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Characterization and gonadal hormone regulation of a sexually dimorphic corticotropin releasing factor receptor 1 cell group

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Abstract

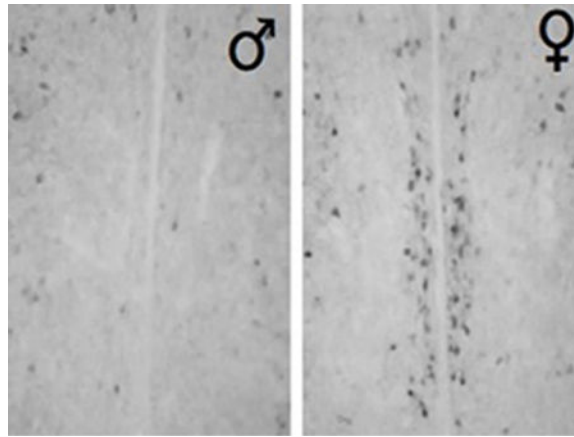
Corticotropin-releasing factor binds with high affinity to CRF receptor 1 (CRFR1) and is implicated in stress-related mood disorders such as anxiety and depression. Using a validated CRFR1-green fluorescent protein (GFP) reporter mouse, our laboratory recently discovered a nucleus of CRFR1 expressing cells that is prominent in the female rostral anteroventral periventricular nucleus (AVPV/PeN), but largely absent in males. This sex difference is present in the early postnatal period and remains dimorphic into adulthood. The present investigation sought to characterize the chemical composition and gonadal hormone regulation of these sexually dimorphic CRFR1 cells using immunohistochemical procedures. We report that CRFR1-GFP-ir cells within the female AVPV/PeN are largely distinct from other dimorphic cell populations (kisspeptin, tyrosine hydroxylase). However, CRFR1-GFP-ir cells within the AVPV/PeN highly co-express estrogen receptor alpha as well as glucocorticoid receptor. A single injection of testosterone propionate or estradiol benzoate on the day of birth completely eliminates the AVPV/PeN sex difference, while adult gonadectomy has no effect on CRFR1GFP cell number. These results indicate that the AVPV/PeN CRFR1 is regulated by perinatal but not adult gonadal hormones. Finally, female AVPV/PeN CRFR1-GFP-ir cells are activated following an acute 30-min restraint stress, as assessed by co-localization of CRFR1-GFP cells with phosphorylated (p) CREB. CRFR1-GFP/pCREB cells were largely absent in the male AVPV/PeN. Together, these data indicate a stress and gonadal hormone responsive nucleus that is unique to females and may contribute to sex-specific stress responses.

Graphical abstract

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Keywords

Corticotropin-releasing factor receptor 1; gonadal hormones; sexual differentiation; anteroventral periventricular nucleus; estrogen receptor alpha; glucocorticoid receptor; RRID: AB_631470; RRID: AB_300798; RRID: AB_2155786; RRID: AB_2296529; RRID: AB_2561044; RRID: AB_390204

1. Introduction

There is a clear discrepancy in the prevalence of stress-related mood disorders such as anxiety and depression, where women are twice as likely as men to get a diagnosis (Nolen-Hoeksma, 1987). Together, anxiety and depression affect approximately 20% of the U.S. population (Greenberg, Fournier, Sisitsky, Pike, & Kessler, 1987; Kessler et al., 1987) and incur \$210 billion in annual costs. The neural mechanisms that underlie the sexually dimorphic prevalence of these disorders are unclear. Cells that produce corticotropin releasing factor (CRF), and those that express the primary receptor for CRF, the G_s-coupled cognate receptor, CRF receptor 1 (CRFR1), represent intriguing cell phenotypes that might regulate these sex differences, given their known role in regulating anxiety, depression, and stress hormone secretion (Chrousos, 2009; Da Silva et al., 2016; Holly et al., 2016; Schussler et al., 2016; Garcia-Carmona JA, Baroja-Mazo, Milanes, & Laorden, 2015).

CRF signaling via CRFR1 regulates hormonal and behavioral stress responses (Heinrichs, Menzaghi, Merlo Pich, Britton, & Koob, 1993; Perrin, Donaldson, Chen, Lewis, & Vale, 1993; Smith et al., 1998; Subbannayya et al., 2013). Global knockout of CRFR1 and pharmacological antagonism of CRFR1 decrease anxiety- and depressive-like behaviors and suppress adrenocorticotropin hormone and glucocorticoid responses to stress (Contarino et al., 1999; Smith et al., 1998; Tran, Schulkin, & Meerveld, 2014; Webster et al., 1996; Zhao et al., 2007). On the contrary, CRF binding to CRFR2 generally produces anxiety/depression *reducing* effects (Bale et al., 2003; Bale & Vale, 2003; Zhao et al., 2007). Interestingly, a global knockout of *both* CRFR1 and R2 in mice results in sex-specific impacts on anxiety-like behavior (Bale et al., 2002), which suggests an important role for CRF receptor signaling in regulating sex differences in stress-associated behaviors.

Sex differences in brain circuitry are generally believed to underlie sexually dimorphic patterns reported for stress-related behaviors and activation of the HPA axis (Handa & Weiser, 2014). In particular, sex differences in CRF and CRFR1 are potential candidates for regulating sexually dimorphic stress functions (Bangasser, Wiersielis, & Khantsis, 2016; Waters et al., 2015). Sex differences in the distribution of CRF, as well as both receptor subtypes, CRFR1 and R2 have been reported. There are more CRF expressing neurons within the preoptic area (POA) (Funabashi, Kawaguchi, Furuta, Fukushima, & Kimura, 2004; McDonald, Mascagni, & Wilson, 1994) and dorsal bed nucleus of the stria terminalis (BNST) of female rats (Funabashi, Kawaguchi, Furuta, Fukushima, & Kimura, 2004), while male rats have a greater number of CRF expressing neurons in the central amygdala (Karanikas, Lu, & Richardson, 2013). CRFR1 immunoreactivity (-ir) is reported to be greater in female rats within the nucleus accumbens, olfactory tubercle, piriform cortex, anterior cingulate (Wealthington, Hamki, & Cooke, 2014). Furthermore, our laboratory recently discovered a dense cluster of CRFR1 expressing cells within the rostral anteroventral periventricular nucleus (AVPV/PeN) that is prominent in female mice but largely absent in males (Rosinger, Jacobskind, Park, Justice, & Zuloaga, 2017). This sex difference in CRFR1 is present by birth and persists through postnatal development.

Development of sex differences in the rodent brain has largely been attributed to perinatal androgen exposure that occurs only in males. Specifically, males are exposed to a prenatal surge at approximately gestation day (GD) 18 followed by a second surge that occurs on the day of birth (McCarthy & Nugent, 2015; Zuloaga, Jordan, & Breedlove, 2010). These androgen pulses serve to either increase or decrease the number of cells expressing specific phenotypes in males (Morris, Jordan, & Breedlove, 2004). In addition, sexually dimorphic nuclei can also be regulated/maintained by *adult* circulating gonadal hormones as has been identified for select sexually dimorphic nuclei (Morris, Jordan, & Breedlove, 2004; Zuloaga, Jordan, & Breedlove, 2010). Corresponding with alterations in brain morphology, gonadal hormone exposure during both the neonatal period and adulthood have been shown to regulate many sexually dimorphic behavioral/hormonal responses, including those associated with stress (Morris, Jordan, & Breedlove, 2004; Zuloaga, Jordan, & Breedlove, 2011). Numerous studies indicate that perinatal testosterone exposure induces lasting effects on anxiety behaviors and the HPA axis (Bingham, Wang, Innala, & Viau, 2012; Seale, Wood, Atkinson, Harbuz, & Lightman, 2005; Seale, Wood, Atkinson, Lightman, & Harbuz, 2005; Zuloaga, Jordan, & Breedlove, 2011). Furthermore, adult testosterone and estrogens dynamically regulate anxiety and depressive-like behaviors as well as glucocorticoid release via actions at their cognate receptors (Borrow & Handa, 2017; Lund, Hinds, & Handa, 2006; Lund, Rovis, Chung, & Handa, 2005; Weiser & Handa, 2009).

The specific brain regions and cell phenotypes involved in gonadal hormone regulation of stress-associated functions are largely unknown (Handa & Weiser, 2014). Therefore, our present study sought to further investigate one potential candidate region (sexually dimorphic CRFR1 in the AVPV/PeN). The described experiments specifically aimed to characterize the phenotypic profile of CRFR1-GFP-ir cells in the AVPV/PeN and determine the influence of neonatal and adult gonadal hormones on the development and maintenance of the nucleus. Due to the absence of selective antibodies for CRFR1, all experiments were performed using a validated CRFR1 reporter mouse line (bacterial artificial chromosome

identified by green fluorescent protein (BAC CRFR1-GFP)) (Justice, Yuan, Sawchenko, & Vale, 2008).

