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The Role of Androgens on Sexually Dimorphic Distributions of Key Corticotropin- Releasing Factor Receptor 1 Populations and Stress Responses within the Brain

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The Role of Androgens on Sexually Dimorphic Distributions of Key Corticotropin-Releasing Factor Receptor 1 Populations and Stress Responses within the Brain

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Abstract

Differences in the prevalence of stress-related disorders between males and females, such as anxiety and depression, are believed to be partly due to sexually dimorphic brain structures, particularly those that regulate the hypothalamic-pituitary-adrenal (HPA) axis. Within the hypothalamus, the paraventricular nucleus (PVN) is a primary structure in the integration of many hormonal inputs and induces the release of corticotrophin-releasing factor (CRF), the primary regulator of the HPA axis as well as behavioral stress responses. A sex difference in the distribution of corticotrophin-releasing factor receptor 1 (CRFR1) containing cells in the PVN has been reported with more in male than in female mice. A previous study performed in our lab shows an adult gonadectomy causes a significant decrease in CRFR1 expressing cells in males, but not in females suggesting a possible mechanism in which gonadal hormones regulate CRFR1 expression. Androgen receptors show high co-expression with CRFR1 and are generally thought to play a role in the downregulation of the stress response and the HPA-axis although the mechanisms are widely unknown. The goal of this study was to gain insight into the role of androgens and potential receptors they may act on within the PVN and related sexually dimorphic structures to produce a sex difference in regulation of the stress response. Specifically, experiments were performed in mice to determine whether androgens, acting through the androgen receptor, regulate CRFR1 expression in the PVN as well as other key CRFR1 cell populations such as the bed nucleus of the stria terminalis (BST). We also determined if alteration of androgen treatment will alter the stress-induced activation of the CRFR1 neurons by having a down-regulation effect. Results from this experiment showed that gonadectomized animals showed a decrease in CRFR1 cell populations in the PVN that was reversed by the addition of dihydrotestosterone (DHT), an androgen that preferentially binds the androgen receptor. In the BSTld there was a decrease in CRFR1 in the GDX-DHT group when compared to GDX-blank and control sham operated groups. No significant findings were reported in the BSTav. Results also showed no differences in neural activation of CRFR1 neurons in the BSTld, BSTav or PVN with addition of DHT. Discovery of the mechanisms in which androgens regulate stress systems in the brain will help us gain a better understanding of why females develop stress-related disorders twice as frequently as males.

Keywords*: HPA-axis, CRFR1, Sexual dimorphism, Androgens*

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Introduction

Throughout their lifetimes, women are twice as likely to experience depression and anxiety disorders including ones such as post-traumatic stress disorder and panic disorder (Donner & Lowry, 2013). Differences in the prevalence of stress-related disorders are largely believed to be due to sexually dimorphic brain structures, particularly those that regulate the hypothalamic-pituitary-adrenal (HPA) axis. The HPA-axis is a complex circuit regulated by feedback mechanisms and is activated in response to a stressor. Therefore, mechanisms that cause sex differences in this important circuitry would have value to the treatment of stress-related disorders. Although, the mechanisms through which these differences potentiate are unknown. It is however known that corticotropin releasing factor (CRF) and its receptors, CRFR1 and CRFR2, play an important role in modulating the stress response through the HPA-axis. Circulating gonadal steroid hormones have been shown in previous studies to influence the regulation of this pathway and therefore provide a possible mechanism through which these sex differences develop (Boivin, Piekarski, Wahlberg, & Wilbrecht, 2017).

