Testosterone's regulation of the HPA axis differs for adolescent and adult male rats

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Abstract

Stress hormones such as corticosterone (CORT), enables rodents to cope and meet the demands of their environment. In adult male rats, CORT release in response to stressors is dampened by the gonadal hormone testosterone. Adolescent male rats secrete more CORT in response to stressors than do adults and our lab has previously reported that gonadectomised pre-pubertal adolescent male rats (postnatal day [P] 35) do not show the dampening effect of testosterone replacement on CORT release in response to restraint stress found in adult rats (postnatal day [P] 75); whereas, post-pubertal adolescent rats (postnatal day [P] 45) show heightened CORT release when given testosterone replacement. Therefore, the main question would be what is the basis of the age difference in response to testosterone? And so, my mechanistic hypothesis that is tested in the thesis is that the greater stress response of adolescents than adults is because of greater conversion of testosterone to estradiol and/or less conversion of testosterone to DHT in adolescents than in adults. In Experiment 1, I replicated the results for P45 and P75 male rats. In Experiment 2, rats were gonadectomised (GDX) and given implants of testosterone, dihydrotestosterone (DHT), or empty, control implants (CTL) and plasma was obtained after 30 minutes of restraint. Although no significant differences were obtained for CORT levels, the pattern of means was consistent with our previous findings. Further, DHT and testosterone-treated rats had lower vasopressin (AVP) and corticotrophin releasing hormone (CRH), and a trend toward lower aromatase-immunoreactive cell counts in the parvocellular paraventricular nucleus (PVN) than did CTL rats, irrespective of age, and there were no group differences in the magnocellular PVN. In Experiment 3, gonadally-intact P45 and P75 males were treated with fadrozole (aromatase inhibitor), finasteride (5a-reductase inhibitor), flutamide (androgen receptor antagonist), or vehicle (VEH). Higher CORT concentrations after restraint

were found in P45 than in P75 only among VEH rats. Among P45 rats, CORT levels were higher in VEH than in fadrozole-treated rats only. Among P75 rats, VEH rats had lower CORT levels than did finasteride-treated rats. These results suggest that the higher stress release of CORT in P45 may involve greater conversion of testosterone to estradiol at the level of the adrenal cortex.

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ACTH (Adrenocorticotropic hormone): a hormone that is released from cells in the anterior pituitary in response to CRH and causes the release of corticosteroids from the adrenals.

AVP (Arginine vasopressin): a neuropeptide that is released from cells in the PVN in response to stress and that potentiates the release of ACTH from the anterior pituitary.

AR (Androgen receptors): the principle receptor for androgens (e.g., testosterone and DHT) that acts primarily as a transcription factor.

BNST (Bed nucleus of the stria terminalis): a brain region that relays stress-related information from other limbic regions (e.g., medial prefrontal cortex, ventral subiculum, amygdale) to the PVN and has modulatory influences on its activity.

CORT (Corticosterone): a steroid hormone produced in the adrenal cortex. It is involved in regulation of energy, immune reactions, and stress responses.

CRH (Corticotropin releasing hormone): a neuropeptide that is released from cells in the PVN in response to stress and that causes the release of ACTH from the anterior pituitary.

DHT (Dihydrotestosterone): a potent androgen that, unlike testosterone, cannot be converted to estradiol via aromatase.

ER (Estrogen receptor): the principal receptor target for estrogens, which come in several sub-types (ERα and ERβ) that act primarily as transcription factors.

FSH (Follicle-stimulating hormone):is a hormone produced in the anterior pituitary gland by gonadotropic cells and regulates the development, growth, pubertal maturation, and reproductive processes of the body.

GDX (Gonadectomy); a surgical procedure to remove the gonads (i.e., ovaries or testes), which is the main site of sex hormones production (i.e., estrogens and androgens).

GR (Glucocorticoid receptors): one of the two main receptors for stress hormones (i.e., corticosteroids) that acts primarily as a transcription factor.

GRE (Glucocorticoid response elements): a region on the DNA where GR can bind to alter gene expression.

HPA (Hypothalamus-pituitary-adrenal) axis: the system that controls the release of stress hormones.

HPG (Hypothalamus-pituitary-gonadal) axis: the system that controls the release of gonadal hormones.

LH (Luteinizing hormone): a hormone produced in the anterior pituitary gland by gonadotropic cells. It triggers ovulation and development of the corpus luteum in females. In males, it stimulates Leydig cells to produce testosterone.

mPOA (Medial preoptic area): a hypothalamic region that is primarily known for its involvement in sexual behavior, and that also modulates HPA activity.

