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Androgen regulation of corticotropin-releasing hormone receptor 2 (CRHR2) mRNA expression and receptor binding in the rat brain

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Abstract

Stress-induced affective disorders, such as depression and anxiety, are more prevalent in females than in males. The reduced vulnerability to these disorders in males may be due to the presence of androgens, which are known to dampen the stress response and reduce anxiety-like behaviors. However, a neurobiological mechanism for this sex difference has yet to be elucidated. Corticotropin-releasing hormone receptor 2 (CRHR2) has been implicated in regulating anxiety-type behaviors and is expressed in stress-responsive brain regions that also contain androgen receptors (AR). We hypothesized that androgen may exert its effects through actions on CRHR2 and we therefore examined the regulation of CRHR2 mRNA and receptor binding in the male rat forebrain following androgen administration. Young adult male Sprague/Dawley rats were gonadectomized (GDX) and treated with the non-aromatizable androgen, dihydrotestosterone propionate (DHTP) using hormone filled Silastic capsules. Control animals received empty capsules. Using quantitative real time RT-PCR, CRHR2 mRNA levels were determined in block dissected brain regions. DHTP treatment significantly increased CRHR2 mRNA expression in the hippocampus, hypothalamus, and lateral septum ($p < 0.01$) when compared to vehicle-treated controls. A similar trend was observed in amygdala ($p = 0.05$). Furthermore, *in vitro* autoradiography revealed significantly higher CRHR2 binding in the lateral septum in androgen-treated males, with the highest difference observed in the ventral lateral region. Regulation of CRHR2 mRNA by AR was also examined using an *in vitro* approach. Hippocampal neurons, which contain high levels of AR, were harvested from E17–18 rat fetuses, and maintained in primary culture for 14 days. Neurons were then treated with dihydrotestosterone (DHT; 1 nM), DHT plus flutamide (an androgen receptor antagonist), or vehicle for 48 hours. CRHR2 mRNA levels were measured using quantitative real time RT-PCR. Consistent with *in vivo* studies, DHT significantly increased CRHR2 mRNA expression in hippocampal neurons ($p < .02$) compared to vehicle treated controls. Flutamide treatment prevented the effect of DHT on CRHR2 mRNA indicating that DHT's effect on CRHR2 expression is AR-mediated. Thus, the CRHR2 gene

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appears to be a target for regulation by AR and these data suggest a potential mechanism by which androgen may alter mood and anxiety-related behaviors.

Keywords

Stress; Anxiety; Gonadal Steroids; HPA Axis; Androgen; CRH

Introduction

Stress-related psychiatric disorders like major depressive disorder and generalized anxiety disorder affect well over 10% of the population in the United States (Kessler et al., 2005). Underlying these pathologies is an apparent dysregulation of stress-responsive neuroendocrine function (Varghese and Brown, 2001; Barden, 2004), suggesting a critical role for stress sensitivity in affective disorders. Furthermore, several studies have consistently reported that major depressive episodes are over twice as common in women as compared to men (Weissman et al., 1993; Kornstein, 1997; Llewellyn et al., 1997), a difference that emerges at puberty (Angold and Worthman, 1993), suggesting a hormonal component. Importantly, numerous studies have confirmed the involvement of hypothalamic-pituitary-adrenal (HPA) axis dysregulation with depression and anxiety disorders (Holsboer and Barden, 1996; Ehler et al., 2001; Varghese and Brown, 2001; Barden, 2004). Taken together, these data indicate a potential interplay between gonadal steroids, stress reactivity, and the development of mood disorders. The mechanism of estrogen's effect on mood and stress sensitivity has been extensively studied. However, the method by which androgens alter mood and HPA axis function has not been as well explored.