The primary gonadal hormones, androgens, and estrogens have been shown to play an important role in the development of these sex differences**.** Androgens or androgen metabolites act at androgen receptors (ARs) to produce a variety of effects on the stress response including a down-regulation. Dihydrotestosterone (DHT), an example of an androgen metabolite, is a potent and non-aromatizable androgen that can also function to inhibit the stress response. A gonadectomy of male rats resulting in a decrease of circulating gonadal hormones will illicit an increase in the stress response, corticosterone (CORT) levels, and adrenocorticotropic hormone (ACTH) levels that can be reversed with addition of either DHT or testosterone (Zuloaga, Heck, Guzman, & Handa, 2020). Subsequently, as opposed to DHT, aromatizable androgens can also

be converted to 17β-estradiol with the addition of the aromatase enzyme (Simpson, 2002). 17β-estradiol can then act on estrogen receptor alpha (ERα) or estrogen receptor beta (Erβ). Actions through these receptors play an important role in the regulation of the HPA-axis and therefore also the prevalence of anxiety and depression. The binding of the ERα causes an increase in the activation of the HPA-axis while ERβ has the opposite effect (Weiser & Handa, 2009). Through actions at ARs, androgens have a similar effect as Erβ since they also down-regulate the HPA-axis response. (McHenry, Carrier, Hull, & Kabbaj, 2013). Therefore, androgens have the potential capability of decreasing the stress response through activation of either ARs or can be converted to estrogens to act at Erβ. A previous study performed in the lab showed that gonadectomized male rodents showed a decrease in the CRFR1 receptors in the paraventricular nucleus (PVN) that are responsible for the suppression of the stress response (Rosinger, Jacobskind, De Guzman, Justice, & Zuloaga, 2019). It is predicted that this decrease in CRFR1 cell expression is primarily due to the prior decrease in androgens or androgen metabolites acting on the ARs or ERs.

Androgens and ARs have also been shown to have an implication in corticotropin releasing factor (CRF) expression. CRF is a hormone released from the hypothalamus and is important in regulation of the HPA axis through binding of the CRFR1 and CRFR2 receptors. CRF and its actions on CRFR1 are crucial to the regulation of stress and the HPA-axis. A decrease in CRFR1, such as in the previous study mentioned, will prevent the down regulation of the stress response, and therefore cause an increase in the release of ACTH and CORT (Rosinger, Jacobskind, De Guzman, Justice, & Zuloaga, 2019; Jiang et al., 2018). Chronic exposure to these hormones increases susceptibility to both anxiety and depression (Wisłowska-Stanek, Lehner, Skórzewska, Skórzewska, & Płaznik, 2016). Sex differences of CRFR1

expressing cells in certain brain regions have been shown to be modulated by androgens and contribute to the differences seen in behavior. Studies utilizing immunohistochemistry have shown that CRFR1 expressing neurons also often co-express AR and ER (Zuloaga et al., 2020). In addition, it has been hypothesized that androgens have a role in regulating CRF levels in an adulthood. One study showed that a GDX in males caused the CRF cell number to increase while a neonatal treatment with testosterone caused the number of CRF cells to decrease (Bingaman, Magnuson, Gray, & Handa, 1994; Fukushima, Furuta, Kimura, Akema, & Funabashi, 2013). These results combined with reversal of effects of GDX on CRF cell number by addition of testosterone, indicate that androgens have a role in modulating CRF-expression. Therefore, brain regions that also modulate CRF-expression such as the PVN, anteroventral periventricular nucleus (AVP) and bed nucleus of the stria terminalis (BST) that modulate the stress response offer potential areas for modulation via androgens and ARs.

Located within the hypothalamus, the PVN is a primary structure in the integration of many hormonal inputs and induces the release of a corticotropin-releasing factor (CRF), the primary regulator of the HPA axis as well as behavioral stress responses (Jiang, Rajamanickam, & Justice, 2019). A dysregulation of this circuit causing more glucocorticoids, such as cortisol, to be released into the body at abnormal rates is a cause of both stress and depressive disorders. Similarly, the bed nucleus of the stria terminalis (BST) is considered crucial in modulating the stress response due to its connections to the amygdala, medial pre-frontal cortex, ventral tegmental area, dorsal raphe and then projections to the hypothalamus (Lebow & Chen, 2016). The BST projections in the hypothalamus reach the PVN and directly inhibit the PVN neuronal activity (Heck & Handa, 2018). Therefore, this is a crucial brain region in the downregulation of the stress response. The goal of this study is to gain insight into the role of androgens and

potential receptors they may act on within the PVN and BST to produce sex difference in regulation of the HPA axis and anxiety-like behaviors during stress. Specifically, we performed experiments in mice to determine whether androgens, acting through the androgen receptor, regulate CRFR1 expression in the PVN as well as key CRFR1 populations within the BST. We also looked at the effects of androgens on the regulation of CRFR1 neural activation. Discovery of the mechanisms in which androgens regulate stress systems in the brain would help us gain a better understanding of why females develop stress-related disorders twice as frequently as males.