MR (Mineralocorticoid receptors): one of the two main receptors for stress hormones (i.e., corticosteroids) that acts primarily as a transcription factor.

mRNA (Messenger ribonucleic acid): a product of gene expression (i.e., transcription) that contains the information for making a protein.

PVN (Paraventricular nucleus): the region in the hypothalamus that regulates HPA axis activity by integrating stress-related input and releasing CRH and AVP.

Introduction

During stress, the hypothalamus-pituitary-adrenal (HPA) axis is activated, releasing hormones that allow the organism to cope with its environment. There are developmental changes in HPA function from early adolescence to adulthood in both people and rodents. The main hypothesis for this age difference involves the changes in adolescence in the hypothalamus-pituitary-gonadal (HPG) axis, which is known to regulate HPA function in adults (Green & McCormick., 2016). Nevertheless, there has been little research to test this hypothesis. Thus, my research investigates the basis of age differences in HPA function in pre-pubertal adolescent, post-pubertal adolescent, and adult male rats. Further, the research described in this thesis will highlight the work conducted on a rodent model (rats), as it is from such models that most of our mechanistic understanding of age-dependent changes in stress reactivity and neurobiological function are derived.

HPA Axis

There are various types of stressors. One main type is psychological stress, which includes emotional stress and cognitive stress that can result from a variety of situations, such as fear of a potential danger when walking alone late at night, coping with the unknown of moving to a new country, or the loss of a loved one. The other main type is physical stress, such as illness, trauma, and pain. The commonality among these stressors is that they typically result in an activation of the HPA axis. In response to stressors, the HPA is activated, releasing a signaling cascade of hormones leading to the release of glucocorticoids, with the main glucocorticoid in rats being corticosterone (CORT) (Sapolsky, 2000). The response to stressors starts with the activation of a population of neurons whose cell bodies are located in the medial

parvocellular portion of the paraventricular nucleus (PVN) of the hypothalamus. This stimulation is generated by impulses from brain regions such as the amygdala and bed nucleus of the stria terminalis (BNST) and from ascending brainstem pathways that respond to physiological challenges such as the nucleus of the solitary tract, periaqueductal gray, raphe nuclei, and locus coeruleus. Although, the specific circuits to the PVN differ depending on whether a psychological or physical stressor is involved (Herman & Cullinan, 1997; Spencer & Deak, 2016; Myers et al., 2017). Psychological stressors are mostly based in higher central nervous circuits involving the cortex and limbic system whereas physical stressors involve to a greater extent circuits in the brainstem. Activation of the medial parvocellular PVN neurons releases corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophyseal portal veins, where they are transported to the anterior pituitary. CRH and AVP binding to receptors on corticotrophs (basophilic cells that produce adrenocorticotropic hormone (ACTH)) of the anterior pituitary which are then activated and secrete a 39-amino acid peptide hormone, ACTH (Spencer & Deak, 2016). Corticotrophs have a low intrinsic activity under baseline conditions (Dallman et al., 1987) and thus, the release of ACTH is mainly controlled by CRH binding to Corticotropin-releasing hormone receptor 1 (CRF1) and AVP binding to Vasopressin receptor 1B (V1bR) (Aguilera & Rabadan-Diehl, 2000). ACTH then binds to the melanocortin 2 receptor on cells located primarily in the zona fasciculata layer of the adrenal cortex; this binding stimulates a series of enzyme-mediated reactions that convert cholesterol into CORT, which is then released into circulation (Spiga & Lightman, 2015) (see Figure 1).

CORT in turn exerts its effects by binding to its receptors, mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), which are ligand-activated transcription factors found in the cytoplasm of cells (Funder, 1997). Binding of the steroid hormone to the unliganded

steroid receptor (GR or MR) induces a conformational change and disassociation from chaperone proteins such as heat shock proteins (HSP90). This modification leads to dimerization of the steroid hormone receptor complex, followed by translocation of the steroid hormone receptor to the target DNA (Oyola & Handa., 2017). The steroid hormone receptor complex is accessible to DNA regulatory regions, which contain specific hormone response elements, in this case glucocorticoid response elements (GREs). These GREs have at their core, a palindromic sequence of nucleotides that can bind the hormone receptor dimer. Coactivators or co-regulatory proteins, such as p160 and p300 are also recruited to uncoil the inactive DNA from histone proteins, exposing regulatory regions and transcriptional initiation sites of target genes (Oyola & Handa, 2017). Transcription or trans-activation of the target gene is initiated through activation of the pre-initiation complex (Beato & Klug, 2000). Corticosteroid receptors can also cause transrepression by binding to a negative GRE or can alter gene expression at composite GREs or by tethering to other transcription factors (Ou et al., 2001).