The HPA axis is the major neuroendocrine axis that responds to stress. Activation of the HPA axis following a physical or emotional stressor is characterized by a series of neuronal and hormonal responses in an attempt to maintain homeostasis [see (de Kloet et al., 2005) for review]. Activity of the HPA axis is controlled by a subset of neurons in the parvocellular part of the paraventricular nucleus (PVN) of the hypothalamus that receive afferent stress-related input, and secrete corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophyseal portal system. Afferents from the brainstem, basal forebrain, and limbic brain regions provide stressor-related input directly and indirectly to these neuroendocrine neurons. At the anterior pituitary, CRH, and to a lesser extent AVP, stimulates the synthesis and secretion of adrenocorticotrophic hormone (ACTH), which in turn drives glucocorticoid production by the adrenal cortex (cortisol in humans, corticosterone (CORT) in rodents). Thus, the rapid activation of the HPA axis in response to a stressor is directed by hypothalamic CRH.

In male rodents, removal of the endogenous source of androgen by gonadectomy (GDX), causes increased anxiety- and depressive-type behaviors which are reversed by systemic testosterone treatment (Slob et al., 1981; Adler et al., 1999; Frye and Seliga, 2001). Moreover, GDX males have higher stress-induced CORT and ACTH, which is also reversible via testosterone or dihydrotestosterone (DHT) treatment (Handa et al., 1994; Viau and Meaney, 2004). Gonadectomy is not accompanied by changes in pituitary sensitivity to CRH (Handa et al., 1994) or changes in levels of circulating corticosteroid binding globulin (CBG; (Lund et al., 2004b)) suggesting that the actions of androgens on HPA axis reactivity to stress are mediated centrally. Similarly, in men, aging is associated with a concomitant decline in androgen levels that may lead to a host of behavioral symptoms that overlap greatly with those of major depression (Amore, 2005).

Dysregulation of CRH signaling, in particular, plays a major role in the development of depression and anxiety (Heuser et al., 1998; Arborelius et al., 1999; Reul and Holsboer, 2002). Both receptors for CRH, CRHR1 and CRHR2, have integral roles in regulating stress sensitivity and alterations in receptor expression can be linked to behavioral disorders [for review see (Bale and Vale, 2004)]. For example, CRHR1 knockout (KO) animals show hyporesponsivity, whereas CRHR2KO animals show hyperresponsivity to a restraint stress. Furthermore, CRHR1KO animals exhibit reduced, whereas CRHR2KO animals display increased anxiety (Smith et al., 1998; Timpl et al., 1998; Bale et al., 2000; Kishimoto et al., 2000). Thus, it appears that CRHR1 and CRHR2 act in an opposing fashion, where CRHR1 is responsible for activation of the HPA axis and anxiety-related behaviors, while CRHR2 may be responsible for attenuating these responses.

Sex hormones play a key role in regulating central CRH expression [for review see (Ni and Nicholson, 2006)]. The CRH promoter contains estrogen receptor response element (ERE) half sites and at least one androgen response element (ARE) (Vamvakopoulos and Chrousos, 1993; Bao et al., 2006). In females, CRH mRNA expression in the PVN is reduced by ovariectomy, and can be restored via estrogen replacement treatment (Roy et al., 1999). Additionally, in gonadectomized males, estradiol benzoate increases restraint-induced CRH hnRNA within the PVN (Lund et al., 2004a). On the other hand, androgens appear to have an inhibitory effect on CRH expression. Basal CRH expression is lower in males than in females, and gonadectomy in males causes an increase in basal CRH expression that can be restored by dihydrotestosterone propionate (DHTP) treatment (Haas and George, 1988; Bingaman et al., 1994). Furthermore, treatment of gonadectomized males with DHTP reduces restraint-induced CRH hnRNA (Haas and George, 1988; Lund et al., 2004a).

It is currently unknown whether sex steroids regulate the expression, function, or activity of CRH receptors. Interestingly, the CRHR2 promoter contains EREs and AREs, which suggests a potential role for sex hormones in the modulation of CRHR2 expression (Catalano et al., 2003). Furthermore, in the male vole, CRHR2 binding is higher in the bed nucleus of the stria terminalis (BST) than in the female vole (Lim et al., 2005). Additionally, the expression pattern for CRHR2 in brain overlaps considerably with that of androgen receptor (AR) (Van Pett et al., 2000). Thus, the connection between androgens, HPA axis sensitivity, and stress-related disorders may be partially explained by androgen regulation of CRHR2 signaling. To explore this possibility, we examined androgen regulation of CRHR2 expression in the male rodent forebrain. The results of these studies demonstrate that androgen upregulates CRHR2 mRNA in most areas of overlap between CRHR2 and AR, and this regulatory function is mediated specifically via AR. Thus, androgen regulation of the CRHR2 gene is a potential mechanism for androgen modulation of stress and stress-related behavioral disorders.