Experimental Procedures

Animals

All animals used in this experiment were CRFR1-GFP transgenic mice. This reporter mouse line has been tested and GFP localization has been confirmed to closely follow the expression of CRFR1 mRNA (Justice, Yuan, Sawchenko, & Vale, 2008). Mice were given food and water as they desired. They were also held at a 12/12 light-dark cycle, with the lights on 0700. All procedures performed in this experiment coincide with National Institute of Health guidelines and have been approved by the University at Albany Institutional Animal Care and Use Committee (IACUC).

Gonadectomy

Gonadectomized and sham surgeries were performed to test the effects of androgens on the number of CRFR1 expressing cells in brain regions of interest. Each of the male CRFR1-GFP 60-70 day old mice were randomly assigned to a treatment group consisting of sham surgery, GDX-blank, or GDX-DHT. Seven males were gonadectomized, seven males underwent

a sham surgery, and the final seven males were gonadectomized and then administered DHT capsules. Animals were then sacrificed, and their brains extracted for visualization.

Restraint Stress

To determine the activation of the CRFR1 cells within the PVN and the BST, restraint stress was used. Mice were restrained inside of a tube for 30 minutes. This tube was placed inside of a bedding cage that was inside of a plastic cage. After restraint within the tube, the animals were placed back into their home cage until they were sacrificed 120 minutes after the onset of the restraint protocol.

Chromogenic Immunohistochemistry

Immunohistochemistry (IHC) procedure was performed as described in the previous paper (Rosinger et al., 2019). Tissue for this experiment was sectioned at a 40-micron thickness using a cryostat before performing IHC. IHC is a widely used technique allowing for the visualization of specific proteins or molecules with a brain region. To visualize co-localization of CRFR1 with AR, tissue was first placed in the primary antibody GFP (Abcam; chicken; 1:10,000) for incubation. From there, tissue was then placed in biotinylated goat anti-chicken antisera (Vector Laboratories; 1:1,000). After being rinsed, the tissue was placed in avidin-biotin complex (ABS Elite kit, Vector Laboratories; 1:1000). Finally, the tissue was placed in the chromogen, diaminobenzidine (DAB), for 10 minutes to label CRFR1.

Dual-Label Fluorescent Immunohistochemistry

To determine the co-expression of CRFR1-GFP cells with c-fos, a dual-labeled fluorescent immunohistochemistry (IHC) was performed. Before addition of antibodies, tissue was rinsed in and incubated with primary antibody c-fos (Santa Cruz; rabbit; 1:250). The following day the tissue was rinsed and the donkey anti-rabbit 594 secondary (Jackson Labs;

1:250) was added. Next, the tissue was incubated in secondary primary antibody GFP (Abcam; chicken; 1:2000). The next day the tissue was incubated in donkey anti-chicken 488 secondary (Jackson Labs; 1:1500). Finally, the tissue underwent additional rinses before being mounted and cover slipped for analyzation.

Microscopy

Images of the stained cells expressing CRFR1-GFP in the bed nucleus of the stria terminalis and the paraventricular nucleus were photographed using a Nikon 80i microscope. ImageJ was used to quantify each of the sections bilaterally. For the PVN, a triangular shaped ROI derived from a rodent brain atlas (Allen Institute Reference Atlas) was used. For the BST, oval shaped ROI was used to quantify neurons within two subdivisions (dorsolateral and anteroventral). For quantification, the CRFR1 cells containing a brown label were counted. To determine co-expression of CRFR1 and c-fos, fluorescent microscopy was used. Co-localized cells appeared as a yellow nuclear label and were also quantified using ImageJ and appropriate ROIs for each section of interest.

