Published in final edited form as:

Brain Res. 2013 September 5; 1529: 74–82. doi:10.1016/j.brainres.2013.07.021.

CENTRAL 5-ALPHA REDUCTION OF TESTOSTERONE IS REQUIRED FOR TESTOSTERONE'S INHIBITION OF THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS RESPONSE TO RESTRAINT STRESS IN ADULT MALE RATS

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Abstract

In rodents, the hypothalamo-pituitary-adrenal (HPA) axis is controlled by a precise regulatory mechanism that is influenced by circulating gonadal and adrenal hormones. In males, gonadectomy increases the adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) response to stressors, and androgen replacement returns the response to that of the intact male. Testosterone (T) actions in regulating HPA activity may be through aromatization to estradiol, or by 5 α -reduction to the more potent androgen, dihydrotestosterone (DHT). To determine if the latter pathway is involved, we assessed the function of the HPA axis response to restraint stress following hormone treatments, or after peripheral or central treatment with the 5 α -reductase inhibitor, finasteride. Initially, we examined the timecourse whereby gonadectomy alters the CORT response to restraint stress. Enhanced CORT responses were evident within 48hrs following gonadectomy. Correspondingly, treatment of intact male rats with the 5 α -reductase inhibitor, finasteride, for 48 hrs, enhanced the CORT and ACTH response to restraint stress. Peripheral injections of gonadectomized male rats with DHT or T for 48 hrs reduced the ACTH and CORT response to restraint stress. The effects of T, but not DHT, could be blocked by the third ventricle administration of finasteride prior to stress application. These data indicate that the actions of T in modulating HPA axis activity involve 5 α -reductase within the central nervous system. These results further our understanding of how T acts to modulate the neuroendocrine stress responses and indicate that 5 α reduction to DHT is a necessary step for T action.

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Keywords

androgen; stress; HPA axis; hypothalamus; 5-alpha reductase; testosterone; dihydrotestosterone

1. INTRODUCTION

In rodents, adrenal corticosterone (CORT) secretion is controlled by the activity of a neuroendocrine axis that involves the hypothalamus, the anterior pituitary and the adrenal gland. This hypothalamo-pituitary-adrenal (HPA) axis represents a cascade of neural and humoral signals driven by both the circadian pacemaker as well as the environment. Changing environmental conditions or perceived threats to homeostasis activate the HPA axis by funneling information through neurons located in the paraventricular nucleus of the hypothalamus (PVN), a critical brain region that integrates positive and negative inputs to achieve a proper endocrine output. Central to HPA axis regulation are selective neurons in the parvocellular part of the PVN that contain corticotropin-releasing hormone (CRH).

Evidence that sex steroid hormones can interact with the regulatory elements of the HPA axis comes from studies showing that gonadectomy of both males and females alters the reactivity of the HPA axis (Handa et al, 1994a). Depending on dose, timing, or age of treatment, estradiol (E) treatment of gonadectomized animals can either enhance or inhibit (Serova et al., 2010, Evuarherhe et al, 2009, Weiser and Handa, 2009, Young et al., 2001) HPA activity, whereas androgen treatment has consistently been shown to inhibit HPA reactivity in rodents, monkeys and humans (Kalil et al, 2013, Williamson and Viau, 2008, Handa et al, 1994b, Toufexis and Wilson, 2012, Rubinow et al, 2005). To date, the mechanism(s) by which T and E act to influence HPA function have not been completely resolved. Evidence for T and E acting in part at the adrenal gland (Kitay 1965), anterior pituitary (Coyne and Kitay, 1969, 1971, Viau and Meaney, 2004) and hypothalamus (Viau and Meaney, 1996; Viau et al., 2002, Handa et al., 1994b) exists. Furthermore, recent studies have also suggested that the actions of E can be differentially mediated by estrogen receptor (ER) alpha and beta. Whereas ERalpha agonists increase the CORT response to stress, ERbeta agonists have been shown to inhibit the response (Lund et al, 2006, Weiser et al., 2009).