Materials and Methods

Animals

Young adult male (300–400g), and timed pregnant female Sprague–Dawley rats were obtained from Charles River Laboratories (Wilmington, MA), caged in pairs (adult males), or individually housed (pregnant dams) in shoebox type cages in the Colorado State University laboratory animal research facility and maintained on a 12:12-h light schedule (lights on at 0700h) with *ad libitum* access to rat chow and water. All animal protocols were approved by the Animal Care and Use Committee at Colorado State University. One week following arrival, the adult male rats were gonadectomized under isoflurane anesthesia. Two weeks following gonadectomy, animals were implanted with two 2.5mm Silastic capsules (Dow Corning, Midland, MI; 0.062" ID, 0.125" OD) containing either crystalline 5 α -androstane-17 β -ol-3-one propionate (DHTP, Steraloids, Newport, RI), or nothing

(blank). One week following capsule implantation, the animals were sacrificed and brains immediately removed and either flash-frozen in isopentane at -30°C (for receptor autoradiography) or chilled on ice and immediately microdissected for subsequent RNA isolation. Trunk blood was collected into prechilled tubes containing 0.5M EDTA. For *in vitro* studies, pregnant dams were halothane anesthetized on gestational day 17 or 18 and the rat fetuses were delivered by Cesarean section. Brains were removed and placed in ice-cold CMF Ringer's-glucose solution until the hippocampi were dissected and cells were dispersed. Primary hippocampal neurons were harvested as described below.

Dihydrotestosterone radioimmunoassay

Plasma concentrations of DHT were measured via radioimmunoassay by a commercially available kit (Diagnostic Systems Laboratories, Inc., Webster, TX). Plasma obtained from each animal was run in duplicate alongside a standard curve of known DHT concentrations ranging from 0 pg to 2500 pg. The sensitivity of the assay was 4 pg/ml and intra-assay variance was 4.2%. All samples were run in a single assay to prevent interassay variation from influencing the results.

Tissue block dissection and RNA isolation

Freshly harvested tissue was dissected on ice from coronal sections, according to the atlas of Paxinos and Watson (1998). For hypothalamus, a coronal slice was made from optic chiasm rostral to the anterior edge of the mamillary bodies caudal and the hypothalamic sulcus lateral to the top of the third ventricle superior. This dissection included the entire rostral-caudal extent of the hypothalamus. The lateral septum was dissected in its entire dorsal to ventral aspect from a coronal slice made of approximately 1.0mm anterior to bregma through -0.5mm posterior to bregma. Medial septum was excluded from this dissection. Hippocampus and amygdala were dissected from the same coronal slice used for hypothalamus. Dissected tissue was placed into microcentrifuge tubes on ice containing GIT extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% N-laurel sarcosine, 0.1 M β -mercaptoethanol) and homogenized. Total RNA was isolated using previously described protocols (Chomczynski and Sacchi, 1987). The concentration of total RNA was determined using a Beckman D6530 Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) (O.D. 260/280). Absolute values of CRHR2 mRNA in the samples were determined by real time RT-PCR (lateral septum, $n=8$; hypothalamus, $n=6$; hippocampus, $n=6$; amygdala, $n=8$).