Results

Results of this experiment revealed statistical differences in the treatment groups within the PVN and the BSTld. A one-way ANOVA was run in order to determine whether CRFR1-GFP was altered by gonadectomy and DHT treatment. For each of the brain regions (PVN, BSTav and BSTld) a post-hoc test was performed to explore some of the differences found between the treatment groups. As seen in Figure 1, the animals that received a gonadectomy as opposed to the sham surgery showed a decrease in the CRFR1 cell expression within the PVN. Figure 1 also shows an increase in the CRFR1 expressing cells when the first gonadectomized animals were treated with DHT capsules.

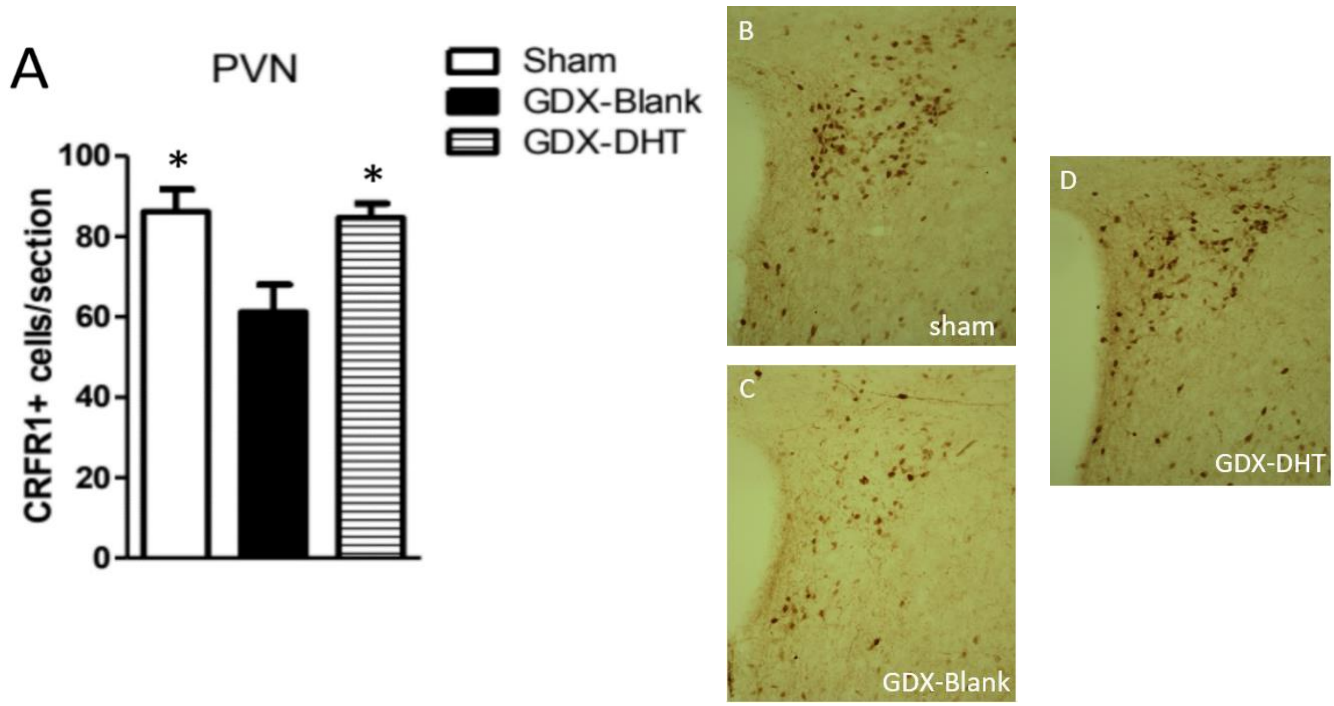

Figure 1
CRFR1-GFP number in the PVN

Note. A one-way ANOVA of CRFR1-GFP was run to determine whether CRFR1-GFP was altered by GDX and DHT treatment. A) This analysis revealed there were significant differences in the PVN ($F= 6.672$, $p \leq 0.05$) A post hoc test was then run to explore the differences between the individual experimental groups (GDX-DHT, GDX-Blank, and sham). This test revealed that a gonadectomy, as opposed to a sham surgery, caused CRFR1 cells in the PVN to decrease. There was also a significant increase in CRFR1 cells in the PVN between the animals that received DHT after being gonadectomized and those that did not. B) Representative image of CRFR1 sham treatment group in PVN C) Image of GDX-blank treatment group in PVN region D) Image of GDX-DHT group in PVN * indicates $p<.05$ compared to GDX-blank group.