There is much overlap in the distribution of GR and MR in the adult rat brain. The cortex and hippocampus express the highest densities of GR mRNA and protein (Ahima & Harlan, 1990). Within the adult rat hippocampus, GR is expressed the highest in the hippocampal regions CA1 and CA2 and to a lesser extent in the granule cell layer of the dentate gyrus, with lowest levels in CA3 (Ahima & Harlan, 1990). Neurons in the PVN also express GR, but importantly, GR expressing PVN neurons also express CRH and AVP (Ceccatelli et al., 1989). Like GR, MR protein is highly expressed in the adult hippocampus in regions CA1 and CA2 and to a much lesser extent in the CA3 and dentate gyrus. Furthermore, MR protein is also expressed at moderate levels in the cortex, including the cingulate cortex, the hypothalamus and subcortical regions (Ahima et al., 1991). MR and GR in turn play a role in energy (glucose) balance,

immune function/anti-inflammation, learning and memory, and dampening activation of the HPA system at each level of its axis (Herman et al., 2005). For example, GR activates pathways back to the PVN, which results in inhibition of CRH production (negative feedback) and thus a termination of the HPA stress response (Gunnar & Quevedo, 2007).

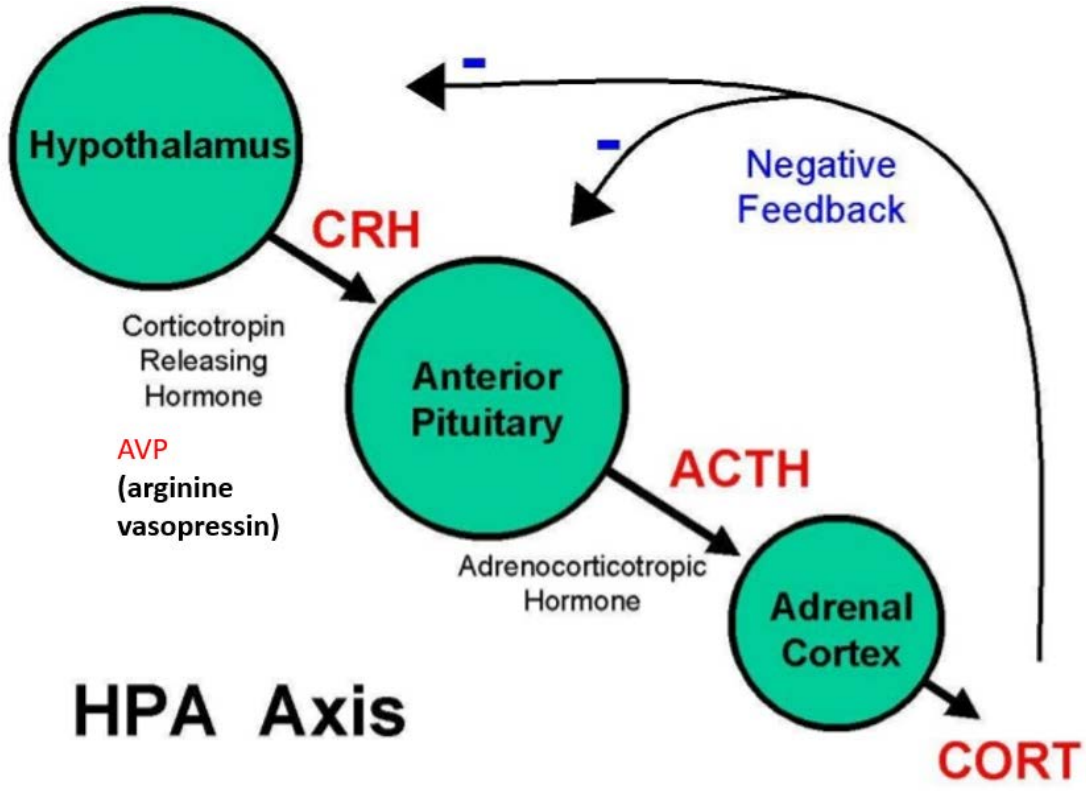

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Fig. 1. Schematic showing the hypothalamic–pituitary–adrenal axis summary (corticotropin-releasing hormone= CRH, arginine vasopressin= AVP, adrenocorticotropic hormone= ACTH, corticosterone= CORT).

