CRFR1-GFP within the PVN at P0, P4, or P21. Relative to adult levels, CRFR1-GFP was low at these postnatal time points. (B) Representative images showing CRFR1-GFP in the P0, P4, and P21 PVN. Dashed lines approximate anatomical borders of the PVN (Paxinos et al., 2007; Allen Institute Mouse Reference Atlas) 3v; 3rd ventricle.

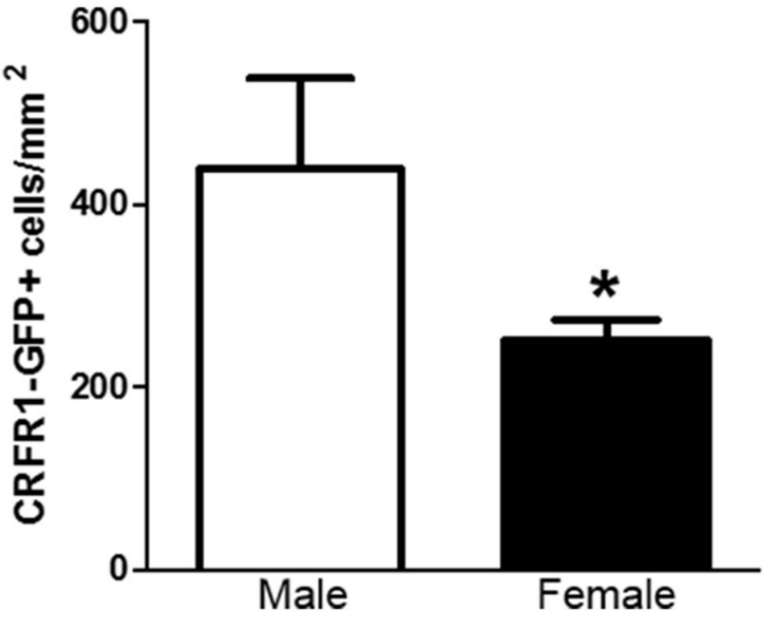

Figure 3. CRFR1-GFP cell number in the male and female 20–24 month old mouse.
In 20–24 month old mice, the adult sex difference in CRFR1-GFP (males > females) persists. *p≤ 0.05.

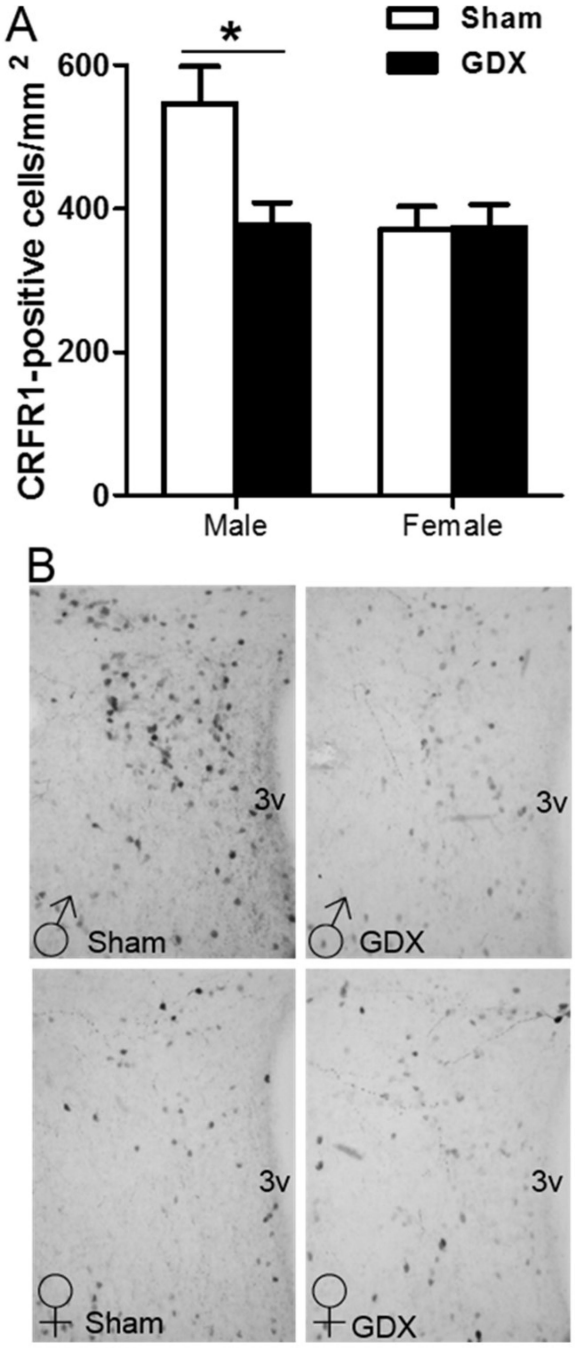

Figure 4. Gonadectomy effects on PVN CRFR1-GFP cell number.