2. Material and Methods

2.1 Animals:

Subjects were male and female BAC CRFR1-GFP mice on a C57BL/6J background. Mice used in the study were adult (60–110 days) or neonates (day of birth). Mice were maintained under a 12/12 L/D cycle (lights on at 0700), with food and water available *ad libitum*. All procedures in this study have been approved by the University at Albany Institutional Animal Care and Use Committee (IACUC) and are in accord with National Institutes of Health (NIH) guidelines.

2.2 P0 Hormone Supplement:

Experiments were performed to assess the role of neonatal gonadal hormones on CRFR1-GFP cell number in the AVPV/PeN. Male and female CRFR1GFP mice were paired and each morning throughout the study, the animal vivarium was checked for new litters. On mornings in which there were new litters, offspring were given sc injection (25 μ l) of either testosterone propionate (TP; 100 μ g dissolved in sesame oil, N = 6 female, 7 male), estradiol benzoate (EB; 20 μ g dissolved in sesame oil, N = 6 female, 6 male), or sesame oil (VEH; N = 7 female, 6 male) as controls. A hypodermic needle (27 gauge) was inserted sc. at the lower portion of the pup's back, and the needle was run until it could be seen roughly between the shoulder blades, where it was then injected, and slowly withdrawn. Animals were then returned to their home cage and left undisturbed until euthanized via rapid cervical dislocation and decapitation at P21. P21 (pre-puberty) was chosen for assessment of neonatal effects in order to avoid any confound of P0 hormone treatments on gonadal hormone production later in life. Brain tissue was extracted for histological analysis of AVPV/PeN CRFR1GFP-ir cells.

2.3 Adult Gonadectomy (GDX):

Experiments were performed to assess the role of adult gonadal hormones on CRFR1 cell number. Adult male and female mice (P60-P70; N= 32, 8 per treatment per sex) were gonadectomized or sham operated. Briefly, adult male mice were anesthetized with isoflurane and the 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 silk suture prior to cutting. Incisions were closed with Vetbond (3M) surgical glue. For removal of ovaries, 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 skin was closed using surgical staples. The analgesic carprofen (5 mg/kg) was injected sc. post operatively. Sham surgery involves only anesthesia and incision in the skin (females) or scrotum (males), post-operative procedures are identical to gonadectomized mice. Brains were collected from mice 6 weeks after gonadectomy or sham surgery for assessment of CRFR1-GFP. Estrus stage was not assessed in intact mice.

This time point was chosen based on a previous study that reported a significant decrease in AVPV kisspeptin at 6 weeks in ovariectomized compared to intact freely cycling mice (Brock & Bakker, 2013).

2.4 Restraint Stress:

To assess whether AVPV/PeN CRFR1 cells are activated during acute psychological stress, gonadal intact adult mice (P60-P80; N= 5 male, 7 female) 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 (2hr from onset of restraint). This time point was chosen based on a previous study which performed a time course and indicated that the pCREB protein response in mice peaks at 2 hours following restraint in the paraventricular nucleus and arcuate nucleus (Kwon et al., 2006). The restraint tube has been validated in our laboratory to induce a robust corticosterone response (~400–500 ng/ml, data not shown).

2.5 Fluorogold; Neurosecretory Cells:

To determine if any CRFR1-GFP-ir neurons in the AVPV/PeN were neurosecretory, gonadal intact adult CRFR1-GFP female mice (P60–65; N=3) were sc. injected with 50µL of 5% fluorogold (Fluorochrome, Denver, CO) in saline (0.9%), based on previous approaches (Kriegsfeld, Korets, & Silver, 2003; Oyola, Thompson, Handa, & Handa, 2017). Animals were perfused with 4% paraformaldehyde 5 days later and used for assessment of immunohistochemical co-labeling of Fluorogold and CRFR1-GFP positive cells (listed within dual-label immunohistochemistry methods section).

2.6 Tissue collection for immunohistochemistry:

All adult animals underwent cervical dislocation and rapid decapitation, except those used for Fluorogold analysis which were perfused. Brains from P0 mice used for phenotypic characterization were collected following rapid decapitation. All brains were excised from the skull and immersed in 4% paraformaldehyde, then stored overnight at 4° C. The following 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 40 µm, into 3 series using a cryostat (Microm HM505E, MICROM international GmbH, Walldorf, Germany). Tissues were placed in cryopreservative and stored at 4° C until immunohistochemistry was performed.

2.7 Immunohistochemistry:

To visualize 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 rinsed in PBS and incubated in 4% normal goat serum (NGS) with PBS-TX for 1 hour. Tissue was incubated overnight in primary GFP antisera (rabbit, Life Technologies, AB221570; 1:7500). The next morning, tissue was rinsed in PBS, and incubated in biotinylated goat anti-rabbit antisera with PBS-TX (Vector Laboratories; 1:500) for 1 hour. After, tissue was rinsed in PBS and then placed in avidin-biotin complex (ABC Elite kit, Vector Laboratories; 1:1000). Tissue was rinsed again in tris-buffered saline (TBS)

and placed for 10 minutes in diaminobenzidine as the chromagen to visualize CRFR1-GFP-expressing cells. Sections from wild type brains were utilized as negative control to assess CRFR1-GFP-ir; no labeling was found in these sections.

2.8 Dual Label Immunohistochemistry:

For further phenotypic characterization of CRFR1GFP+ cells, we performed several dual-label fluorescence IHC studies to determine CRFR1GFP-ir co-localization patterns. 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 (phosphorylated CREB (pCREB; Cell Signaling; anti-rabbit; AB2561044; 1:500), tyrosine hydroxylase (Millipore; anti-rabbit; AB390204; 1:500), kisspeptin (Millipore; anti-rabbit; AB2296529; 1:500), estrogen receptor alpha (Santa Cruz; anti-rabbit; AB631470; 1:250), or glucocorticoid receptor (Santa Cruz; anti-rabbit; AB2155786; 1:250)) and incubated at room temperature overnight. The following day, tissue was rinsed in PBS and then placed into secondary antisera (anti-rabbit, 594; 1:500) in 4% NDS and PBS-TX for 2.5 hours. Following rinses, tissue was then transferred to the second primary antisera (chicken, GFP; Abcam; AB300798; 1:1000 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 (donkey anti-chicken, 488; 1:1000) 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.

2.9 Antibody characterization

See Table 1 for description of antibodies.

2.9.1 Phosphorylated CREB (pCREB)—The pCREB antibody (RRID: AB_2561044) was generated by immunizing rabbits with a synthetic phosphopeptide that corresponds to residues that surround Ser133 of human CREB (129–137). Using Western blotting, this antibody recognizes a 43 kDa band selective for pCREB (manufacturer information). This antibody has been utilized to detect neural activation within CRFR1-GFP labeled cells using the CRFR1-BAC mouse line (Uribe-Marino et al., 2016). Our studies also indicate that little pCREB is present in the hypothalamus of resting mice while it is abundant following psychogenic stress (Figure 8c,d).

2.9.2 Estrogen Receptor Alpha (ER α)—This ER α antibody (RRID: AB_631470) was generated against a peptide at the C-terminus of mouse ER α which corresponds to amino acids 580–599 (manufacturer information). Peptide preabsorption studies have been used to confirm specificity of this antibody (Quesada, Romeo, & Micevych, 2007). An absence of ER α signal has also been confirmed in ER α knockout mice (Omoto, Imamov, Warner, & Gustafsson, 2005). We also confirmed regional ER α distribution patterns in our own tissue to known distributions in mice and found high concordance (Allen Institute ISH data, Merchenthaler, Lane, Numan, & Dellovade, 2004).

2.9.3 Green fluorescent protein (GFP)—The GFP antibody (RRID: AB_300798) was generated against the full length GFP protein. Using Western blotting, this antibody recognizes a 27–30 kDa band selective for GFP (manufacturer information). Studies in our laboratory using this antibody confirm no labeling in brain tissue from wild type mice.

2.9.4 Tyrosine hydroxylase (TH)—This polyclonal antibody (RRID: AB_390204) is generated from denatured tyrosine hydroxylase from rat pheochromocytoma with rabbit as host species. Western blotting shows it selectively labels a single 62-kDa band in PC12 cell lysate (manufacturer's information). Specificity was confirmed in our laboratory based on specific distributions of known TH-positive cells based on previous work (Scott, Prigge, Yizhar, & Kimchi, 2015) and the Allen Institute ISH data. Specifically, high levels of TH expressing cell bodies are found in the AVPV, arcuate nucleus and locus coeruleus.

2.9.5 Kisspeptin (KISS)—This polyclonal antibody (RRID: AB_2296529) is generated using the mouse kisspeptin 10 peptide with rabbit as host species. Specificity for the KISS antibody was confirmed in our laboratory again by comparing our regional labeling distributions against published IHC (Poling & Kaufmann, 2013) and ISH (Allen Institute ISH data). Similar to previous work, we found labeled cell bodies in the AVPV/PeN and arcuate nucleus and few labeled cells elsewhere in the hypothalamus.