Regulation of HPA function by the HPG axis

The HPG axis is a neuroendocrine axis that regulates reproductive functions (Handa & Weiser, 2014). Gonadotropin-releasing hormone (GnRH), a central neuropeptide, is released from neurons that are dispersed throughout the rostral hypothalamus and medial septal-diagonal

band complex (Jennes & Conn., 1994). GnRH is secreted into the hypothalamic-hypophyseal portal system found in the median eminence, and then binds to its receptor on gonadotropic cells of the anterior pituitary to stimulate the release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Handa & Weiser, 2014). LH stimulates the secretion of steroid hormones from the gonads. In the testis, the receptors for LH are found on Leydig cells, which synthesize testosterone after LH stimulation (Handa & Weiser, 2014). In the ovary, steroidogenic cells of the follicle are under the control of pituitary LH and FSH, which act by binding to their respective high affinity receptors on granulosa and thecal cell membranes. The actions of FSH are restricted to the granulosa cells found in the follicle, where FSH receptors work to activate the aromatase enzyme and allow synthesis of estrogens from aromatizable androgens (testosterone and androstenedione) (Handa & Weiser, 2014). However, granulosa cells rely upon the thecal cell, whose leading role appears to be the production of androgens that act as a substrate for estrogen synthesis by the granulosa cells. Thecal cell production of androgens is under the control of LH (Handa & Weiser, 2014). Androgens, mainly testosterone and dihydrotestosterone (DHT) exert their actions by binding to androgen receptors whereas estradiol actions involve mainly the binding to two different estrogen receptor isoforms (ERα & ERβ).

Evidence of sex differences in HPA function suggests a role for gonadal hormones in the regulation of the HPA axis. Under basal conditions, adult females' CORT concentrations in the bloodstream are higher than are those of adult males (Handa et al., 1994; Atkinson, 1997), and there are sex differences in how the HPA axis responds to stress (Bangasser & Valentino, 2014). Females have higher blood stream concentrations of corticosteroid binding globulin (CBG), to which approximately 95% of CORT is bound (Gala & Westphal, 1965), although the functional

consequences of bound CORT are not well understood. Stress-induced activation of PVN neurons is higher in females than in males (Larkin et al., 2010; Seale et al., 2004; Viau et al., 2005; Babb et al., 2013). Moreover, after an acute stress, females release higher concentrations of ACTH and CORT than do males, and their return to basal levels takes more time than in males (Viau et al., 2005; Seale et al., 2004). Males also have less expression of mRNA for AVP and CRH in the PVN than do females after an acute stress exposure (Viau et al., 2005).

Consistent with the sex differences, most of the results from studies involving gonadectomy (GDX) and hormone replacement in adult rats show that estrogens increase whereas androgens decrease HPA function (Goel et al., 2014). For example, GDX males had increased post-stress concentrations of ACTH and corticosterone and had a slower return to basal stress levels compared with GDX males given androgen replacement (Handa et al., 1994; Seale et al., 2004). In contrast, in females, GDX decreased post-stress concentrations of ACTH and corticosterone and caused a quicker return to basal stress levels, effects that were reversed by estradiol treatment (Seale et al., 2004; Kalil et al., 2013; McCormick et al., 2002). There is an overlap of sex hormone and glucocorticoid receptors in the PVN and nearby brain regions that have direct inputs to the PVN, such as the BNST, medial preoptic area, and hippocampus (Handa& Weiser, 2014). For example, there are region-specific effects of testosterone on AVP. In the BNST and medial amygdaloid nucleus, vasopressin-immunoreactive cell bodies are increased in adult rats after gonadectomy, which can be reversed by testosterone replacement (Leeuwen et al., 1985), not only for AVP cell bodies, but also their projections (De Vries et al., 1985). In the PVN, the effect of testosterone is opposite; male rats that were GDX and replaced with a testosterone implant show reduced AVP levels compared to rats that were GDX and did not receive testosterone replacement (Viau et al., 1999). However, to date the mechanisms by

which gonadal steroid hormones may act to influence HPA function have not been completely resolved.

Although the dampening effects of testosterone on stress-induced HPA function primarily involve actions at androgen receptors (AR) and the enhancing effects of estradiol primarily involve actions at estrogen receptor alpha (ERα) (Handa & Weiser, 2014; Liu et al., 2012; Lund et al., 2006), there is regulation of the HPA axis by metabolites of testosterone that can involve actions beyond androgen and estrogen receptors (see Figure 2). Testosterone can be converted to DHT by the 5α-reductase enzyme (Lephart, 1993). Although both testosterone and DHT bind to AR with high affinity, DHT is a more potent and selective agonist for ARs (Handa & Weiser, 2014). Handa and colleagues (2013) showed that preventing the conversion of testosterone to DHT by blockade of 5α-reductase with the 5α-reductase inhibitor, finasteride, enhanced the CORT and ACTH response to restraint stress. Further, the effects on HPA function of testosterone, but not DHT, administered to GDX males, could be blocked by finasteride (Handa et al., 2013), indicating that DHT is necessary for decreasing the stress responses.