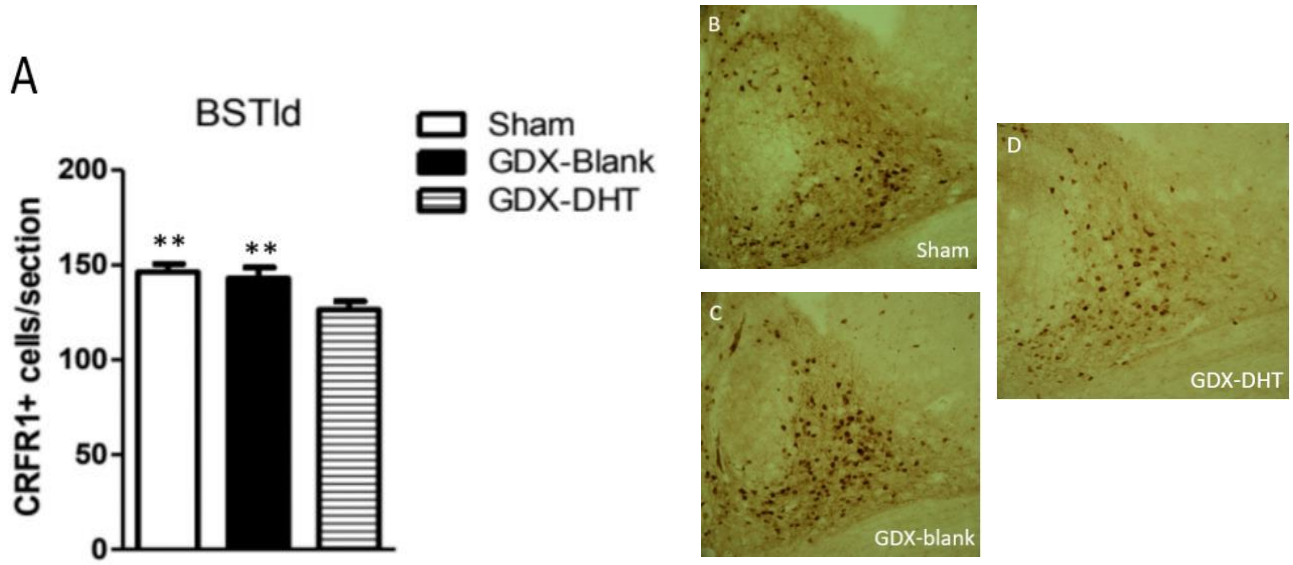

Figure 2
CRFR1-GFP number in the BSTav

Note. A one way ANOVA of CRFR1-GFP was run to determine whether CRFR1-GFP was altered by GDX and DHT treatment. This analysis revealed there were significant differences in the BSTld ($F=4.921$, $p \leq 0.05$). A post hoc test was then run to explore the differences between the individual experimental groups (GDX-DHT, GDX-Blank, and sham). Post hoc tests revealed there were decreases in CRFR1 cells in the BSTld when animals were administered DHT compared to both sham operated and GDX-Blank animals. B) Representative image of sham treatment group in BSTld C) Image of GDX-blank treatment group in BSTld region D) Image of GDX-DHT group in BSTld * indicates $p<.05$ compared to GDX-DHT group.

As seen in Figure 2, significant differences were seen among treatment groups within the BSTld. The sham operated and the gonadectomized animals that did not receive DHT showed a significant increase in CRFR1 expressing cells when compared to the treatment group that received the DHT. Finally, as seen in Figure 3, there were no statistic differences found in the BSTav.