2.9.6 Glucocorticoid receptor (GR)—The GR polyclonal antibody (RRID: AB_2155786) was generated against a peptide at the N-terminus of GR α of mouse origin (5–24) (manufacturer's information). Western blotting shows a specifically labeled band at 110 kDa in adrenal tissue (Damjanovic et al., 2012). Peptide preabsorption studies have been used to confirm specificity of this antibody (Damjanovic et al., 2012). Brain distributions of GR in our labeled tissue also match those reported in Allen Institute ISH data, with highest levels found in the paraventricular hypothalamus, central amygdala, and hippocampus.

2.10 Microscopic analysis:

Analysis of CRFR1-GFP-ir distribution was conducted on a Nikon 80i microscope equipped with a digital camera. The AVPV/PeN was identified using the Allen institute mouse brain reference atlas for adults and an atlas of the developing mouse brain for analysis of neonatal tissue (Paxinos, Halliday, Watson, Koutcherov, & Wang, 2007). Images were collected using a 20X objective. Following image collection, cells were bilaterally quantified within two atlas-matched sections using rectangular ROIs placed around the AVPV/PeN (P0, 138000 μm^2 ; P60, 166000 μm^2). Dual-labeled cells were quantified using image J and considered co-localized when green (CRFR1-GFP-ir) and pseudo-colored magenta cells (secondary labels) overlapped, appearing white. Nuclear proteins (ER α , pCREB, GR) were counted as co-localized if they appeared within the nuclear region of CRFR1 cells. Cell numbers are shown as immunoreactive cells per mm 2 .

2.11 Statistical Analyses:

Analysis of CRFR1-GFP-ir and co-localization patterns within the AVPV/PeN was performed using 2-way ANOVA, 1-Way ANOVA, or Student's t-Tests as appropriate. All analysis was performed using GraphPad Prizm v.4. For neonatal hormone experiments we

hypothesized treatments would affect females but not males. Therefore, we separated these analyses by sex and performed 1-way ANOVAs. 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 \pm SEM.

3. Results

3.1 Phenotypic Characterization of CRFR1-GFP-ir Cells in the AVPV/PeN

3.1.1 Tyrosine Hydroxylase (TH).—Numbers of CRFR1-GFP, TH, and CRFR1-GFP/TH-ir cells were assessed in male and female mice at P0 and P60. As previously reported we found a sex difference in CRFR1-GFP-ir cells at P0 (females > males; $t(8)=2.329$, $p < .05$; Figure 1). No sex difference was found in the number of TH cells at P0 (Figure 1). 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 1). At P60, CRFR1 ($t(8)=2.787$, $p < .05$), TH ($t(8)=3.755$, $p < .01$), and CRFR1/TH ($t(8)=4.165$, $p < .01$) cells were more abundant in females than males (Figure 2). The percentage of CRFR1 cells that co-express TH ($t(8)=5.210$, $p < .001$) and the percentage of TH cells that co-express CRFR1 ($t(8)=5.802$, $p < .001$) were also greater in females than males (Figure 2).

3.1.2 Kisspeptin (KISS).—Numbers of CRFR1-GFP, KISS, and CRFR1-GFP/KISS-ir cells were assessed at P60 in male and female mice. CRFR1 ($t(8)=5.048$, $p < .01$), KISS ($t(8)=3.285$, $p < .05$), and CRFR1/KISS ($t(8)=4.435$, $p < .001$) cells were more abundant in females than males (Figure 3). There was a trend toward a greater percentage of CRFR1 cells that co-express KISS in females compared to males ($t(8)=2.168$, $p = .07$). The percentage of KISS cells that coexpress CRFR1 was significantly greater in females than males ($t(8)=6.458$, $p < .001$; Figure 3).

3.1.3 Estrogen Receptor Alpha (ER α).—Quantification of dual label CRFR1-GFP-ir/ER α colocalization showed that females had significantly more CRFR1-GFP-ir/ER α colocalized cells than males (Age P60–70; Figure 4a). In addition, the male AVPV/PeN had significantly fewer ER α expressing cells ($t(8)=2.691$, $p < .05$), few of which co-express CRFR1, as indicated by a significantly higher percentage of CRFR1-GFP-ir cells that co-localize ER α in the female AVPV/PeN ($t(8)=5.775$, $p < .001$; Figure 4b). Co-localization of CRFR1 and ER α is also found in the AVPV/PeN of P0 female mice (Figure 4f).

3.2 Neonatal Hormone Treatment on CRFR1 Expression Pattern in the P21 Female and Male AVPV/PeN

One-way ANOVA of CRFR1-GFP-ir cells within the female AVPV/PeN revealed a main effect of hormone supplementation on CRFR1-GFP expression ($F(2,14)=188$, $p < .001$; Figure 5) where TP and EB injection significantly reduced CRFR1-GFP cells compared with female vehicle-injected animals ($p < .001$). *Post-hoc* analysis further revealed a significant difference between treatments, where EB reduced CRFR1 cell number to a greater extent than TP ($p < .05$). One-way ANOVA of CRFR1-GFP-ir cells within the male AVPV/PeN revealed no significant effect of treatment on CRFR1-GFP-ir cell number (Figure 5).

3.3 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 (($F(1,26)=75.50$, $p < 0.001$) matching our previous data. However, gonadectomy had no effect on CRFR1 cell number in either sex (Figure 6).

3.4 AVPV/PeN CRFR1-GFP-ir activation (pCREB co-expression) following a 30-minute restraint stress and CRFR1-GFP-ir co-localization with glucocorticoid receptor (GR)

To determine whether acute psychogenic stress (30min restraint) would elicit activation of CRFR1-GFP-ir 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-GFP/pCREB co-localized cells in females compared to males ($t(10)=5.994$, $p < .001$; Figure 7). The percentage of AVPV/PeN CRFR1 cells co-expressing pCREB was also greater in females ($t(10)=2.241$, $p < .05$; Figure 7). Quantification of dual-label CRFR1-GFP/GR indicated 75% co-localization in the female AVPV/PeN (Figure 8).

3.5 AVPV/PeN CRFR1 co-localization with fluorogold.

Quantification of dual label CRFR1GFP-ir/FG co-localization for adult (P60–65) mice indicated that none of the AVPV/PeN CRFR1GFP expressing cells are neurosecretory (See Figure 9). Little fluorogold was found near the sexually dimorphic CRFR1 AVPV/PeN cluster (Figure 9a-d), although abundant fluorogold and CRFR1/fluorogold co-labeling was found in more caudal periventricular sections (Bregma -0.40 ; Figure 9e-h).

4. Discussion

4.1 Chemical composition of AVPV/PeN CRFR1-GFP-ir cells

The current study characterized a novel cluster of CRFR1-ir cells that previous work from our lab has shown to be sexually dimorphic from P0-P21 mice (Rosinger, Jacobskind, Park, Justice, & Zuloaga, 2017). Other AVPV/PeN cell phenotypes such as TH and KISS are known to be more abundant in female than male rodents (Poling & Kauffman, 2013; Semaan et al., 2010; Scott, Prigge, Yizhar, & Kimchi, 2015). These findings prompted us to investigate whether these CRFR1 cells represent the same or distinct cell populations. We found that the majority of CRFR1-GFP+ cells in the female AVPV/PeN do not co-express TH (~22% co-expression), and conversely approximately 18% of TH+ cells co-express CRFR1-GFP. Similar low percentages of CRFR1/TH co-localization are found at P0. TH-expressing cells in the rodent AVPV have been directly implicated in regulation of maternal behaviors and TH cells are dynamically affected by maternal experience (Scott, Prigge, Yizhar, & Kimchi, 2015). The presence of CRFR1 on a subpopulation of these cells indicates that CRF signaling can potentially influence dopamine transmission and associated functions such as maternal behaviors.

A greater percentage of KISS+ cells in the AVPV/PeN co-express CRFR1-GFP (~35%), which opens the possibility for CRF signaling within the AVPV/PeN to disrupt KISS function, potentially contributing to stress-induced alterations in reproduction. CRF signaling in the medial preoptic area and the arcuate nucleus has been shown to inhibit KISS signaling to gonadotropin releasing hormone neurons, thereby affecting luteinizing hormone

pulsatile secretion (Kinsey-Jones et al., 2009). In addition, intracerebroventricular administration of CRF can delay the onset of puberty in female rats (Kinsey-Jones et al., 2010), therefore CRF binding to AVPV/PeN CRFR1 cells may be key in this process. Our data also show that the majority of CRFR1-GFP-ir cells in the AVPV/PeN *do not* co-express KISS (~21%). In general, CRFR1 cells appear to be more abundant than KISS cells in this region which suggests that CRFR1 within the AVPV/PeN likely serves multiple functions. However, even in instances where these cells are not colocalized, the close physical proximity of CRFR1-GFP-ir cells to both TH+ and KISS+, may suggest communication between these cells, though further investigation is needed. Rodent AVPV cells project to several forebrain and hindbrain structures involved in reproductive behaviors, stress-related behaviors, and hormone release (e.g., vascular organ of the lamina terminalis, medial preoptic area, ventral lateral septum, arcuate nucleus, paraventricular hypothalamus, and periaqueductal gray; Gu & Simerly, 1997; Scott, Prigge, Yizhar, & Kimchi, 2015), further supporting the potential involvement of the AVPV/PeN in an array of sexually dimorphic responses. Whether AVPV/PeN CRFR1 cells project to these same brain regions is currently unknown, though our data involving fluorogold injections indicate that these cells are not neurosecretory, and therefore do not directly project outside of the brain.