DHT can be reduced intracellularly to 5α-androstane-3α,17β-diol (3α-diol) or 5α-androstane-3β,17β-diol (3β-diol) (Jin & Penning, 2006). Oxidative 3α-hydroxysteroid dehydrogenase (3α-HSD) activity can convert 3α-diol back to DHT, and this represents an alternative pathway for DHT synthesis (Ishizaki et al., 2013). However, unlike 3α-diol, 3β-diol is a potent inhibitor of HPA axis reactivity, similar to the actions of DHT (Lund et al., 2004). Treatment of GDX mice or rats with 3β-diol can reduce plasma CORT and ACTH responses to stress by binding and activating estrogen receptor beta (ERβ), and thereby dampening stress-induced HPA activity (Lund et al., 2004). This effect is not present in ERβ null mice (Handa & Weiser, 2014). In further support of an ER-mediated mechanism, the effects of 3β-diol and DHT

are not blocked by the AR antagonist, flutamide, but rather, are blocked with tamoxifen, an ER antagonist (Lund et al., 2006).

Testosterone, unlike DHT, can be converted to estradiol by the aromatase enzyme (Roselli et al., 1985). There are at least two populations of aromatase-positive cells in the adult brain; a steroid-dependent and steroid-independent population (Roselli et al., 2009). In the steroid-dependent population, regulation of aromatase expression in the brain is region-specific (Roselli et al., 1985). For example, within the preoptic area and hypothalamus, androgens regulate aromatase mRNA via AR-mediated transcription (Abdelgadir et al.,1994). Extensive co-localization of AR and aromatase is observed in the neuroendocrine regions of the brain (Veney et al., 2000). Aromatase activity and mRNA levels in the preoptic area and hypothalamus decreased by 7 days after castration, which could be prevented by treatment with testosterone and DHT, but not with estradiol (Roselli et al., 1997). In contrast, androgens do not regulate aromatase in most other brain areas, including the amygdala and hippocampus (Abdelgadir et al., 1994); neither aromatase activity nor mRNA levels in the amygdala are affected by castration or hormone replacement (Roselli et al., 1997).

Estradiol can bind to two different receptor isoforms, ERα and ERβ; each has an opposing action on HPA function (Handa et al., 2008). Administration of the agonist pylprazoletriol (binds with greater affinity to ERα than to ERβ) into the PVN increased post-stress plasma ACTH and CORT concentrations and c-fos expression (a marker of neural activity) in the PVN in male and female rats, whereas an ERβ-selective agonist, diarylpropionitrile, had dampening effects on the HPA axis (Liu et al., 2012; Lund et al., 2006;). ERβ is expressed in some populations of CRH and AVP containing neurons in the hypothalamus (Hrabovszky et al., 2004). ERβ is also expressed by CRH-immunoreactive neurons in the medial parvocellular PVN

and in the caudolateral PVN (Laflamme et al., 1998). ERβ is also expressed in vasopressin-immunoreactive neurons of the PVN (Suzuki &Handa, 2004). These data suggest that ERβ is a cellular mediator of the direct actions of estradiol on PVN function. On the other hand, ERα is not expressed much in PVN neurons (Suzuki & Handa, 2005), but rather is found at high levels in brain regions that send direct and indirect projections to the PVN, such as the peri-PVN, BNST, medial preoptic area, lateral septum, and hippocampus (Suzuki & Handa, 2005). Despite the expression of ERβ in the PVN, estradiol typically increases HPA responses to stressors (Green & McCormick., 2016; Handa & Weiser, 2014), which suggests greater actions of estradiol at ERα receptors in other regions.