Although androgens suppress HPA axis reactivity (Handa et al., 1994b) and reduce CRH-immunoreactivity (ir) in the PVN (Bingaman et al., 1994a), androgen receptors (ARs) are not expressed in neuroendocrine neurons within the PVN (Bingham et al, 2006, Bingaman et al. 1994b). PVN neurons that express AR are found in the dorsal and the ventral medial parvocellular parts of the PVN, which are non-neuroendocrine neurons that project to spinal cord and brainstem pre-autonomic nuclei (Bingham et al. 2006). Consequently, it has been hypothesized that androgens regulate PVN neuropeptide expression and secretion transsynaptically. In support of this, implantation of testosterone (T) into the medial preoptic area (MPOA) and bed nucleus of the stria terminalis (BnST), brain regions that provide afferent input to the PVN, can reduce the CORT response to acute stress (Williamson and Viau, 2008, Viau, 2002; Viau and Meaney, 1996). Further, retrograde tracing studies show that AR-ir can be specifically found in neurons of the BnST, MPOA and anteroventral periventricular nucleus that project to the PVN (Williamson and Viau, 2007, Suzuki et al, 2001). By contrast, these areas may not be the only brain sites mediating the inhibitory effect of androgens on HPA reactivity since stereotaxic application of dihydrotestosterone (DHT) to a region just above the PVN (to prevent mechanical disruption of the PVN) was as effective as peripherally administered DHT in inhibiting HPA function (Lund et al, 2006). Such results raise the possibility that androgens may work at multiple brain sites to regulate the gain of the HPA axis.

It is now well established that T, the principle circulating androgen in males, can be intracellularly converted to E in brain tissue by the aromatase enzyme (Roselli et al., 1997), or to DHT by the 5- α reductase enzyme (5 α R; Lephart 1993). Although both T and DHT bind the AR with high affinity, DHT has classically been used in studies of androgen action as it is considered to be a more potent and selective agonist for ARs and is not a substrate for aromatization to estradiol. However, whether central 5- α reduction of T to DHT is a necessary step for the inhibitory effects of androgens on HPA function has not been determined. Consequently, in these studies, we have examined the role of the central 5 α R enzyme in the androgenic modulation of the HPA reactivity to restraint stress by using centrally or peripherally administered finasteride, an inhibitor of 5 α R enzyme activity. In addition, as a prelude to this exploration, we also characterized the time course of changes in HPA stress responsiveness following gonadectomy of male rats.

2. RESULTS

2.1. Time course of changes in stress-reactive levels of CORT following gonadectomy of male rats

Restraint stress-responsive levels of plasma CORT were measured at 1, 2, 3, 5 and 7 days following gonadectomy of adult male rats. The effect of gonadectomy over days was first analyzed by comparing the CORT responses to stress using a 2 way ANOVA with gonadal state (GDX, Sham) and days post-gonadectomy as main factors. The analysis yielded a main effect of gonadectomy ($F_{1,88} = 10.64$; $p < 0.002$). As shown in Figure 1, a one-way ANOVA including intact animals (0 days GDX) and all times following GDX revealed increased levels of CORT at day 2, 3, 5 and 7 days following gonadectomy compared to those of intact animals. A one-way follow-up ANOVA in sham animals showed no significant increase over days following sham surgery compared to intact animals, indicating that surgery alone is an unlikely contributor to changes in stress-induced CORT observed in GDX males.

2.2. Effects of peripheral finasteride treatment on stress-reactive CORT and ACTH secretion

To determine if the 5- α reduction of T to DHT is an essential part of the androgenic inhibition of the HPA response to restraint stress, we next examined the ability of two doses of the 5- α reductase inhibitor, finasteride, to alter post-restraint stress levels of CORT and ACTH. Two-way ANOVA (stress condition $\times$ finasteride condition) revealed a significant main effect of stress on CORT ($F_{1,34} = 335.1$, $p < 0.01$) and ACTH ($F_{1,33} = 17.7$, $p < 0.05$). There was also a significant main effect of finasteride treatment on plasma CORT ($F_{2,34} = 3.53$, $p < 0.05$) as well as a significant interaction effect ($F_{2,34} = 3.25$, $p = 0.05$) on plasma CORT. Specifically, finasteride treatment, at both doses, significantly increased post-stress plasma CORT levels (Figure 2; one-way ANOVA: $F_{2,19} = 4.11$, $p = 0.03$), whereas there was no effect of finasteride on baseline (non-stress) CORT levels.

2.3. Effects of peripheral finasteride treatment on circulating T levels

Plasma T levels were measured following peripheral finasteride treatment in order to verify that the changes observed occurred independently from changes in endogenous circulating T levels (Table 1). One-way ANOVA showed no significant changes in plasma T levels in intact male rats following either dose of finasteride.