4.2 Gonadal hormone regulation of AVPV/PeN CRFR1-GFP-ir cells

We next investigated whether the male-typical perinatal androgen surge was responsible for sexual differentiation of the CRFR1-GFP-ir nucleus. Previous research has shown that perinatal androgen secretions are responsible for masculinizing a number of hypothalamic and other brain structures (Morris, Jordan, & Breedlove, 2004; Raisman & Field, 1971). Our findings show that P0 treatment with testosterone, or its aromatized metabolite 17 β -estradiol, results in a defeminized AVPV/PeN CRFR1-GFP-ir nucleus in P21 females (Figure 6). Females treated with testosterone or 17 β -estradiol were indistinguishable from their male counterparts, while having significantly fewer CRFR1-GFP cells than vehicle-treated female controls (Figure 6B-E). Furthermore, 17 β -estradiol treatment in P0 females caused a slightly greater reduction in CRFR1-GFP cells than did testosterone. Although estrogens can potentially act at both ER α and β subtypes to induce these effects, ER α is the likely mechanism. We demonstrate that CRFR1 cells at P0 co-express ER α , while previous work has shown that the P0 AVPV/PeN is largely devoid of ER β (Zuloaga, Zuloaga, Hinds, Carbone, & Handa, 2014). In addition, androgen receptor (AR) levels are barely detectable at P0 (Juntti et al., 2010; Kanaya et al., 2014). Apoptosis is potentially responsible for the observed sex difference in the AVPV as others have shown that perinatal androgen secretions trigger cell death in the developing AVPV (Forger et al., 2004; Kelly, Varnum, Krentzel, Krug, & Forger, 2013). However, whether CRFR1 cells specifically undergo apoptosis is unknown. Interestingly, the dimorphic pattern of CRFR1GFP+ cells within the AVPV/PeN appears earlier than other known dimorphic cell phenotypes, such as TH and KISS (Figure 1; Poling & Kauffman, 2013; Waters & Simerly, 2009), and might potentially influence the development of the mouse AVPV/PeN. CRF signaling through CRFR1 has been implicated in normal brain developmental processes. Specifically, CRF/CRFR1 signaling has been shown to facilitate dendrite and synapse formation in the developing olfactory bulb (Garcia et al., 2014). Notably, AVPV/PeN CRFR1 cell number also appears to decrease between P0 and P60 in both sexes, a decline we have previously reported

(Rosinger, Jacobskind, Park, Justice, & Zuloaga, 2017). This decrease in CRFR1 cell number might reflect ongoing apoptosis in the AVPV during the postnatal period.

We next sought to determine the role of circulating gonadal steroid hormones on the *maintenance* of the AVPV/PeN CRFR1-GFP-ir nucleus in adulthood. We gonadectomized P60 female and male mice, and following a 6-week recovery period, found no alterations in CRFR1GFP cells in animals of either sex (Figure 7). The lack of effect following gonadectomy suggests that this nucleus is *organized* during the perinatal period, and not sensitive to circulating androgens and estrogens in adulthood. These results contrast those reported for other sexually dimorphic cell phenotypes in the AVPV such as TH and KISS. Ovariectomy increases TH-ir in rats and decreases KISS-ir in mice (Brock & Bakker, 2013; Simerly, 1989). Estrus phase was not accounted for in the present study, therefore it is possible that a difference could be observed in proestrus (when estrogens are elevated) compared to ovariectomized females. However, a similar comparison of freely cycling and ovariectomized mice (6 weeks) revealed a decrease in kisspeptin within the same region (Brock & Bakker, 2013). Furthermore, our findings reveal near exact CRFR1-GFP cell numbers between OVX and intact mice, indicating that circulating estrogens likely have little effect on this cell phenotype. It remains possible that other hormones such as those elevated during the maternal period (e.g. prolactin, glucocorticoids) might alter CRFR1 levels, however, further investigation is required. Regardless, in males, the organizational influence of perinatal testosterone exposure is sufficient enough to maintain an absence of expression in adulthood, even after removal of the primary source of androgens.

4.3 Potential role of AVPV/PeN CRFR1 in sexually dimorphic stress regulation

Considering the well-established sex differences in behavioral and hormonal responses to stress, we sought to investigate the involvement of CRFR1-GFP-ir in the AVPV/PeN during a single acute psychogenic stressor. CRFR1-GFP containing cells showed extensive activation illustrated through high levels of pCREB co-expression following a single 30-min restraint. Additionally, the few CRFR1-GFP cells present in males were not activated following restraint. This finding indicates a discrete population of cells that is engaged in the female, but not the male, brain and may therefore represent a unique site of regulation for the female stress response. The sustained pCREB response (2 hours after stress onset) appears to reflect a prolonged stimulation of pCREB which is induced by phosphorylation of calcium/calmodulin dependent protein kinase II (CaMKII). The temporal induction of hypothalamic pCREB following restraint mirrors that of phosphorylated CaMKII indicating that a sustained increase in CaMKII might drive the sustained pCREB response (Kwon et al., 2006). The resulting CREB activation might therefore regulate expression of various CREB-associated genes. It is important to note that increases in pCREB within CRFR1 cells doesn't necessarily indicate that pCREB elevations are due to activation of CRFR1. Future studies utilizing CRFR1 antagonists are warranted to determine whether pCREB induction is indeed due to CRF actions at CRFR1. CRFR1 receptor antagonists have been shown to reduce immediate early gene responses following restraint in the paraventricular nucleus (Imaki et al., 1995), however whether similar effects occur for AVPV/PeN pCREB is currently unknown.

The potential CRF inputs that might contact/activate AVPV/PeN CRFR1 cells remain unknown. Anatomical studies in the rat indicate several preoptic, hypothalamic, amygdala regions project to the AVPV. These include the medial preoptic area, paraventricular hypothalamus, bed nucleus of the stria terminalis, central amygdala, and medial amygdala (Canteras et al., 1992; 1995; Hutton et al, 1998; Simerly & Swanson, 1988). Each of these regions contain CRF-expressing cells and represent potential sites for CRF regulation of AVPV/PeN R1 cells.

We further demonstrate that the majority of CRFR1-GFP cells co-localize GR, indicating a site at which circulating glucocorticoids can influence this cell group in female rodents. Another CRFR1 cell group in the paraventricular hypothalamus has been shown to be upregulated by circulating glucocorticoids and glucocorticoid actions on CRFR1 cells during chronic stress have been proposed as a mechanism of hormonal and potentially behavioral adaptation to chronic stress (Ramot et al., 2017). Whether AVPV/PeN CRFR1 cells are similarly regulated by glucocorticoids and their involvement in adaptation to chronic stress is currently unknown.

Gonadal hormones and actions at their cognate receptors strongly influence HPA axis tone and anxiety/depression-like behaviors. Androgens including testosterone or metabolite 5 α -dihydrotestosterone, effectively reduce the adrenocorticotrophic hormone and CORT responses to stress (Handa et al., 1994; Lund, Hinds, & Handa, 2006) while the opposite is true of estrogens, which enhance glucocorticoid responses and impair glucocorticoid-mediated negative feedback (Burgess and Handa, 1992; Handa & Weiser, 2014; Goel, Workman, Lee, Innala, & Viau, 2014; Viau, 2002; Weiser & Handa, 2009). ER β and AR binding induce anxiolytic and HPA axis suppressing effects (Lund, Hinds, & Handa, 2006; Zuloaga, Morris, Jordan, & Breedlove, 2008). On the contrary ER α binding generally produces anxiogenic effects and impairs HPA axis negative feedback, shown through studies utilizing ER α -specific agonists (e.g., propylpyrazoletriol; Rossi et al., 2010; Weiser & Handa, 2009). However, what remains largely unknown are the specific brain regions and cell phenotypes upon which gonadal hormones can induce effects on stress-related functions.

Given the known role of CRFR1 in regulation of behavioral and hormonal stress responses, the presence of ER α in CRFR1 cells represents an intriguing potential site for estrogen regulation of these functions. Furthermore, the virtual absence of ER α /CRFR1 cells in the male AVPV/PeN indicates this is a key site for female-specific regulation of stress functions via ER α . Although the AVPV/PeN has not traditionally been considered a central region for regulation of stress responsivity, it projects to other hypothalamic and limbic regions involved in the regulation of behavioral and hormonal stress functions including the paraventricular hypothalamus, arcuate nucleus, and periaqueductal gray (Gu & Simerly, 1997). Future studies are warranted to determine the specific connectivity of AVPV/PeN CRFR1-GFP-ir cells with other hypothalamic and limbic structures. It is also possible that these cells play a lesser role in mediating stress effects on other neural systems and are primarily a stressor-responsive circuit. Potential functions noted above may include CRF regulation of the luteinizing hormone surge, ovulation, and maternal behaviors which are functions known to be controlled by AVPV cells (Herbison, 2008; Scott et al., 2015). Additionally, neurons in the AVPV have also been shown to regulate feeding and drinking

behaviors and may therefore serve as a site through which stress induced CRF might mediate these functions (Bealer & Johnson, 1980; Johnson & Buggy, 1978).