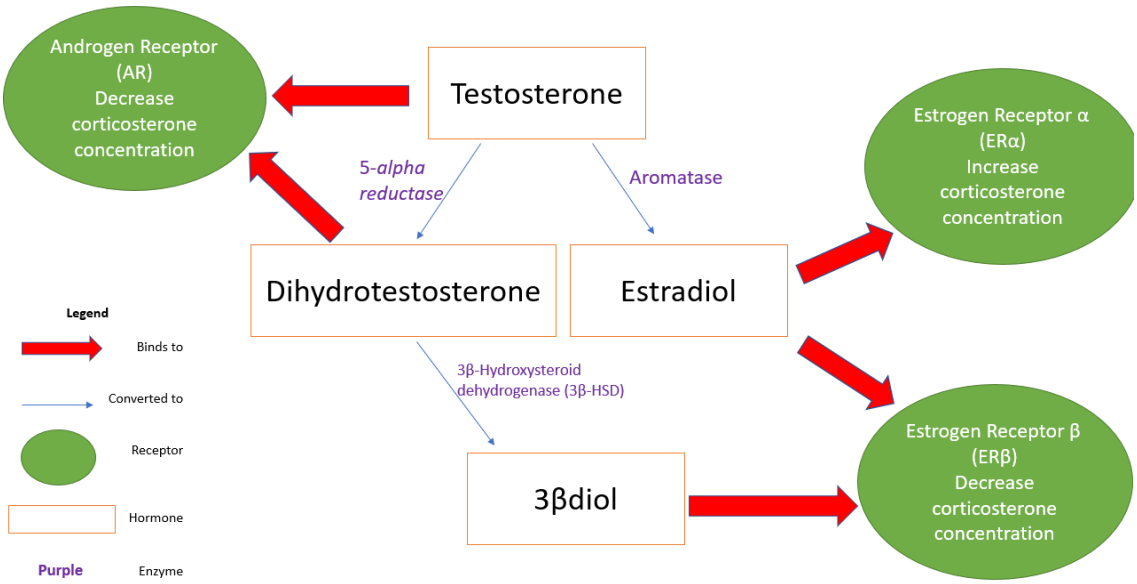

Fig. 2. Schematic showing the regulation of HPA axis by gonadal hormones.

Developmental differences

The HPA axis functions differently in rats as they develop, although less is known about the adolescent period (Lui et al., 2012). Adolescence in rats is a transitional phase between

childhood and adulthood, in which puberty is a defining characteristic that is marked by an increase in blood testosterone level at about 40-42 days of age in males (McCormick & Green, 2013; Green et al., 2016). The HPA functions differently in pre-pubertal adolescence compared to adults, and this change is well documented in rats (Green & McCormick., 2016). Pre-pubertal rats have higher ACTH and CORT concentrations than do adults after an acute stress, whether the stressor is physical or psychological in nature (Romeo et al., 2005). Further, the time required for concentrations to return to baseline, is longer in pre-pubertal than in adult rats (Romeo et al., 2005). Romeo and colleagues (2007) found no difference in volume, somal size, and cell number in the parvocellular and magnocellular subdivisions of the PVN in pre-pubertal (28 days of age) compared with adult (77 days of age) male rats. Also, pre-pubertal adolescent and adult males had similar numbers of anterior pituitary projecting neurosecretory neurons in the parvocellular region of the PVN. Nevertheless, in the same study, they found that in response to acute stress, pre-pubertal males had greater CRH expression than did adults, which might explain the greater release of ACTH and CORT seen in pre-pubertal rats.

One reason for the age difference in the stress response could be immature HPA negative feedback regulation in adolescence (Goldman et al., 1973). Dziedzic and colleagues. (2014) found no differences between pre-pubertal adolescents and adults in GR protein levels in regions that are important in negative feedback, including the medial prefrontal cortex and PVN of the hypothalamus. These data indicate that the extended hormonal stress response exhibited by pre-pubertal adolescent rats is likely independent of significant pubertal changes in GR protein levels. However, Green and colleagues (2016) investigated GR in the hippocampus because of its high density of corticosteroid receptors and its role in HPA negative feedback (Herman et al., 2012), which is mediated in part by classical GR actions (Feldman & Weidenfeld, 1999). They

found no age differences in baseline expression of GR in the hippocampus within the cellular compartments examined (cytosolic and nuclear). However, pre-pubertal adolescent males had greater expression of nuclear GR immediately after restraint stress, which corresponded with, and likely reflects, the age difference in the release of corticosterone. GR translocation to the nucleus in post-pubertal adolescents was intermediate to that of pre-pubertal adolescents and adults.