(A) Orchidectomy in adult males results in a decrease in CRFR1-GFP cell number while ovariectomy in females produces no effects on CRFR1-GFP cell number. (B) Representative images of PVN CRFR1-GFP cells in sham and gonadectomized males and females. * indicates $p \leq 0.01$. 3v; 3rd ventricle. GDX; gonadectomy.

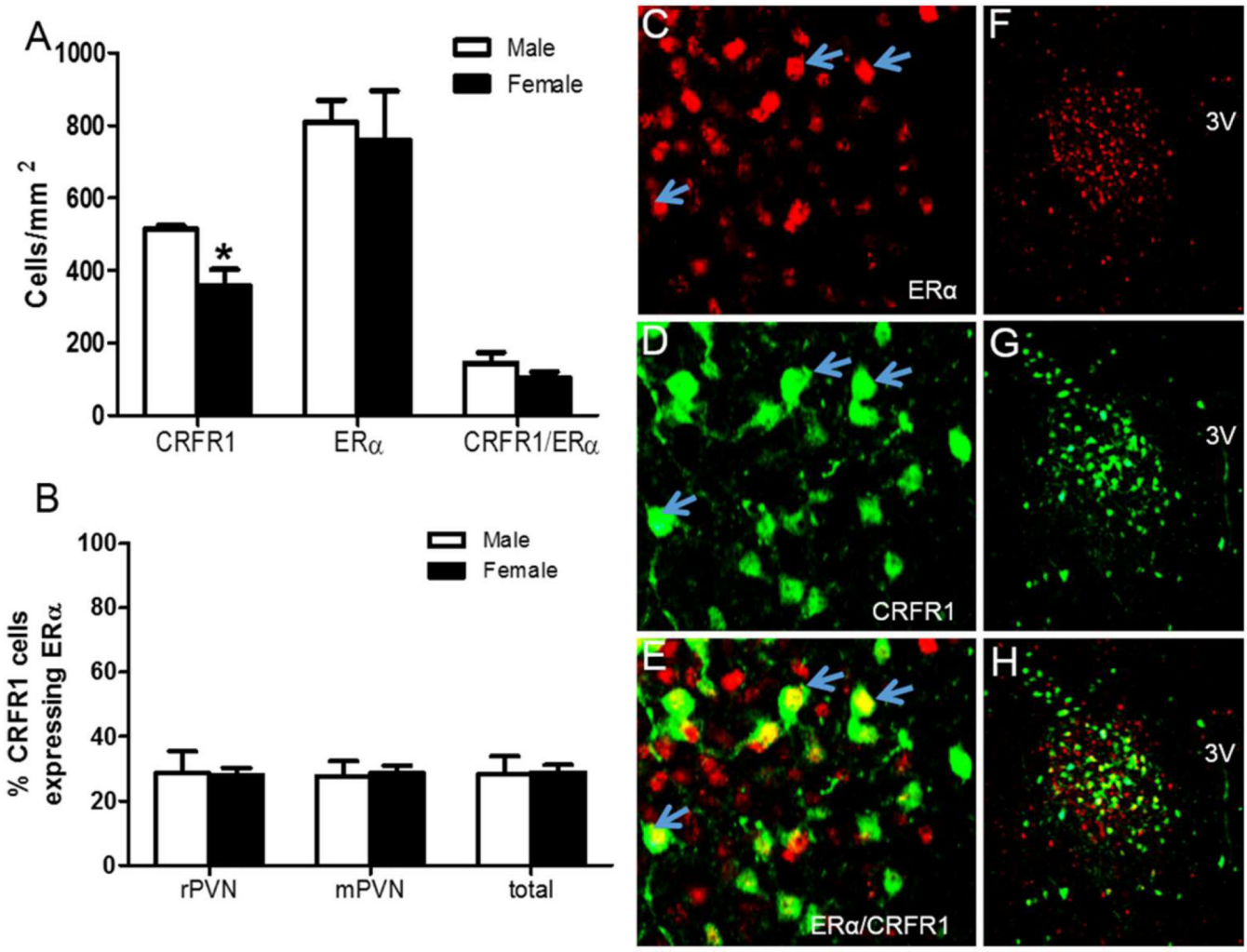

Figure 5. Co-localization of CRFR1-GFP with estrogen receptor alpha (ERα) in the PVN.

(A) A male/female comparison of CRFR1-GFP, ERα, co-localized CRFR1-GFP/ERα, and (B) the percentage of CRFR1-GFP/ERα co-localized cells in the PVN. Approximately 29% of CRFR1 cells were found to be co-localized