4.4 Summary

Overall, we report a novel sexually dimorphic expression of CRFR1-GFP in the female AVPV/PeN. Sexual differentiation of this nucleus occurs via testosterone conversion to estrogen, likely via actions at ER α . On the contrary, this nucleus is unaffected by removal of circulating gonadal hormones via gonadectomy in adulthood. These cells are also activated by psychological stress and co-express GR indicating the potential for circulating glucocorticoids to alter their function. The function of this sexually dimorphic CRFR1 population is currently unknown, although co-localization patterns with ER α indicate a potential role in the estrogen regulation of stress functions. Lower levels of co-localization with KISS and TH also suggest these cells as potential sites for stress/CRF regulation of reproductive and maternal functions.

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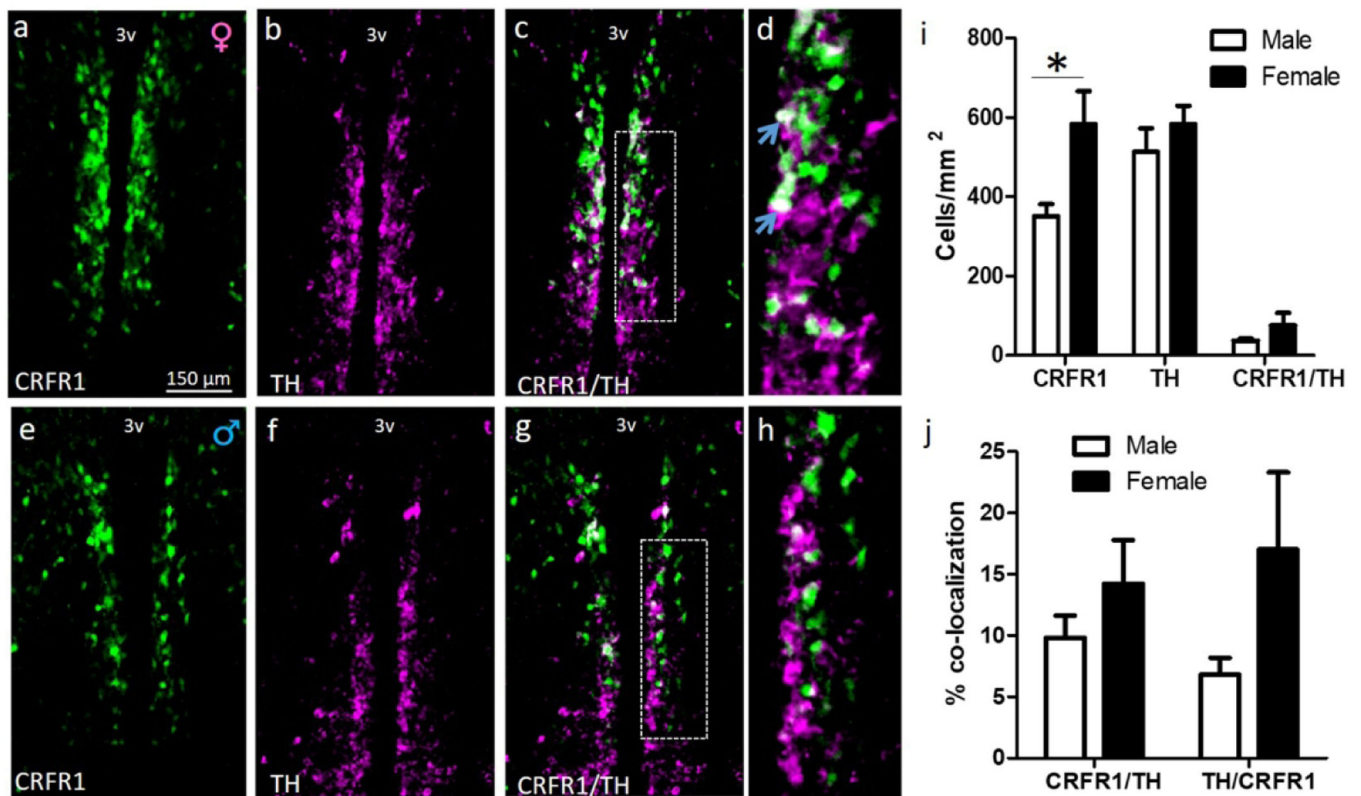


Figure 1. Dual-label fluorescent immunohistochemistry comparing female (a-d) and male (e-h) AVPV/PeN CRFR1-GFP, tyrosine hydroxylase (TH), and co-localization of CRFR1/TH on the day of birth (d, h; high-magnification display of images c, g respectively). (i) Comparison of male versus female CRFR1-GFP- and TH-expressing cells (green, magenta, respectively), and co-expression of TH/CRFR1-GFP (white) 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 \pm SEM. Inset boxes indicate regions that were further magnified. Arrows indicate examples of co-localized cells. N=5 per sex. 3V, third ventricle.

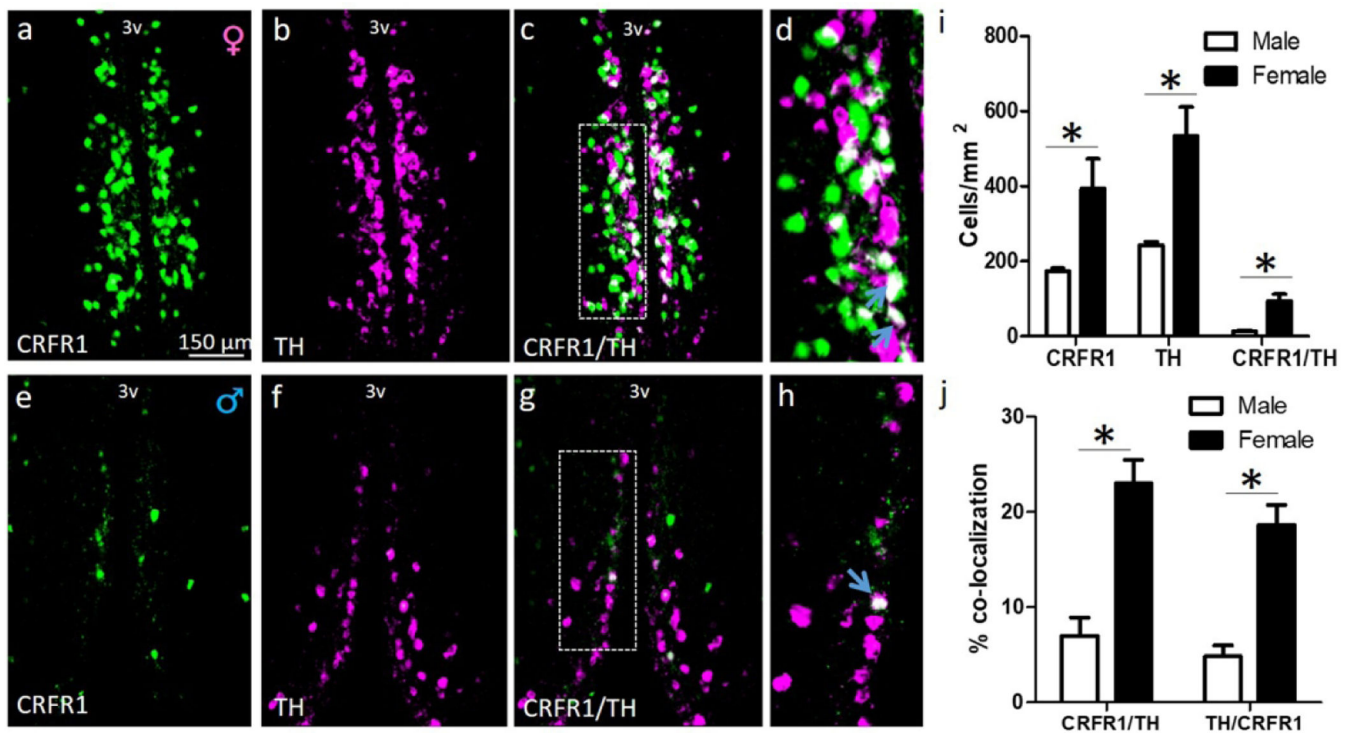


Figure 2.

Dual-label fluorescent immunohistochemistry comparing the adult (P60) female (a-d) and male (e-h) AVPV/PeN CRFR1-GFP, 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-GFP- (green), TH-expressing cells (magenta), and co-expression of TH in CRFR1-GFP cells (white) illustrating significant sex differences in P60 CRFR1-GFP-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 \pm SEM. Inset boxes indicate regions that were further magnified. Arrows indicate examples of co-localized cells. N=5 per sex. 3V, third ventricle.