2.4. Effects of T and DHT on stress-reactive CORT and ACTH secretion

To determine if the changes in stress-reactive CORT secretion observed in experiment 1 were due to the removal of testicular androgens, we treated gonadectomized male rats with DHTP and TP, and examined pre- and post-stress levels of ACTH and CORT. As shown in

Figure 3, when examined 2 days after gonadectomy, both DHT and T treatment reduced post-stress levels of plasma CORT and ACTH but had no effect on pre-stress levels of either hormone. Two way ANOVA revealed significant main effects of stress condition on plasma CORT ($F_{1,43}=295$, $P<0.0001$) as well as a treatment by stress interaction ($F_{2,43}=4.49$, $p=0.017$). Similarly, for ACTH levels, there was a significant main effect of stress condition ($F_{1,43}=97.9$, $p<0.0001$) and a hormone treatment by stress condition interaction ($F_{2,43}=4.0$, $p=0.026$).

2.5. Effects of central finasteride treatment on the effects of peripheral T or DHT administration

The results of experiment 2 suggested that blocking the conversion of T to DHT could prevent the inhibitory effects of endogenous androgens on HPA axis reactivity to restraint stress. We addressed the possibility that this inhibitory effect of T is mediated centrally by the reduction of T to DHT. To assess this, gonadectomized male rats were treated peripherally with TP or DHTP, and then received daily infusions of finasteride into the 3V to inhibit the central reduction of T to DHT by 5 α -reductase.

For CORT values, three-way ANOVA (stress condition $\times$ finasteride treatment $\times$ hormone treatment) revealed significant main effects of stress condition ($F_{1,72}=301.4$, $p<0.0001$), finasteride treatment ($F_{1,72}=8.81$, $p=0.04$) and hormone treatment ($F_{2,72}=3.41$, $p=0.03$) as well as a trend for a finasteride treatment $\times$ stress condition interaction ($p=0.07$). Multi-way ANOVA of data for plasma ACTH using the same factors showed a significant effect of stress condition ($F_{1,72}=50.61$, $p<0.0001$), finasteride treatment ($F_{1,72}=5.69$, $p=0.02$) and a strong trend for a finasteride $\times$ stress interaction ($p=0.055$). Further, two-way ANOVA across non-stress or post-stress values (hormone $\times$ finasteride) revealed a significant effect of 3v infusion of finasteride on non-stress levels of CORT ($p=0.012$), but not on post-stress levels, whereas there was a significant effect of finasteride on post-stress ACTH levels ($p=0.028$). Moreover, one-way ANOVA of post-stress CORT levels revealed significant group effects where T and DHT treatment inhibited CORT secretion in vehicle-treated animals only. Following central 3V finasteride infusion, CORT levels were increased in the T-group relative to the vehicle-group (Figure 4), and the T group was no longer significantly reduced compared with the stressed, vehicle-treated animals. There was no effect of 3V infusion of finasteride on CORT levels of DHT-treated animals, although this group (finasteride/DHT) was also no longer different compared to the 3V finasteride/Veh group. *Post hoc* analyses showed no significant effects of 3V infusion of finasteride on plasma ACTH levels of individual groups.

3. DISCUSSION

In these studies, we have examined the biochemical pathway by which T acts to regulate the response of the HPA axis to restraint stress. Our studies have demonstrated that the 5- α reduction of T is a requisite step for HPA axis modulation by T. Inhibition of 5 α R by peripheral administration of finasteride increased the ACTH and CORT response to restraint stress in intact male rats. Moreover, central administration of finasteride prevented the inhibitory effects of TP treatment on HPA responsivity. Taken together, these findings implicate central 5- α reduction as an important player in the control of stress-responsive neuroendocrine function by gonadal steroids.

In rodents, basal and stress-induced adrenal GC secretion is greater in females than in males (Kitay 1963, Critchlow et al, 1963, Handa et al, 1994a) and stimulation of the hypophysiotrophic stress response and PVN neuron activation are correspondingly higher in females (Larkin et al, 2010, Seale et al, 2004, Viau et al, 2005). These sex differences arise due to differences in the adult gonadal steroid environment and are attributed to changes in

E2 that occur across the estrous cycle of females (Iwasaki-Sekino et al, 2009, Viau and Meaney 1991), or due to circulating T levels in males (Viau and Meaney 2004, Handa et al, 1994b). Because T can serve a dual purpose, as a substrate for estradiol synthesis, or as a precursor for DHT which is a potent ligand for the androgen receptor, it is important to understand the mechanisms by which T might act to inhibit HPA function.