with ERα although no sex differences in the number or percentage of co-localized cells were found. No differences were found for the percentage of CRFR1 cells co-expressing ERα between the rostral (r) and middle (m) PVN. (C-H) High and low magnification representative images of a male mPVN showing co-localization of CRFR1-GFP and ERα. Note the distinct distribution overlap for CRFR1-GFP and ERα in the low magnification images (F-H). Arrows indicate examples of co-localized cells. * indicates $p \leq 0.05$. 3v; 3rd ventricle.

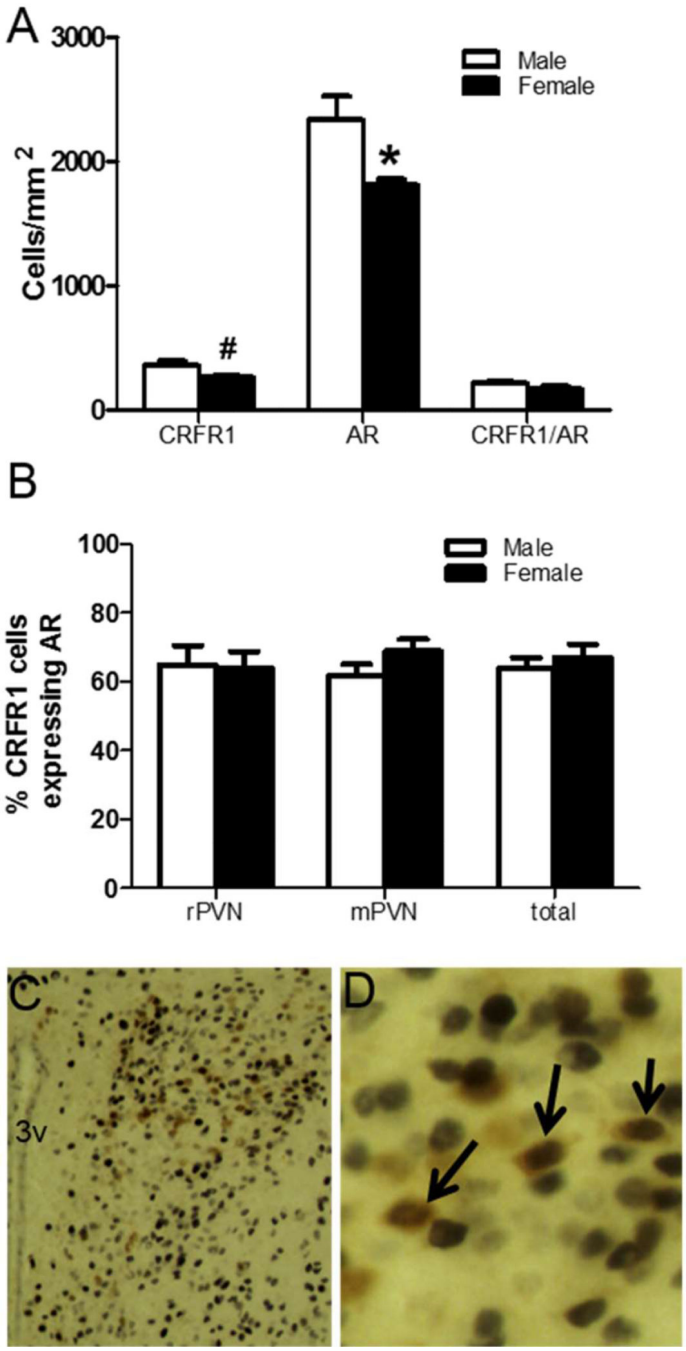

Figure 6. Co-localization of CRFR1-GFP with androgen receptor (AR) in the PVN.