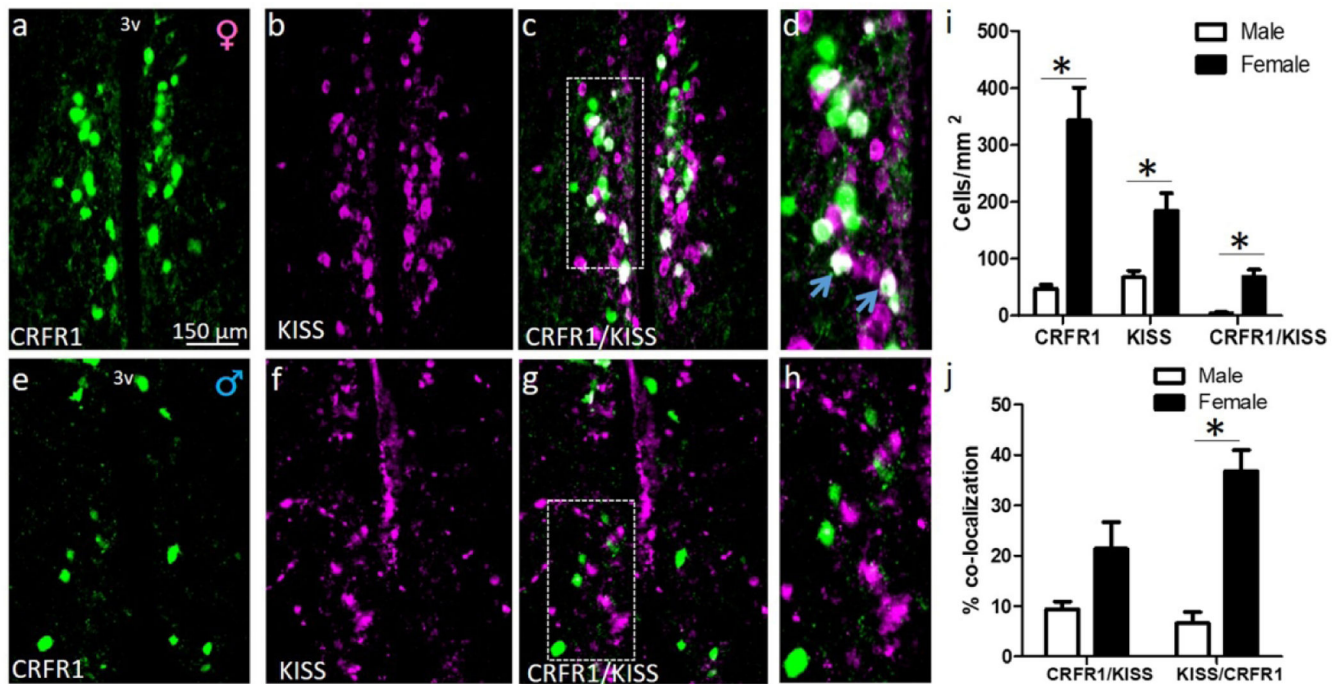


Figure 3.

Dual-label fluorescent immunohistochemistry comparing adult (P60) female (a-d) and male (e-h) AVPV/PeN CRFR1-GFP (a, e), kisspeptin (KISS) (b, f), and co-expression (c, g). (d, h) high magnification display of images c, g respectively. (i) Comparison of female and male CRFR1-GFP-ir and KISS-ir, with significant differences in the number of CRFR1-GFP-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 \pm SEM. Inset boxes indicate regions that were further magnified. Arrows indicate examples of colocalized cells. N=5 per sex. 3V, third ventricle.

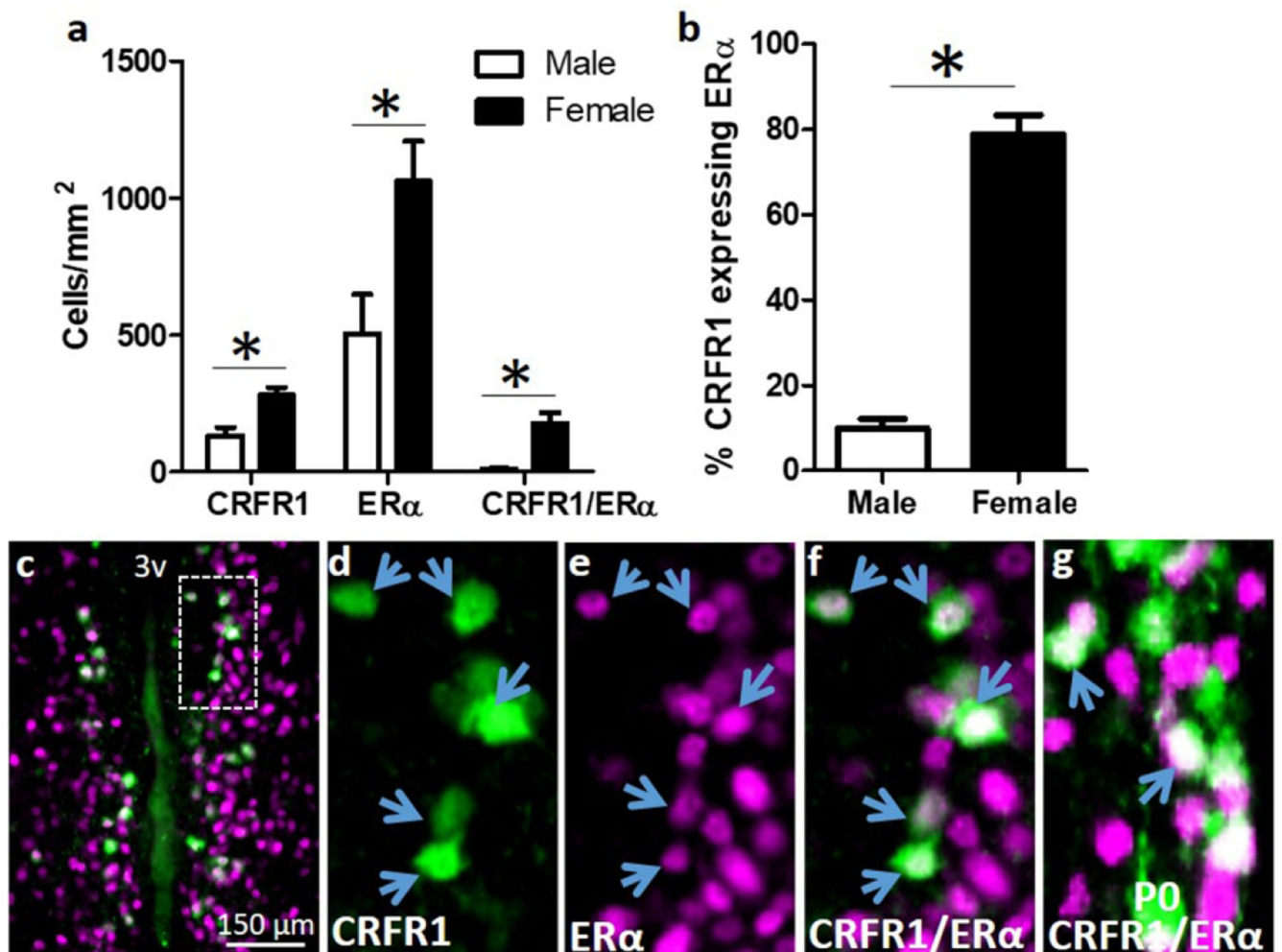


Figure 4.

(a,b) Co-localization of CRFR1-GFP and ER α . Dual-label fluorescent immunohistochemistry showing that females have significantly more CRFR1-GFP, ER α , CRFR1-GFP/ER α co-localized, and a higher percentage of CRFR1-GFP cells that co-express ER α compared to males. (c-f) Representative images from a female AVPV/PeN showing a low magnification image of co-localized cells (c), CRFR1-GFP (d; green), ER α (e; magenta), and coexpression of CRFR1/ER α (f; white). (g) Image showing co-expression of CRFR1 and ER α on the day of birth (P0) in the female mouse AVPV/PeN. Inset box indicates region that was further magnified. Arrows indicate examples of co-localized cells. * Indicates statistical significance ($p < 0.05$), data are reported as mean \pm SEM. N=5 per sex. 3V, third ventricle.