Quantitative real time RT-PCR

Total RNA (0.5 μg) was reverse transcribed in duplicate using MMLV reverse transcriptase and oligoDT primers (Invitrogen, Carlsbad, CA). Concentration of the resulting cDNA was measured using OliGreen ssDNA quantification and reagent kit (Molecular Probes, Inc., Eugene, OR). Quantification of CRHR2 mRNA was determined by real time RT-PCR using the LightCycler 2.0 system (Roche Applied Science, Indianapolis, IN) using forward (5' GGC CTC AAG GAT CAA CTA CTC A 3') and reverse (3' AAT GAT AGG GCA GGG TAT GCA 5') primer pairs specific for the rat CRHR2 coding sequence. Primer design was based on the GenBank sequence (GenBank accession no. [NM022714](#), forward primer position 482 and reverse primer position 932) and analyzed using Oligo software, ver. 6.51 (Molecular Biology Insights, Cascade, CO). A nucleotide BLAST search (NCBI Entrez Pubmed) confirmed specificity of the designed CRHR2 primers. Duplicate PCR reactions were run with each cDNA sample and included 0.5 U Platinum Taq antibody (Invitrogen, Carlsbad, CA, USA), 100 mM Tris-Cl, 0.5 U Taq polymerase, 2 μl of 10x SYBR green I (Roche Inst. Indianapolis, IN, USA), 0.5 μM of forward primer and reverse CRHR2 primer. The reaction involved an initial melting step at 95°C for 10 min followed by 45 cycles of 95°C (denature) for 10 s, 61°C (annealing) for 10 s, and 72°C (elongation) for 6 s. Samples

were assayed alongside a CRHR2 cDNA standard curve to determine the absolute CRHR2 mRNA concentration present. The CRHR2 cDNA used to prepare a standard curve was generated by RT-PCR of rat hypothalamic mRNA using the aforementioned CRHR2 primers using a Hybaid Omnigene PCR Thermocycler (Thermo Electron Corp. Waltham, MA, USA). The initial melting step was 94 °C for 2 min followed by 45 cycles of 94 °C (denature) for 45 s, 64 °C (annealing) for 30 s, and 72 °C (elongation) for 2 min. The size of the amplified CRHR2 cDNA was confirmed by 1% agarose gel electrophoresis. The cDNA concentration was calculated from an absorbance reading at wavelength 260 (OD260). The CRHR2 cDNA was diluted to a stock concentration of 10 fg/ml then subsequently serially diluted from 1 pg/ml to .001 fg/ml in PCR grade sterile water (Roche Inst. Indianapolis, IN) to generate seven working standards for each primer pair. Negative controls, where water was used in place of template, were used in all experiments. Levels of CRHR2 mRNA expression for each sample was determined by comparison to the standard curve and reported as the absolute concentration of CRHR2 (fg/ng cDNA). Specificity was confirmed via thermal melting curve analysis ($T_m = 84.8^\circ \text{C}$).

Receptor autoradiography

Whole brains were sectioned at 20 μm on a Leitz cryostat and mounted on Superfrost Plus slides (Fisher Scientific). Tissue sections were incubated in ^{125}I -sauvagine (2.67nM; PerkinElmer Inc., Waltham, MA) in buffer (50mM Tris, 120 mM NaCl, and 5 mM KCl at pH 7.6) for 60 minutes at 25°C and subsequently washed in PBS + 0.01% Triton X-100 at 4°C four times for 5 min each, dipped in distilled water, rapidly dried under cool air, and apposed to Kodak BioMax film for 3 days. Films were analyzed for mean optical density of signal using IPLab Scientific Image Processing (BD Biosciences, San Jose, CA) such that anatomically matched sections for each animal were compared. Two adjacent brain sections containing the caudal portion of the lateral septum were analyzed and averaged for each animal (n=8). Sauvagine binding was analyzed in the following subregions of the lateral septum: caudal dorsal, caudal ventral, and ventral caudal. Two adjacent brain sections from each animal were similarly analyzed and averaged for sauvagine binding in the rostral hippocampus and VMH (hippocampus, n=3; VMH, n=5 rats per treatment group). A background reading of similar area and adjacent to the region of interest was taken and subtracted from the specific signal.