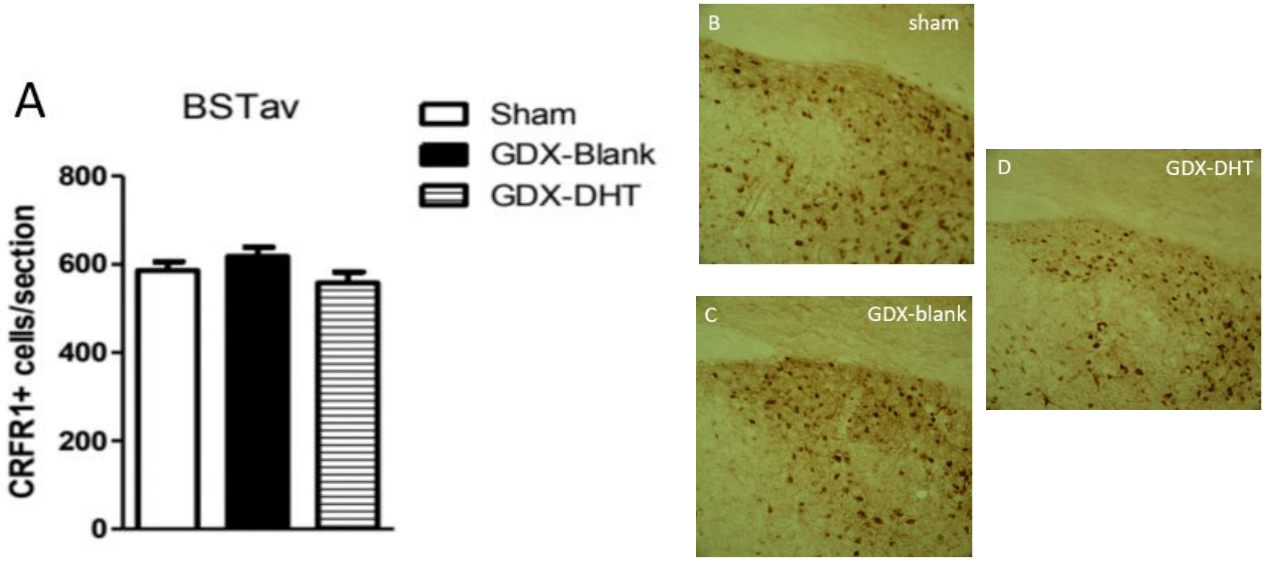

Figure 3
CRFR1-GFP in the BSTld

Note. A one-way ANOVA of CRFR1-GFP was run to determine whether CRFR1-GFP was altered by GDX and DHT treatment. This analysis revealed there was no statistical significance in the BSTav ($F=1.904$, $p \geq 0.05$). No significant differences were found in the BSTav. B) Representative image of sham treatment group in BSTav C) Image of GDX-blank treatment group in BSTav region D) Image of GDX-DHT group in BSTav.