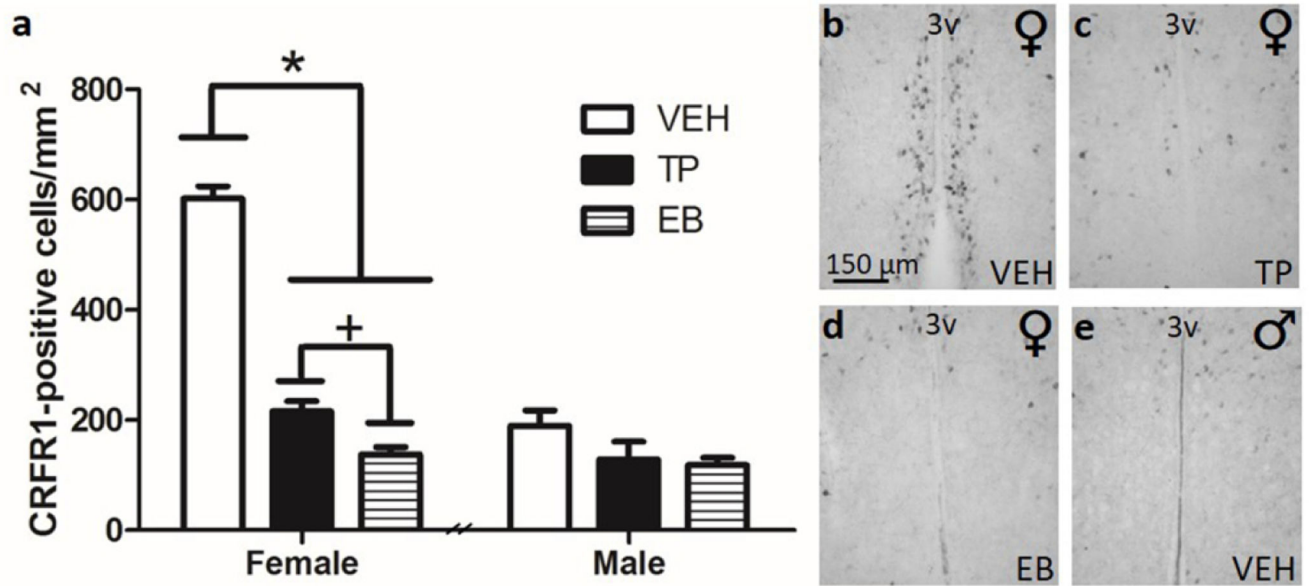


Figure 5.

Neonatal (P0) hormone supplement influence on CRFR1-GFP+ 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-GFP+ cells.

Treatment with EB reduced CRFR1-GFP to a greater extent than TP. Representative images from females treated with VEH (b), TP (c), EB (d), and a male treated with VEH (e). *

Indicates statistical significance ($p < 0.001$), + indicates $p < .05$. Data are reported as mean \pm SEM. N=6–7 per sex/treatment. 3V, third ventricle.

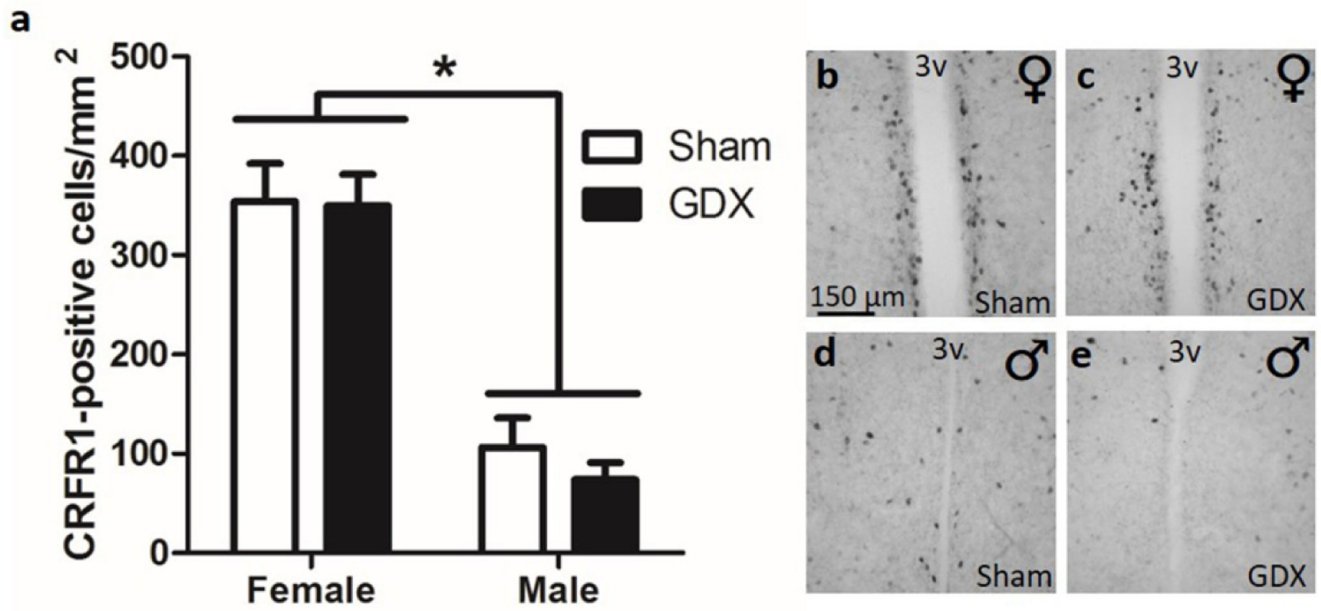


Figure 6. Adult gonadectomy effects on CRFR1-GFP+ cells. (a) Gonadectomy did not alter CRFR1-GFP+ cell number in either males or females. Representative images from female (b) and male (d) sham surgery compared with female (c) and male (e) gonadectomy, showing that removal of adult gonadal hormones does not change the expression pattern. * Indicates significant sex difference ($p < 0.001$), data are reported as mean \pm SEM. $N=8$ per sex/treatment. 3V, third ventricle.

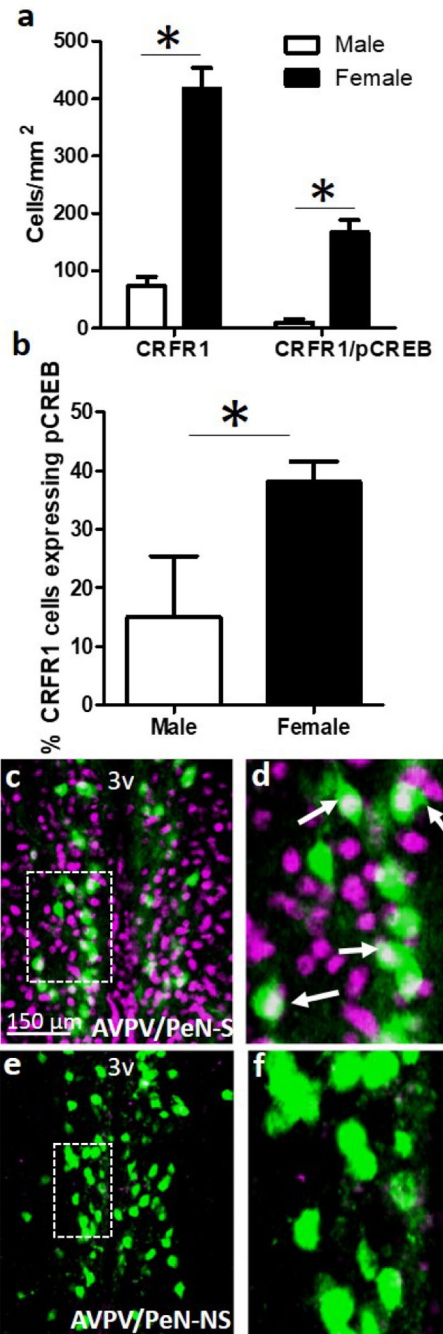


Figure 7. Dual-label fluorescent immunohistochemistry showing (a) female versus male expression levels of CRFR1-GFP (F>M), and CRFR1-GFP/pCREB colocalization (F>M). (b) Comparison between female and male co-expression, showing a sex difference in the percentage of activated CRFR1-GFP+ cells (pCREB co-expression) following a 30-minute restraint stress (F>M). Low and high magnification representative images of a female AVPV/PeN from a (c-d) s; stressed and (e-f) ns; non-stressed mouse. * Indicates statistical significance ($p < 0.05$), data are reported as mean \pm SEM. Inset boxes indicate regions that

were further magnified. Arrows indicate examples of co-localized cells. N= 5 male, 7 female mice. 3V, third ventricle.