Previous studies have shown that treatment of gonadectomized male rats with T or DHT can reduce the ACTH and CORT response to an acute stressor (Viau and Meaney, 1996, Handa et al, 1994b; Lund et al, 2004). This effect has been shown in multiple species including primates (Toufexis and Wilson, 2012). In order to demonstrate that the elevation in stress-reactive CORT secretion following orchidectomy occurs within a timeframe that was amenable to study by pharmacological blockade of 5 α R, we first identified the time course for the gonadectomy effect. Our studies show that enhanced stress-responsive CORT secretion was present within two days of gonadectomy and that this effect persisted for at least seven days. Thus, in the absence of gonadal steroids, HPA axis reactivity to acute restraint stress was elevated. In support of previous studies, which have demonstrated an inhibitory effect of T or DHT treatment on HPA function (Lund et al, 2004, Handa et al, 1994b), we also showed that T or DHT treatment for 48 hrs was sufficient to prevent the gonadectomy-induced increased secretion of ACTH and CORT secretion in response to restraint stress, and that the effects of these two hormone treatments were equivalent in potency. These data provide further evidence that circulating androgens can profoundly inhibit neuroendocrine stress responses in males. Therefore, in subsequent studies, we utilized the 48 h time point as the minimal time period during which we predicted that manipulation of androgen metabolism would result in changes in HPA axis reactivity to restraint stress.

Evidence that the 5- α reduction of T to DHT is involved in the actions of androgen on HPA axis stress reactivity was demonstrated by the elevations in the ACTH and CORT response to stress that occurred in intact male rats following peripheral treatment with finasteride. Finasteride treatment enhanced stress-reactive ACTH and CORT in intact male rats in a similar fashion to that observed following castration. Although CORT secretion was elevated following both doses of finasteride, ACTH increases were marginal and noted only in the group receiving the highest finasteride dose. This lack of effect of finasteride on ACTH secretion is perhaps due to the timing of blood collection since ACTH elevations occur very rapidly following the onset of stress and may no longer be maximal at the time that animals were sacrificed in this study (20 min after the onset of restraint). This may also be reflected by the greater variation seen in the ACTH measurements. Furthermore, in this study, we observed that finasteride treatment did not alter circulating levels of T, thus ruling out changes in the androgen substrate as causative to the effects on HPA axis reactivity.

The 5- α reductase enzyme can also convert progesterone to the neurobiologically active metabolite (Feder and Marrone 1977, Kubli-Garfias and Whalen 1977), 5 α -dihydroprogesterone (5 α DHP). Thus, it remains possible that inhibition of 5 α DHP synthesis could be in part responsible for the changes in HPA axis reactivity that we describe in these studies. However, previous studies examining the effects of progesterone treatment on CORT responses to restraint stress failed to show a significant effect of progesterone alone on HPA reactivity (Carey et al, 1995), thereby suggesting that the metabolism of progesterone to 5 α DHP is not involved. Therefore, available evidence suggests that the peripheral effects of finasteride on the HPA axis describe in this study are not due to inhibition of progesterone metabolism.

To address whether the 5- α reduction of T to DHT occurs in the brain, we also delivered finasteride centrally and examined the effects on subsequent T or DHT modulation of the

HPA axis. Our results show that, when delivered to the 3V, finasteride can block the actions of T on CORT secretion, thus implicating a role for central 5-alpha reduction of T in HPA reactivity to stress. Unfortunately, the hypothalamic site for reduction of T to DHT cannot be determined by these results. Studies examining the distribution of 5αR in the hypothalamus have demonstrated highest levels of activity is found in the lateral preoptic area and in the lateral hypothalamus (Melcangi et al, 1985), suggesting that reduction of T may occur locally within the hypothalamus. Moreover, immunocytochemical studies have localized 5αR protein to glia and ependymal cells (Pelletier et al, 1994). Thus, a possible scenario is that T metabolism to DHT occurs in glial cells that then deliver DHT to neighboring neurons. In these studies, we did not observe an effect of finasteride on DHT actions, and this might be expected given that DHT is downstream of the actions of finasteride. Finasteride treatment did not alter ACTH levels in these studies, although in all groups there was a trend to elevated levels of ACTH following stress. The lack of specific treatment effects of finasteride may be due to the high individual sample variance in the ACTH assay used for these studies.