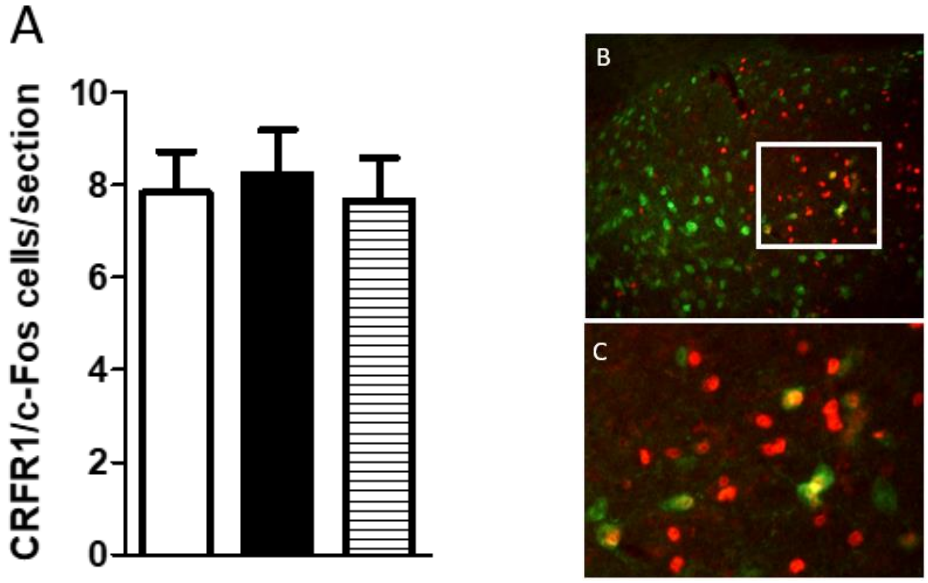

Figure 4
Co-localization of CRFR1 and c-fos within the BSTav

Note. Graph comparing CRFR1 c-Fos colocalization revealed no significant differences were reported in Sham, GDX-blank, GDX-DHT treatment groups in neural activation of CRFR1 within the BSTav region. A low magnification (B) and high magnification (C) show CRFR1 represented by red fluorescence, c-fos by green fluorescence and co-localization represented by yellow labeled fluorescence.

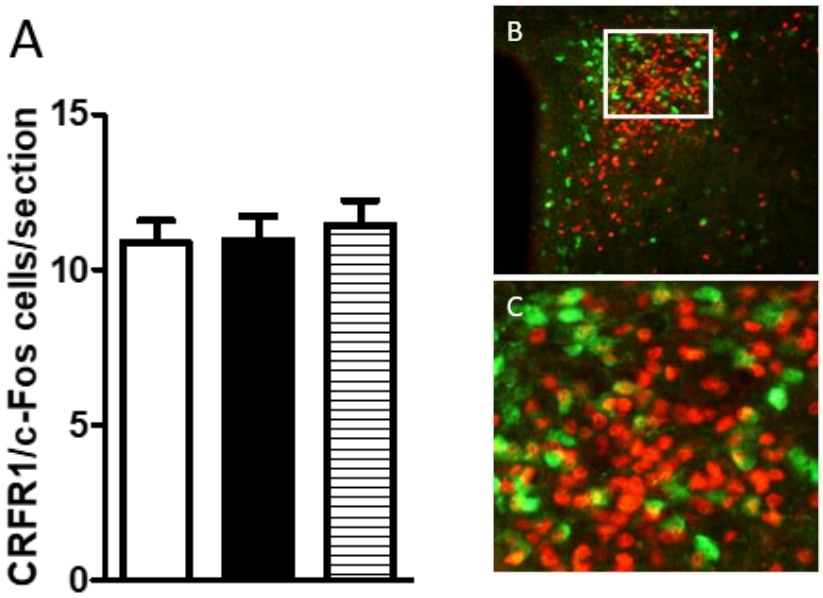

Figure 5
Co-localization of CRFR1 and c-fos within the PVN

Note. Graph comparing CRFR1 c-Fos colocalization revealed no significant differences were reported in Sham, GDX-blank, GDX-DHT treatment groups in neural activation of CRFR1 within the PVN region. A low magnification (B) and high magnification (C) show CRFR1 represented by red fluorescence, c-fos by green fluorescence and co-localization represented by yellow labeled fluorescence

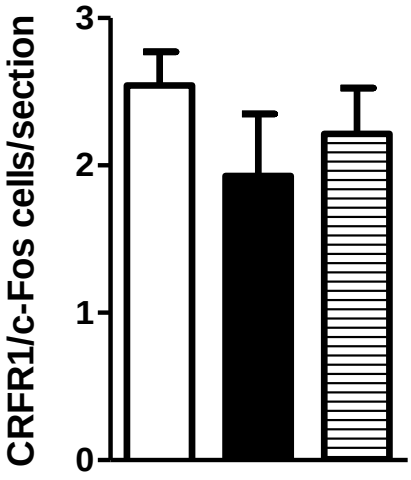

Figure 6
Co-localization of CRFR1 and c-fos within the BSTld

Note. Graph comparing CRFR1-cfos colocalization revealed no significant differences were reported in Sham, GDX-blank, GDX-DHT treatment groups in neural activation of CRFR1 within the BSTld region.