There is no evidence for differences between pre-pubertal adolescents and adults in densities of AR, ERα, ERβ, or progesterone receptors (PR) mRNA in the preoptic area (part of the anterior hypothalamus) (Walker et al., 2009). The preoptic area is implicated in the control of HPA function by sex steroids. Replacement of androgens in the medial preoptic area was sufficient to dampen CORT concentrations after a stressor in gonadectomized male rats (McCormick et al., 2002; Viau & Meaney, 1996). When it comes to the PVN, ARs are not localized in neuroendocrine neurons within the PVN (Bingaman et al., 1994; Bingham et al., 2006), but rather, AR-immunoreactive neurons 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 (Handa & Weiser., 2014). Similarly, the PVN lacks ERα (Suzuki & Handa., 2005), indicating that the age difference in the stress response cannot be explained by differences in ARs and ERα within the PVN. However, there is more PR expression in pre-pubertal rats, compared to adults (Romeo et al., 2005). It remains unclear how progesterone regulates the HPA axis in adolescence, which is an important question taking into account that progesterone dampens HPA function in adult males (Viau & Meaney, 1999) and that pre-pubertal and post-pubertal adolescents secrete more progesterone in response to stress compared to adults (Romeo et al., 2005; Green et al., 2016). Certain enzymes that may be

involved in age-related differences in HPA response show changes in expression across development; for example, aromatase and 5α-reductase expression in the brain decreased to adult-typical levels from mid to late adolescence (Ivanova & Beyer, 2000; Lephart et al., 2002). The role of these enzymes in age differences in HPA function, however, has not been investigated.

Gonadal immaturity (i.e., lower testosterone concentrations in adolescents) is one hypothesis for the difference between pre-pubertal and adult male rats in HPA responses to stressors. Nevertheless, a study done by Romeo and colleagues (2004) showed that providing GDX pre-pubertal and adult male rats with equivalent, adult-like concentrations of testosterone did not eliminate the age difference in HPA stress responses. This study, however, did not include a GDX group that did not receive testosterone replacement, thus whether there is any inhibitory action of testosterone at this age is unknown. A study on non-operated (gonadally-intact) rats spanning from 30 to 70 days of age (pre-pubertal to adult) reported that adult-like ACTH stress response develops between 50 and 60 days of age, whereas the CORT response to restraint stress changes between 30, 40, and 70 days of age; mid-adolescent rats had higher CORT concentrations than adult rats, despite no difference in plasma testosterone concentrations (Foilb et al., 2011; Green et al., 2016).

To further examine the effects of testosterone on the stress system throughout adolescence and how it compares to the effects in adults, Green (2017) investigated male rats from three age groups (P35, P45, and P75). All rats were GDX and given either an empty implant (vehicle; VEH) or a testosterone implant for five days before undergoing 30 min of restraint stress. In P35 rats, CORT concentrations did not differ between the VEH and testosterone-treated rats, whereas, in P75 rats, testosterone treatment dampened CORT levels

relative to VEH rats. In P45 rats, there was an unexpected result: an increase in CORT concentrations in testosterone-treated rats compared with VEH rats. This result contradicts the typical dampening effect of testosterone treatment on CORT release seen in adult males.

Goals of thesis

My research is primarily focused on examining this unexpected result, with the overarching goal of investigating the basis behind the age differences in response to testosterone treatment. The main mechanistic hypothesis that is tested in the thesis is that the greater stress response of adolescents than adults is because of greater conversion of testosterone to estradiol and/or less conversion of testosterone to DHT in adolescents than in adults. In Experiment 1, I predicted that regulation of the stress response by testosterone treatment at P45 would be different compared to P75. The rationale behind this is because Green, (2017) found that at P45, testosterone treatment increased the stress response, which opposes the typical dampening effect of testosterone treatment on the stress response at P75. Therefore, my first objective of this experiment was to confirm the findings of increased CORT release in response to testosterone treatment at P45, in contrast to the dampening effect of testosterone treatment at P75. Further, Experiment 1 had a second part in which I tested the hypothesis that age difference in response to stressors involves age difference in adrenal sensitivity. The rationale behind this is because a study by Romeo et al., (2004) showed that lower levels of ACTH were required to achieve higher CORT concentrations in P35 rats than in P75 rats (P45 rats were not tested). Therefore, the second objective of Experiment 1 was to investigate whether adrenal sensitivity to ACTH explains the age differences between P45 and P75 in CORT release in response to stress. In Experiment 2, my prediction was that providing GDX adolescents with the non-aromatizable androgen,

dihydrotestosterone (DHT) would attenuate age differences in HPA function. The rationale was that if the age difference in testosterone effects on HPA function is because of greater aromatization of testosterone to estradiol in adolescents, then the age difference may be eliminated by using a non-aromatizable androgen. Thus, the objective for Experiment 2 was to test whether DHT (a non-aromatizable androgen) treatment would abolish the age difference in HPA function in response to a stressor. In Experiment 3, I hypothesized that preventing testosterone's aromatization to estradiol would eliminate the age differences in HPA function. The rationale for this hypothesis was that if the age difference in testosterone effects on HPA function is because of greater aromatization of testosterone to estradiol in adolescents, then inhibiting the action of the aromatase enzyme at P45 would eliminate the age difference. Consequently, the objective for Experiment 3 was to investigate whether the aromatization of testosterone to estradiol at P45 is the reason behind the age difference in HPA function in response to stress.