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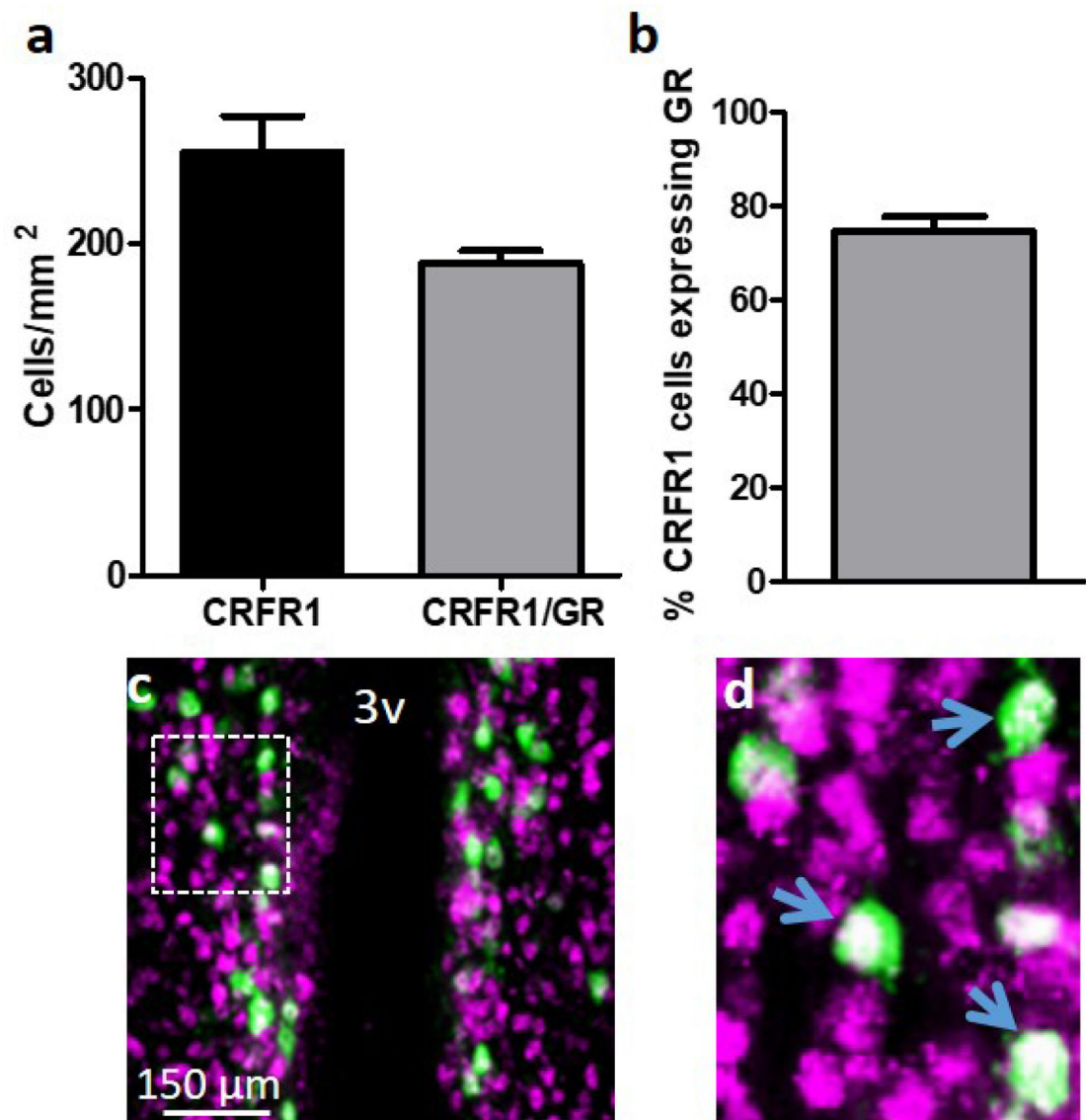


Figure 8. Dual-label fluorescent immunohistochemistry showing the number of (a) CRFR1-GFP and CRFR1-GFP/GR co-expressing cells, and (b) the percentage of female AVPV/PeN CRFR1GFP+ cells that express GR (~75%). (c) Representative image of CRFR1 (green) and GR (magenta) co-expression (white), and (d) high magnification image of co-localized cells in the AVPV/PeN. Inset box in (c) indicates region further magnified in (d). Arrows indicate examples of co-localized cells. N= 4 female mice. 3V, third ventricle.

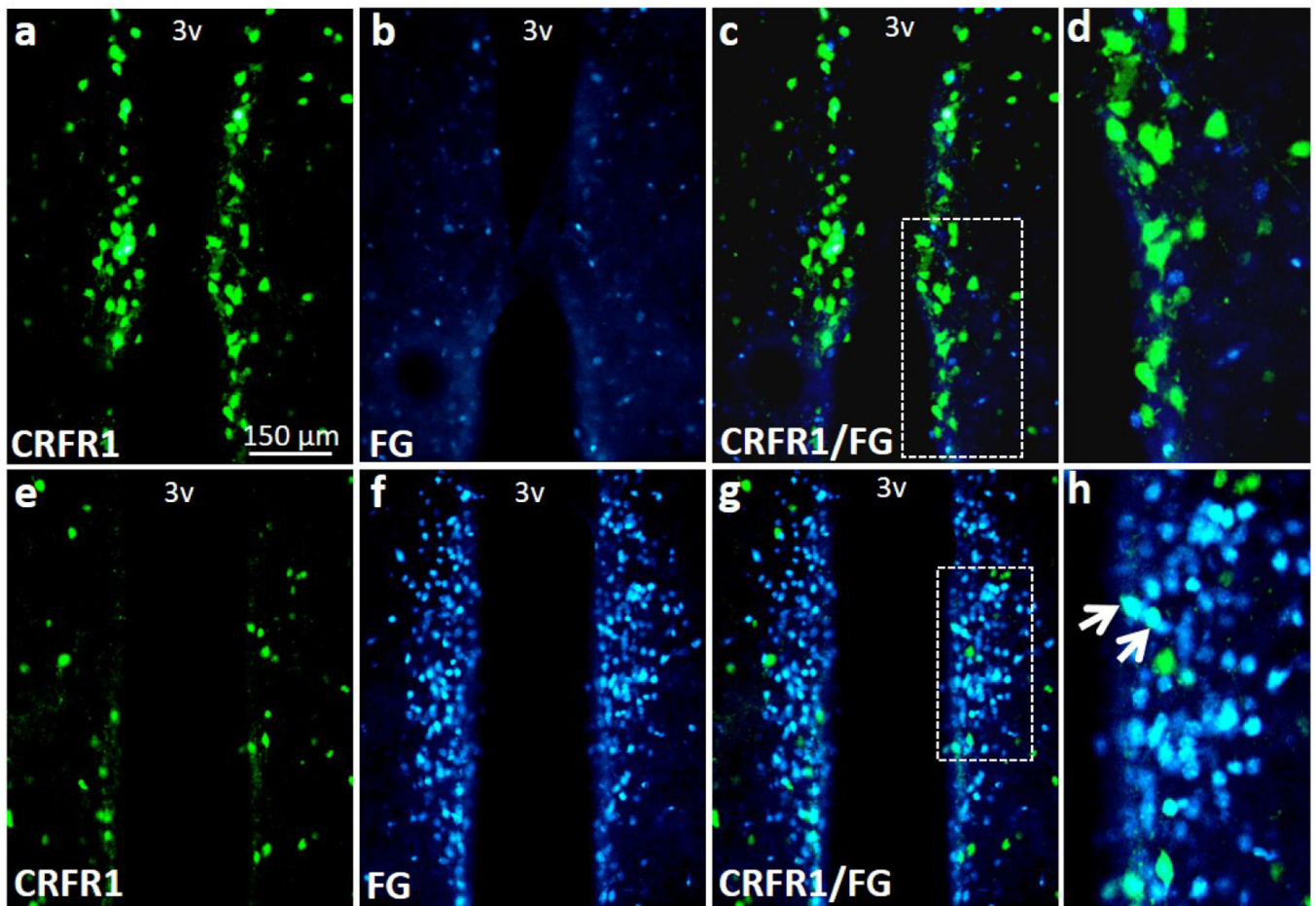


Figure 9. Images showing fluorogold and CRFR1-GFP labeling. Few neurosecretory (fluorogold) were found near the sexually dimorphic CRFR1 AVPV/PeN nucleus and no CRFR1GFP/fluorogold co-labeled cells were found in this region (a-d). However, high levels of fluorogold and CRFR1/fluorogold co-labeling was found in further caudal periventricular sections (e-h). Inset boxes indicate regions that were further magnified. Arrows indicate examples of colocalized cells. 3V, third ventricle.

Table 1.

Primary Antibodies

Target	Antigen	Source	Working dilution	Reference
ER α	ER α C-term (580–599)	Santa Cruz sc-542	1:250	Quesada et al. (2007) Omoto et al. (2005) RRID: AB_631470
GFP	Full length recombinant GFP protein	Abcam ab13970	1:1000	Milosevic et al. (2017) RRID: AB_300798
GR	N-terminus of GR α (5–24)	Santa Cruz sc-1004	1:250	Sarabdjitsingh et al. (2010) Damjanovic et al. (2012) RRID: AB_2155786
KISS	Mouse kisspeptin 10 peptide	Millipore AB9754	1:500	Brock and Bakker (2013) RRID: AB_2296529
pCREB	synthetic phosphopeptide that corresponds to residues that surround Ser133 of human CREB (129–137)	Cell Signaling 9198	1:500	Dimitrov and Usdin (2010) Uribe-Marino et al. (2016) RRID: AB_2561044
TH	Denatured tyrosine hydroxylase from rat pheochromocytoma	Millipore AB152	1:500	Scott et al. (2015) RRID: AB_390204