The site of androgen action in inhibiting HPA axis activity is currently under investigation. Our previous studies have demonstrated that T and DHT can inhibit HPA reactivity to stress when they are administered near the PVN (Lund et al, 2006), implicating an action upon neurons within or adjacent to neuroendocrine neurons of the PVN. Other studies have administered T and DHT directly into the preoptic area and BnST and reported an inhibition of HPA axis activity (Viau and Meaney, 1996, Williamson and Viau, 2007). Thus, it is possible that there are multiple sites of androgen action which can affect neuroendocrine neurons of the PVN either through direct or transsynaptic actions.

The ability of T or DHT to inhibit the HPA axis strongly suggests an involvement of the androgen receptor. Previous studies have demonstrated that androgen receptors are found in areas such as the MPOA and BnST that project to the PVN and provide inhibitory control of the HPA axis (Williamson and Viau, 2007). However, while placement of T and DHT adjacent to the PVN can also inhibit HPA axis activity, androgen receptors are not found in neuroendocrine neurons of the PVN (Bingham et al, 2006, Bingham et al, 1994b). This suggests the possibility that several receptor-signaling pathways are involved. Studies by Lund et al. (2006) have demonstrated that peripheral treatment with androgen receptor antagonists cannot block the central actions of T and DHT on HPA reactivity, whereas an estrogen receptor antagonist was effective. These data raise the intriguing possibility that DHT can be further metabolized to compounds that bind estrogen receptor beta to further mediate its actions (Handa et al, 2011). In support of this hypothesis, 5-alpha androstane 3β, 17β diol (3β-diol), a direct metabolite of DHT has been shown to bind ERbeta (Kuiper et al, 1997, Weihua et al, 2002), and can also inhibit HPA activity in a manner consistent with the actions of DHT (Lund et al, 2006). Consequently, two parallel pathways for androgen inhibition of HPA activity are suggested: one involves the direct activation of AR containing neurons in the MPOA and/or BnST by T or DHT; a second involves the further conversion of DHT to an ERbeta binding metabolite (3α-diol), which can act on neurons within the PVN to inhibit HPA function. As these two mechanisms of action employ different receptor types, this then allows for regional specificity of T-mediated actions based on the distribution density of AR vs. ERbeta.

In summary, the results of these studies have further characterized the actions of androgens in modulating HPA axis responsiveness to stressors. These studies have determined that central conversion of T to DHT is a necessary pathway for the inhibitory actions of T. It remains to be determined whether further metabolism of DHT to other steroid molecules is involved.

4. Experimental Procedures

4.1. Animals

Adult male Sprague–Dawley rats (250–300 g body weight, Charles River Laboratories, Wilmington, MA) were received at the Laboratory Animal Facility at Colorado State University and allowed to acclimate for at least one week after arrival. Animals were housed at 22° C under a 12:12 h light/dark cycle with lights on at 0600 h. Animals were provided *ad libitum* access to water and standard rat chow. All animals were housed in pairs except for those receiving stereotaxic surgery. Following stereotaxic surgery, animals were single housed. All animal protocols followed NIH guidelines and were approved by the Animal Care and Use Committee at Colorado State University.

4.2. Experimental design

Experiment 1. Time course of changes in stress-induced ACTH and CORT levels following gonadectomy of male rats—This study was used to establish the minimal time required following gonadectomy to allow visualization of castration-dependent increases in the stress-reactivity of the HPA axis. This data was necessary for later comparison to studies employing finasteride treatment. Adult male Sprague–Dawley rats were either gonadectomized, underwent a sham gonadectomy, or were left gonad-intact and were then returned to their home cage. At 1, 2, 3, 5 or 7 days following surgery, half of the animals were subjected to a 20 minute restraint stress in the morning (0900–1200 h) using plexiglass restraint tubes (Stoelting Co., Wood Dale, IL), while the other half of the animals remained undisturbed in their home cages until the time of euthanasia. All rats were killed by rapid decapitation immediately after the termination of restraint stress or upon removal from their home cage. Trunk blood was collected into pre-chilled tubes containing EDTA (ethylenediaminetetraacetic acid; 100 µl, 0.1 M) on ice (4 °C). Plasma samples were collected after centrifugation at 5000 rpm at 4 °C and were stored at –20 °C until assayed for plasma CORT.