Primary neuronal cell cultures

Primary hippocampal neurons were harvested from embryonic day 17–18 rat fetuses using a modified version of previously described protocols (Banker and Cowan, 1977; Brewer et al., 1993) that preferentially select for neuronal growth over glial growth (Brewer, 1997). During the dissection, brain tissue was kept on ice in a CMF Ringer's–glucose solution (0.155 M NaCl, 5.0 mM KCl, 10.0 mM Hepes, 11.0 mM d-glucose). Dissected hippocampal tissue was incubated at room temperature in a solution consisting of 0.5% trypsin in CMF for 15 min. The tissue was washed once with Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA [DMEM]) containing 15% fetal bovine serum then dissociated by trituration in the same media. Hippocampal neurons were plated in Neurobasal medium (Gibco) supplemented with $1 \times \text{B27}$ (Gibco), 100 $\mu\text{g/ml}$ penicillin–streptomycin (Gibco), 0.5 mM L-glutamine (Cellgro) and 0.025 mM L-glutamate (Sigma). Viable cell number and concentration was determined by trypan blue exclusion and counted using a hemacytometer. Primary hippocampal neurons were plated at a density of 5×10^5 cells/9.4 cm^2 . All cultures were maintained for 3 days in vitro (DIV) in plating media, and then maintained an additional 11 DIV in Neurobasal media, and supplemented with $0.1 \times \text{B27}$, 100 $\mu\text{g/ml}$ penicillin–streptomycin and 0.5 mM L-glutamine. Experiments began after 14 DIV, at a time the primary hippocampal neurons have attained an adult phenotype (Banker and Cowan, 1977). Neurons were treated with 1nM dihydrotestosterone (DHT),

1nM DHT plus 0.1 μ M flutamide, 0.1 μ M flutamide, or vehicle (0.005% EtOH in previously described media) for 48 hours. Following treatment, medium was then removed and the hippocampal neurons were washed once with PBS. Hippocampal neurons were collected into PBS by manually scraping the cells from the culture dish with a rubber policeman. Cells were pelleted by centrifugation at 3000xG for 2 min. The neurons were then resuspended and homogenized in GIT extraction buffer. Total RNA was isolated from the primary hippocampal neurons using the previously described method (Chomczynski and Sacchi, 1987). Total RNA concentration was determined using a Beckman DU 530 spectrophotometer (O.D. 260/280). Absolute values of CRHR2 mRNA in the cultures were determined by real time RT-PCR (n=10/treatment group).

Statistical Analysis

Data were analyzed via ANOVA using the Statview data analysis software (Abacus Concepts, Inc., Berkeley, CA, USA). Analysis of variance was performed for each experiment. Student's *t*-test procedures were used post hoc where appropriate. Differences were considered significant when $p < 0.05$. Data are expressed as group means $\pm$ SEM.

Results

Androgen increases CRHR2 mRNA expression

CRHR2 mRNA levels were determined in animals implanted with either a Silastic capsule containing dihydrotestosterone propionate (DHTP), or a blank capsule. Radioimmunoassay showed that plasma levels of DHT were at normal physiological levels for total androgens (Bingaman et al., 1994) in the DHTP implanted animals (2.07 ± 0.2 ng/ml versus 0.03 ± 0.01 ng/ml for controls). Quantitative real-time RT-PCR showed that animals treated with DHT had significantly higher CRHR2 mRNA levels in the lateral septum ($F_{(1,14)} = 13.411$; $p < 0.01$), hippocampus ($F_{(1,10)} = 10.119$; $p < 0.01$), and hypothalamus ($F_{(1,9)} = 37.861$; $p < 0.001$) (Figure 1A, 1B, and 1C respectively). Moreover, there was a trend for higher CRHR2 mRNA expression in the amygdala of DHTP treated animals than in controls ($F_{(1,10)} = 4.831$; $p = 0.053$). No difference was observed in the cortex.

Androgen increases CRHR2 binding capacity

To determine if androgen's effect on CRHR2 mRNA expression translates into functional receptor differences, we utilized *in vitro* autoradiography using ^{125} I-sauvagine, a high affinity CRHR ligand (Grigoriadis et al., 1996), to measure relative changes in CRHR2 binding (Figure 2A). Analysis of autoradiographs indicated no significant treatment effect in the hippocampus ($F_{(1,4)} = 0.547$; $p = 0.501$), or ventral medial hypothalamus (VMH) ($F_{(1,8)} = 0.070$; $p = 0.799$). However, ^{125} I-sauvagine binding in the lateral septum of DHTP-treated animals was significantly higher than controls ($F_{(1,14)} = 6.277$; $p < 0.05$) (Figure 2B). A subregion-specific analysis of the lateral septum revealed higher CRHR2 binding in the caudal dorsal ($F_{(1,14)} = 9.187$; $p < 0.01$) and ventral ($F_{(1,14)} = 12.763$; $p < 0.01$) divisions in DHTP-treated rats (Figure 2C). In the caudal ventral lateral septum, there was a trend for DHTP to increase ^{125} I-sauvagine binding ($F_{(1,14)} = 4.435$; $p = 0.054$).