To determine effects of GDX and DHT on restraint stress-induced neural activation of CRFR1 cells within the BSTld, BSTav, and PVN, immunohistochemistry was performed, and co-localization was quantified. No significant differences were reported in any of the above brain regions.

Discussion

Results of this study suggest a possible mechanism in which testosterone plays a role in the regulation of CRFR1 expression and in turn, the regulation of the HPA-axis. In the BSTld, a gonadectomy followed by the administration of DHT caused the CRFR1 cells to decrease. Gonadectomized animals experienced a decrease in the number of CRFR1 expressing cells in the PVN. Also, in the PVN, the administration of DHT was shown to increase the number of CRFR1 cells. Although GDX and DHT had an effect on CRFR1 levels within these brain regions, it did not affect the activation of these neurons after restraint stress.

The PVN of the hypothalamus has been shown to play an important role in modulating the stress response. There is a subset of neurons within this region that are responsible for the release of corticotropin releasing factor (CRF) which then stimulates the pituitary gland and activates the HPA-axis. (Herman & Takser, 2016). CRFR1 receptors within the PVN have been shown to be important in the regulation of the stress and behavioral responses (Rosinger et al., 2019). Activation of CRFR1 neurons in the PVN plays a role in the negative feedback loop that causes a decrease in activation of the CRF neurons. Therefore, a decrease in CRFR1 in the PVN would cause an increase in HPA-axis activation (Jiang, Rajamanickam, & Justice, 2018).

Androgens, such as testosterone and DHT, have been shown to have a negative feedback effect on the HPA-axis. Previous studies performed in our lab have shown that there is a high level of androgen receptors in CRFR1 expressing PVN neurons (Rosinger et al., 2020). Co-expression between CRFR1 and AR has also been reported suggesting a possible mechanism in which androgens produce their anxiolytic effects. Therefore, the results of this study propose a possible mechanism to explain the differences in anxiety prevalence between males and females.

A gonadectomy caused the androgens to decrease and therefore the number of CRFR1 cells were also decreased. This reduces the activity of CRF on CRFR1 receptors of the PVN and prevents the deactivation of the HPA-axis.

Another potential site at which androgens will bind to ARs to down-regulate the HPA-axis and the stress response is the BST. Previous studies have shown that BST projects into the PVN and regulate the HPA-axis activation. Evidence shows that the BST, more specifically, functions to integrate forebrain and limbic information after receiving a stressful stimulus (Choi, Furay, Evanson, Ostrander, Ulrick-Lai, & Herman, 2007). It has been previously reported that the BSTav contains high levels of both androgen and estrogen receptors (Rosinger et al., 2020). AR containing cells within the BST extend into the PVN and therefore are thought to play a possible role in the regulation of anxiety and depressive behaviors. The BSTld was shown to have a sexually dimorphic distribution of CRF; with more reported in females than in males (Rosinger et al., 2020) The effects of the gonadectomy followed by DHT in the BSTld caused CRFR1 expressing cells to decrease. This may be due to the administration of more DHT than is naturally found in the body causing an increase in AR binding and a decrease in CRFR1. In contrast, there was no effect on the number of CRFR1 cells in the BSTav.

Understanding sex differences in key brain regions associated with the stress response helps unveil possible mechanisms behind the higher prevalence of anxiety disorders in females versus males. Results of this study revealed that a gonadectomy and a decrease in circulating androgens causes a decrease in CRFR1 cell populations in the PVN. Decreasing CRFR1 expressing cells results in a lack of down regulation of the HPA-axis. Therefore, it is possible that a function of testosterone in males is to down regulate the stress response. If these mechanisms are studied and understood it is possible that a treatment can be developed that

closes the gap between males and females when it comes to being diagnosed with an anxiety or depressive disorder.

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