4.2.1. Experiment 2. Effects of peripheral finasteride treatment on post stress plasma CORT and ACTH levels in intact male rats—This experiment examined the role of 5 alpha reduction of T to DHT on HPA reactivity to stress by assessing whether peripherally delivered finasteride, a 5alpha-reductase inhibitor has a similar effect on HPA axis reactivity as that seen following gonadectomy (expt. 1). For this study, all animals were left intact. Animals received a peripheral injection of finasteride (FIN, 1 or 10 mg/kg in safflower oil vehicle; Tocris Bioscience, Bristol, UK; n= 13 - 14) or VEH (sesame oil; n=13). Two days later, groups were divided and half of the animals in each group were subjected to 20 min of restraint stress as described above between 0900 and 1200h, when CORT levels are at their diurnal nadir, while the other half remained undisturbed in their home cages until time of euthanasia. All rats were euthanized immediately upon termination of restraint stress or upon removal from their home cage. Trunk blood was collected into tubes containing EDTA (100 l, 0.1 M) on ice (4 °C) and plasma was collected and stored at –20 °C for later measurements of plasma ACTH, CORT and T by radioimmunoassay.

4.2.2. Experiment 3. Effects of TP and DHTP on stress-reactive CORT and ACTH secretion—This study compared the ability of the aromatizable androgen, T, and the non-aromatizable androgen, DHT, to inhibit the stress-induced ACTH and CORT response of gonadectomized male rat, when given for two days prior to restraint stress. For this study, 45 males rats were gonadectomized and allowed to recover from surgery for 4 days. On day 5 and 6, all rats received a daily s.c. injection of either TP or DHTP (1 mg/kg body weight in safflower vehicle). Control animals received oil vehicle alone. On day 7, animals (n=8/group) were subjected to a 20 min restraint stress as described above, while

control animals (n=7/group) remained unstressed and in their home cage until time of euthanasia. All collections were performed in the morning, between 0900 and 1200 h. Following decapitation, trunk blood was collected into polypropylene tubes containing 100 μ l 0.1M EDTA. Plasma was collected and stored at -80°C for later assessment of plasma ACTH and CORT concentrations via radioimmunoassays.

Experiment 4: Effects of central finasteride administration on the HPA axis stress response after peripheral administration of TP and DHTP—In this experiment, the conversion of peripherally delivered TP or DHTP was blocked via central, infusion of finasteride into the 3V, to determine if central 5 α reduction was a necessary pathway underlying androgen mediated restraint of HPA axis reactivity. To reduce endogenous levels of T, all animals underwent gonadectomy four days prior to cannulation surgery. All animals were then stereotactically implanted with guide cannulae directed at the 3V, followed by four days of handling to habituate them to subsequent infusion procedures. On days 5 and 6, all rats received a s.c. injection of either TP or DHTP (1 mg/kg in safflower oil), or vehicle, between 0700 and 0900 h. Between 1000 and 1200 h on days 5 and 6, rats also received an icv infusion of vehicle (artificial CSF), or finasteride (10 μ g/kg; both delivered in a volume of 2 μ l over the course of 2 min). On day 7, half of the animals in each group were subjected to a 20 min restraint stress as described above, while the other half of each group served as unstressed control animals. All rats were euthanized immediately after termination of restraint stress. Trunk blood was collected into tubes containing EDTA (100 μ l, 0.1 M) on ice (4°C) and plasma was stored at -80°C for later measurements of plasma ACTH and CORT. This study resulted in six treatment groups (number in parenthesis indicates n/group): Veh/Veh (14), Veh/TP (11), Veh/DHTP (14), finasteride/Veh (13), finasteride/TP (11) and finasteride/DHTP (14) which were then divided into stressed and unstressed conditions.

4.3. Surgical procedures

Gonadectomy—When indicated by study design, one week following arrival in the Colorado State University laboratory animal facility, animals were bilaterally gonadectomized under deep isoflurane anesthesia. Following gonadectomy, animals were allowed 4 days to recover if they were scheduled for stereotaxic surgery.