Androgen increases CRHR2 mRNA expression in an androgen receptor dependent manner

To establish whether androgen's effect on CRHR2 expression is AR-mediated, we utilized an *in vitro* approach using primary cultures of hippocampal neurons. We chose primary hippocampal neurons since we saw significant differences in CRHR2 mRNA levels in the microdissected hippocampus of DHTP treated animals and because hippocampal pyramidal neurons express high levels of androgen receptor (Kerr et al., 1995). Treatment of hippocampal neurons with DHT for 48 h significantly increased levels of CRHR2 mRNA

compared to vehicle treated cultures ($F_{(3,36)} = 28.776$; $p < 0.0001$) (Figure 3). Co-treatment of hippocampal neurons with flutamide, an AR antagonist, blocked the effects of DHT, supporting the hypothesis that DHT acts via the androgen receptor to increase CRHR2 mRNA expression. Treatment with flutamide alone had no effect on CRHR2 mRNA levels ($F_{(3,36)} = .157$; $p = 0.786$).

Discussion

With these studies we have demonstrated that exposure to the potent androgen, dihydrotestosterone, can increase CRHR2 mRNA and binding in stress-responsive regions of the male rat brain, and that DHT acts specifically via an AR mechanism to modulate CRHR2 expression. In these studies, all animals were gonadectomized in order to remove endogenous testosterone, and animals treated with DHT were dosed via Silastic capsules which resulted in constant levels of DHT that were similar to total androgen levels found in normal male rats (Bingaman et al., 1994). For these studies, DHT, rather than T, was chosen for the replacement hormone since it is a non-aromatized androgen with a high affinity for the androgen receptor. However, it should be mentioned that in some tissues, including the brain, DHT can be metabolized to 5α -androstane- $3\beta,17\beta$ -diol, a weak androgen that has been reported to bind and activate estrogen receptor beta (ER β ; for review see (Handa et al., 2007)). Such data indicate the possibility that the DHT effects that we report here may be mediated by ER β , however, the fact that flutamide was capable of blocking the actions of DHT *in vitro* indicates an AR-mediated mechanism.

Sex differences in stress reactivity and stress-related disease vulnerability may be, in part, due to the influence of gonadal hormones. Evidence from gonadectomized and hormone-replaced male animals suggests that androgens have an inhibitory effect on HPA activity (Handa et al., 1994; Viau and Meaney, 2004). Treatment with DHT decreases stress-responsive corticosterone and ACTH release, decreases stress-induced cellular activation in the hypothalamus, and decreases CRH levels in the hypothalamus (Bingaman et al., 1994; Lund et al., 2004a; Seale et al., 2004). Administration of DHT also leads to reductions in anxiety- and depressive-like behaviors (Frye and Wawrzycki, 2003; Edinger and Frye, 2005). The mechanism for androgens' effects on stress physiology and behavior is not fully known. However, given that CRHR2 plays a role in reducing anxiety- and depression-like behaviors as well as HPA activity (Bale et al., 2000; Kishimoto et al., 2000; Bale and Vale, 2003; Isogawa et al., 2003), we have hypothesized that androgen may work in part, through the regulation of CRHR2 expression. In these studies, we have found that DHT increases CRHR2 binding and mRNA in brain regions involved in modulating stress responses.