(A) CRFR1-GFP, AR, co-localized CRFR1-GFP/AR, and (B) the percentage of CRFR1-GFP/AR colocalized cells in the male and female PVN. Approximately 65% of CRFR1 cells co-localized with AR with no sex differences in the number or percentage of co-localized cells found. No differences were found for the percentage of CRFR1 cells co-expressing AR between the rostral (r) and middle (m) PVN (B). A low magnification (C) and high magnification image of middle PVN showing co-localized cells containing black AR label

within brown CRFR1-GFP cells (D). Arrows indicate co-localized cells. # indicates p=0.08 compared to males. * indicates p≤0.05 compared to males. 3v; 3rd ventricle.

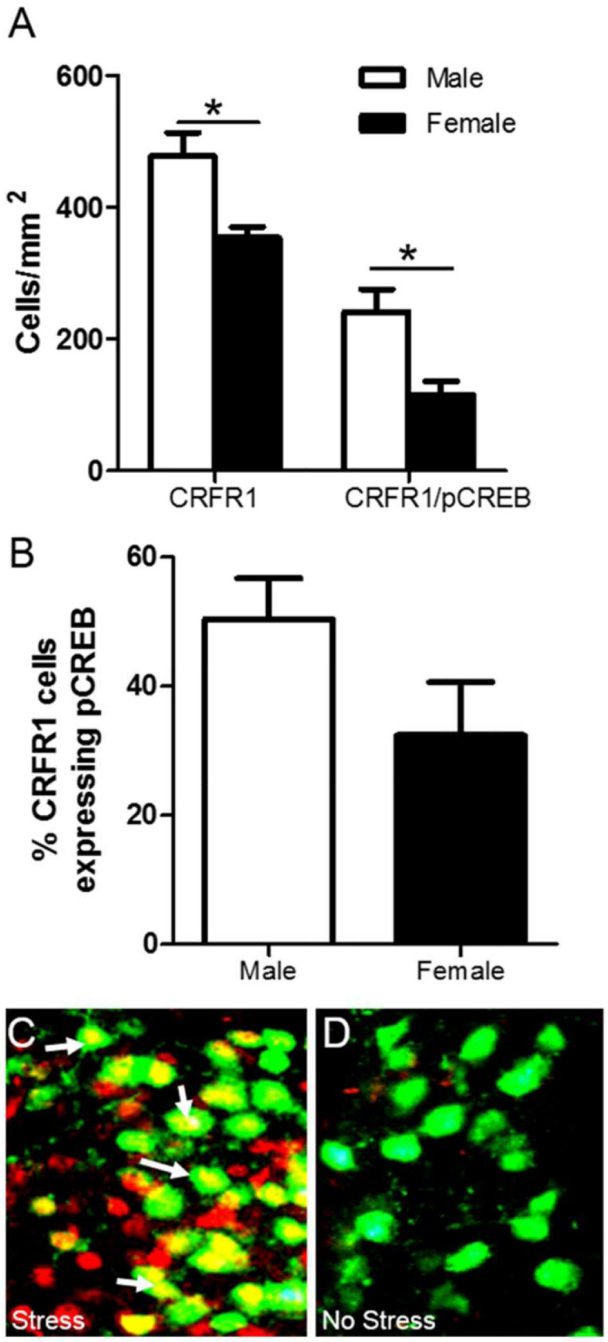

Figure 7. Co-localization of CRFR1-GFP with phosphorylated Creb (pCREB) following restraint stress.

(A) Males show a greater number of CRFR1-GFP cells that co-express pCREB following a 30 minute restraint stress. (B) The percentage of cells that co-express CRFRI-GFP/pCREB did not differ by sex. (C-D) Representative images of a male PVN showing co-localization of CRFR1-GFP and pCREB in a stressed and unstressed mouse. Arrows indicate examples of co-localized cells. * indicates $p \leq 0.01$. 3v; 3rd ventricle.