Stereotaxic surgery & Third ventricle (3V) infusion—All stereotaxic cannulation surgeries were performed under anesthesia using a ketamine/xylazine/acepromazine cocktail (31.25, 6.25, and 1.25 mg/kg, respectively, subcutaneous, s.c.). For stereotaxic surgery, the incisor bar was set at 3.3mm below horizontal and a midsagittal, skin incision was made through the scalp. The skull surface was cleared of connective tissue and vasculature using sterile cotton swabs and was dried with 0.3% hydrogen peroxide in 0.9% sterile saline. Three small stainless steel screws (Small Parts Inc., Miami Lakes, FL, USA) were placed around the burr hole for the cannula to stabilize the guide cannula once acrylic was applied. A 22 gauge guide cannula (Plastics One Inc., Roanoke, VA) was lowered into the third ventricle at the following coordinates with reference to bregma: 0.00 mm (lateral), 0.8 mm (anterior/posterior), and 6.0 mm (depth) and was secured using dental acrylic (Stoelting, Wood Dale, IL, USA), with the stainless steel screws anchoring the placement. A stainless steel dummy cannula (28 gauge, Plastics One Inc.) was then inserted into the guide cannula to seal the opening and to prevent contamination until time of infusion. During a recovery period of four days the animals received daily subcutaneous injections of antibiotic (Baytril, 5 mg/kg BW; Bayer, Pittsburgh, PA) followed by two minutes of handling to prepare them for subsequent treatment injections. Correct cannula placement was verified visually during cryosectioning of each brain.

For infusion of either finasteride or VEH (artificial cerebrospinal fluid; aCSF, Tocris Bioscience, Bristol, UK), into the 3V, the dummy cannula was removed and a sterilized 28ga internal infusion cannula (Plastics One) with a 1.0 mm protrusion past the end of the guide cannula was inserted. The respective volume of each treatment solution (2 μ l over the course of 2 min) was injected manually using a 10 μ l Hamilton precision syringe (Hamilton Company, Reno, NV,) connected to the internal injection needle via polyethylene medical grade tubing. Between treatment days, the guide cannula was re-capped with the dummy cannula which was manipulated daily and re-tightened as needed during post-operative checks to prevent clogging or removal by the subject.

4.4. Drugs

In experiment 2, the 5- α reductase inhibitor finasteride (Tocris Bioscience) was delivered s.c. at doses of 1 mg/kg or 10 mg/kg in safflower oil. These doses bound those used in previous studies (Bandivdekar et al, 2000, Amini and Ahmandiani, 2002). In experiment 3, either testosterone propionate (TP, Steraloids, Newport, RI) or dihydrotestosterone propionate (DHTP, Steraloids) was delivered by once daily s.c. at a dose of 1 mg/kg. In experiment 4, finasteride was delivered into the third ventricle via an injection cannula at a dose of 10 μ g/kg (delivered in a volume of 2 μ l over the course of 2 min) 48 hrs prior to restraint stress exposure. This dose has previously been shown to be effective in modifying rodent behaviors (Frye and Vongher, 2001).

4.5. Radioimmunoassay

4.5.1. Corticosterone (CORT)—Plasma levels of CORT were measured as previously described (Weiser and Handa, 2009). Briefly, samples were diluted 1:40 in phosphate-buffered saline (PBS) and heated to 60°C for 30 min to denature serum-binding proteins that might interfere with the assay. Plasma samples were assayed using 3 H-CORT (Perkin Elmer, Boston, MA, USA) as the tracer. An anti-CORT antiserum (MP Biomedicals, Sonoma, OH) was used at a final tube dilution of 1:2,000 in 0.01 M PBS containing 0.1% gelatin and bound and free tracer were separated by adding dextran coated charcoal. Experimental samples were quantified by comparison to a standard curve of corticosterone ranging from 5pg to 750 pg/tube (Steraloids, Newport, RI, USA). The intra-assay coefficient of variation, as measured by internal quality controls, was 4.9%.

4.5.2. Adrenocorticotrophic hormone (ACTH)—Plasma levels of ACTH were measured as previously described (Weiser and Handa 2009). Briefly, 50 μ l plasma aliquots were assayed using 125 I-ACTH as the tracer and an anti-ACTH antibody (Immunostar, Hudson, WI) at a final tube dilution of 1:10,000. Each sample was compared to a standard curve of increasing doses of rat ACTH (National Hormone and Pituitary Program, Torrance, CA) for quantification. The intra-assay coefficient of variation was 8.2%.

Testosterone: Plasma levels of T were measured using an RIA kit purchased from Diagnostic Systems Laboratories (Webster, TX). The assay was run according to the manufacturer's directions. Intra-assay coefficient of variation was 8.6%.