Our studies demonstrate that within the lateral septum, CRHR2 mRNA expression and receptor binding is increased by DHT treatment. CRHR2 binding was increased in specific subregions of the lateral septum. There is substantial evidence for the involvement of the lateral septum in regulating stress responses, coping behaviors, and antidepressant efficacy. Moreover, sex differences in the hormonal and behavioral responses to stress may involve dichotomies in this brain region. For example, exposure to stressful stimuli has been shown to activate lateral septal neurons (Duncan et al., 1993; Campeau and Watson, 1997). Stress-induced activation of lateral septal neurons is decreased in animals that exhibit behavioral learned helplessness whereas chronic antidepressant treatment increases activation, suggesting that lateral septal activity may be important in coping responses to stress (Steciuk et al., 1999; Lino-de-Oliveira et al., 2006). Furthermore, the caudal dorsal subregion of the lateral septum receives dense inputs from the hippocampus, dorsal raphe, locus coeruleus, and ventral tegmental area, all of which are brain areas that synthesize monoamine neurotransmitters whose synthesis or release are believed to be dysregulated in depression (Duman et al., 1997; Risold and Swanson, 1997a, b). The reported sex difference in the

prevalence of depression and the corresponding change in stress responsivity may involve the effects of gonadal hormones on lateral septum neurochemistry. In our studies, CRHR2 binding was increased in specific subregions, namely the caudal dorsal and the ventral portions. The caudal dorsal subregion receives dense inputs from monoaminergic brainstem nuclei, suggesting that this region may be involved in depression- and anxiety-like behaviors (Risold and Swanson, 1997a). The largest increase in CRHR2 binding occurred in the ventral subregion, which is the only septal region with bidirectional connections to the PVN and therefore may play a role in HPA axis activity (Risold and Swanson, 1997a). This region also expresses high levels of AR mRNA, making it plausible that DHT binds to the AR and increases CRHR2 in the same cells. Furthermore, increased CRHR2 in these lateral septum areas by androgens may lead to decreased stress sensitivity and increased coping behaviors. Thus, these data provide a possible mechanism by which androgens may have a protective effect on stress-related disease susceptibility in males.

Our results also show that *in vivo*, DHT treatment leads to an increase in CRHR2 mRNA within block-dissected hypothalamus, but this change was not reflected by changes in CRHR2 binding in the ventromedial hypothalamus (VMH), a region with high expression of both CRHR2 and AR. The lack of correlation between mRNA and binding could be due to differences in other hypothalamic areas included in the dissection used for measuring mRNA levels. Alternatively, it is possible that changes in mRNA do not correspond well with changes in receptor protein. Nonetheless, in the VMH, activation of CRHR2 is predominantly known to produce an anorexic effect and CRHR2 mRNA expression is highly correlated with leptin levels (Makino et al., 1998; Nishiyama et al., 1999). In addition to its known function in satiety, the VMH may play a role in controlling basal activity of the HPA axis (Suevaru et al., 1995). Whether this function is mediated by CRHR2 is unknown, but CRHR2 expression in the VMH is altered following acute and chronic stress and glucocorticoid administration (Makino et al., 1999; Chen et al., 2005). Although our data cannot rule out a direct effect of androgen on hypothalamic CRHR2 mRNA levels, the possibility remains that the effect of androgen may be secondarily related to HPA axis feedback or to the changes in other hormones such as leptin.

Within the hippocampus, another AR-rich brain region (Simerly et al., 1990; Van Pett et al., 2000), we also observed a DHT-dependent increase in CRHR2 mRNA. Again, these changes in mRNA levels did not correspond with changes in sauvagine binding, which may be due to the expression of CRHR1 and R2 in the rat hippocampus, both of which have an affinity for sauvagine (Grigoriadis et al., 1996; Van Pett et al., 2000). Furthermore, there may be differences in sensitivity between quantitative real-time RTPCR and *in vitro* receptor autoradiography and although every precaution was taken, small portions of choroid plexus, a tissue with high CRHR2 expression, may have been included in the hippocampal block dissection, thus washing out any effect of androgen treatment. This would not have affected our results using primary hippocampal neurons (see below). Importantly, discrepancies between mRNA levels and receptor binding may also be attributed to region specific changes in mRNA stability or expression of cell-surface receptor protein. For example, sex hormones can autoregulate their own receptor mRNA stability. Both androgens and estrogens can increase or decrease AR and ER mRNA stability, respectively (Ing, 2005). It is plausible that androgens may also alter CRHR2 mRNA stability. Furthermore, cell-surface CRHR2 β levels decrease significantly with CRH or urocortin II treatment (Markovic et al., 2008). Androgens are known to alter CRH peptide levels, and this may in turn modify cell-surface expression of CRHR2.