4.6. Statistical Analysis

Between group Analysis of Variance was performed using Graphpad Prism software (San Diego, CA). Within group contrasts to compare hormone response patterns after gonadectomy and sham operations were performed using BMDP software (Statistical Solutions, Cork, Ireland) software. *Post hoc* analysis was performed using Fisher's Protected Least Significant Difference test. Differences were considered significant when $p < 0.05$.

Acknowledgments

Supported by USPHS grant R01-NS039951 (RJH)

List of Abbreviations

5αR	5-alpha reductase
3β-diol	5-alpha androstane 3 β ,17 β diol
3V	third ventricle
5αDHP	5 α -dihydroprogesterone
ACTH	adrenocorticotrophic hormone
BnST	bed nucleus of the stria terminalis
CORT	corticosterone
CRH	corticotropin releasing hormone
CSF	cerebrospinal fluid
DHT	dihydrotestosterone
DHTP	dihydrotestosterone propionate
E	estradiol
ER	estrogen receptor
HPA	hypothalamo-pituitary-adrenal
icv	intracerebroventricular
ir	immunoreactivity
MPOA	medial preoptic area
PBS	phosphate-buffered saline
PVN	paraventricular nucleus
s.c.	subcutaneous
T	testosterone
TP	testosterone propionate
Veh	vehicle

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HIGHLIGHTS

- Restraint stress-induced increases in plasma corticosterone are augmented after orchidectomy.
- Treatment of intact males with finasteride enhances restraint stress-induced corticosterone.
- Androgen treatment reduce the restraint stress-induced increase in plasma corticosterone
- Finasteride treatment blocks testosterone's ability to reduce corticosterone.

Figure 1.

Changes in post-stress plasma corticosterone levels following gonadectomy (GDX) of male rats. Animals were GDX'd or sham GDX'd and subjected to a 20 min. restraint stress 1-7 days later. An additional group was left intact. Each bar represents the mean $\pm$ SEM of 8-14 animals. Two way analysis revealed a significant effect of gonadectomy. Post hoc analysis using Fisher's protected LSD after one way ANOVA for GDX or sham groups including the intact animals (0 day GDX) showed # = $p < 0.05$ compared to intact control group. * = $p < 0.05$ compared to day 1 GDX group..

Figure 2.

Effect of 5 α reductase inhibition on plasma corticosterone (CORT; upper panel) or adrenocorticotrophin (ACTH; lower panel). Intact male rats were given finasteride (1 or 10 mg/kg bodyweight, SC) for two consecutive days and then subjected to a 20 min restraint stress. Non-stress animals were euthanized directly from their home cage. Each bar represents the mean $\pm$ SEM of 6-8 individuals. Fisher's protected LSD showed * = significantly different from non-stress group of same treatment. # = significantly different from stress/vehicle group.

Figure 3.

Effect of testosterone propionate (TP) or dihydrotestosterone propionate (DHTP) treatment of gonadectomized male rats on plasma corticosterone (left panel) and ACTH (right panel) following a 20 min restraint stress. Non-stressed animals were euthanized immediately after removal from their home cage. Each bar represents the mean $\pm$ SEM of 7-8 animals per group. Significant differences by Fisher's protected LSD are shown by lower case letters that are different from each other ($p < 0.05$).

Figure 4.

Effect of 3rd ventricle infusion of finasteride on testosterone propionate (TP) or dihydrotestosterone propionate (DHTP) effects on plasma corticosterone or ACTH. Upper panel shows plasma corticosterone levels in non-stress or stress (20' restraint) conditions. Lower panel shows plasma ACTH levels in non-stress or stress (20' restraint) conditions. Each bar represents the mean $\pm$ SEM of 5-7 animals per group. Posthoc analysis using Fisher's protected LSD shows: * = stressed groups that are significantly different from vehicle / stress group. # = finasteride groups that are significantly elevated compared to the similar vehicle control group. @ = groups showing significant elevation in CORT or ACTH compared to non-stress groups.

Table 1

Plasma testosterone levels in intact male rats following treatment with finasteride (1 or 10 mg/kg BW). Values represent mean and standard error of the mean (SEM). n= number of animals per group.

Treatment Group.	Testosterone (ng/ml)	S.E.M.	n
Vehicle	3.9	0.6	12
Finasteride (1mg/kg BW)	2.5	0.4	13
Finasteride (10mg/kg BW)	3.7	0.8	14

1 way ANOVA showed no significant group differences (p=0.25)