The role of CRHR2 within the hippocampus has not yet been described. In general, the hippocampus does play an integral role in regulating the neuroendocrine response to stress and in particular, psychogenic stressors [for reviews please see (Herman et al., 2003;

Herman and Mueller, 2006)]. The hippocampus is critical in mediating glucocorticoid-dependent negative feedback and thus helps to control the duration of the stress response (Jacobson and Sapolsky, 1991). Hippocampal influences on the HPA axis are indirect via a trans-synaptic mechanism where glutamatergic projections from the hippocampus target GABAergic neurons in the amygdala, lateral septum, bed nucleus of the stria terminalis, and prefrontal cortex, which then project to and alter secretion of CRH and AVP from the PVN (Herman et al., 2003; Herman and Mueller, 2006). Furthermore, the hippocampus is not directly involved in mediating the onset or initial magnitude of the stress response, which has been shown to be higher in females than males. Thus, androgen's effect on HPA axis function may not be mediated by regulation of CRHR2 in the hippocampus, but rather through other brain regions primarily involved in the activation of the axis.

In order to determine whether androgen's effect on CRHR2 expression is AR-mediated, we utilized an *in vitro* approach. Because in our initial study, we saw significant increases in hippocampal CRHR2 mRNA following DHT treatment and hippocampal pyramidal neurons express high levels of AR, we exploited these findings to determine if DHT acts directly on hippocampal neurons through an AR dependent mechanism to regulate CRHR2 mRNA. That hippocampal pyramidal neuron cultures highly express AR (87% $\pm$ 3.4 of total cells are AR-immunoreactive), makes them a useful tool for examining the effect of androgen on cellular processes (Foradori et al., 2007). Our studies confirm that androgen increases CRHR2 mRNA expression, an effect that is blocked by the AR antagonist, flutamide. Thus, our data supports the concept that the DHT effects on CRHR2 are AR-mediated.

In summary, the studies described here indicate that androgen modulates CRHR2 mRNA in specific stress-responsive brain regions. Further, these changes in CRHR2 mRNA correspond with changes in CRHR2 binding within the lateral septum. It appears that this regulation is mediated specifically through androgen receptor activation, at least in the hippocampus. Thus, the CRHR2 gene may be a target for AR-mediated regulation and these data suggest a potential mechanism for androgen modulation of stress and stress-related disorders.

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Fig. 1.

Androgen treatment of gonadectomized male rats increases levels of CRHR2 mRNA in block dissected brain regions. CRHR2 mRNA levels are shown in lateral septum (A), hypothalamus (B), and hippocampus (C) as measured by real-time RTPCR. * indicates significant differences ($p < 0.01$) versus control group ($n=6-8$). Each bar represents the mean $\pm$ SEM. DHTP, dihydrotestosterone propionate.

Fig. 2.

(A) Photomicrographs of representative autoradiograms showing *in vitro* ^{125}I -sauvagine binding. Outlined areas were examined for quantitation by densitometry. (B) Androgen treatment of gonadectomized males significantly increased ^{125}I -sauvagine binding in the lateral septum. (C) Androgen treatment of gonadectomized males significantly increased ^{125}I -sauvagine binding within the caudal dorsal and ventral caudal subregions of the lateral septum. * indicates significant differences ($p < 0.05$) versus control group. Each bar represents the mean $\pm$ SEM (lateral septum, $n=8$; hippocampus, $n=3$; VMH, $n=5$ per treatment group). LS, lateral septum; Hippo, hippocampus; VMH, ventral medial hypothalamus; DHTP, dihydrotestosterone propionate; cc, corpus callosum; cd, caudal dorsal; cv, caudal ventral; vc, ventral caudal; MS, medial septum; ac, anterior commissure; 3V, third ventricle; Arc, arcuate nucleus.

Fig. 3.

Androgen treatment of primary hippocampal neurons in culture increases CRHR2 mRNA. Concomitant treatment of DHT with flutamide blocked the effects of DHT. CRHR2 mRNA levels were measured by real-time RTPCR. * indicates those groups that were significantly different from control ($p < 0.001$, $n=10$). Each bar represents the mean $\pm$ SEM. DHT, dihydrotestosterone; flu, flutamide.