Experiment 1

Introduction

Testosterone, a key gonadal hormone in regulating HPA function, seems to affect the stress response differently between post-puberty adolescence (P45) and adult (P75) male rats, with P45 rats showing increased and P75 rats showing decreased plasma CORT release after testosterone treatment (Green, 2017). Therefore, the first objective of this experiment was to replicate this preliminary finding of Green, (2017) given the novelty of the findings. The second objective was to investigate whether adrenal sensitivity to ACTH explains the age differences in CORT release in response to stress. A previous study reported higher expression of the ACTH receptor, melanocortin 2 receptor in the adrenals, in pre-pubertal compared to adult rats, and that lower levels of ACTH were required to achieve higher CORT concentrations in pre-pubertal rats than in adult rats (P45 were not tested) (Romeo et al., 2014). Therefore, I tested the hypothesis that testosterone increases adrenal sensitivity to ACTH in P45, but not in P75 rats. I predicted that GDX P45 and P75 rats will not differ in CORT release to injection of ACTH, and that among rats given testosterone replacement, P45 rats will show higher CORT concentrations after injection of ACTH, than will P75 rats.

Methods

Animals

For Experiment 1, male Long-Evans rats were obtained at P36 (n = 24; 90–115 grams) and P66 (n = 24; 280–320 g) from Charles River (St. Constant, Quebec) and housed in same-aged pairs. Rats were given free access to food and water and kept on a 12 h light-dark cycle (lights on at 09:00). All procedures were approved by the Brock University Animal Care and Use

Committee and were in keeping with the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) as well as the Canadian Council on Animal Care guidelines.

Surgery

Four days after the arrival of the animals to the facility, all rats were weighed and then underwent GDX surgery under isoflurane anesthetic. Rats from each age group were implanted subcutaneously with silastic tubing (0.015 mm i.d., 0.031 mm o.d.) containing either crystalline testosterone propionate (TP: Sigma, T-1875; 2.0 cm long tubes; n= 12 per age as in McCormick et al., 1998) or left empty (blank tubes were 2.0 cm long; n= 12 per age). Silastic tubes were sealed with medical grade silastic glue.

Acute stressor procedure and sample collection

Restraint a form of psychological stress was conducted five days after surgery. Rats were thus P45 (±1 day) or P75 (±1 day) at collection. On each collection day, all age and treatment groups were represented. Within ~2–5 h after lights on, tail blood was collected immediately after 30 min of restraint stress (post-restraint) in Plexiglas® restrainers. This range in time of day was chosen for the experiments so that samples were collected during the phase of the light cycle when basal plasma CORT concentrations are low. Tail blood was collected into 2 ml microcentrifuge tubes containing EDTA and centrifuged at 3000 RCF for 10 min. Plasma was collected and stored at −20◦ C until hormone assays were conducted.

ACTH injection

To test whether age differences in CORT release in response to stress involve a higher adrenal sensitivity to ACTH, three days after the restraint stress procedure, the same rats were weighed and injected with 6.25 mg/kg of rat ACTH (Sigma, St. Louis, MO) in a 0.9% sterile

saline vehicle (1 ml/kg). Trunk blood was collected 60 min after injection. Intraperitoneal injections were chosen as the route of ACTH administration, based on a previous study assessing HPA reactivity in adult male rats (Cole et al., 2000). Moreover, the dose of ACTH was chosen based on studies that indicated that the 6.25 mg/kg dose of ACTH provided physiologically relevant levels of ACTH (mean of 400 pg/ml) (Romeo et al., 2014). Nevertheless, these doses result in significant individual differences in plasma concentrations of ACTH, which allows for the association between ACTH and CORT to be investigated across the four experimental groups. 60 min after ACTH injection, all rats were decapitated, and trunk blood was collected into ice chilled glass tubes containing EDTA and centrifuged at 3000 RCF for 15 min. Plasma was collected and stored at -20^{o}C until hormone assays were conducted. In addition, brains and adrenal glands were extracted, snap frozen on powdered dry ice, and stored at -80^{o}C until further processed.

Hormone assays

Steroids were extracted from plasma (except for ACTH samples) using diethyl ether for the measurement of CORT. Extracted samples were reconstituted in buffer provided in the enzyme-linked immunosorbent assay kits (Neogen, Lansing MI for CORT and testosterone; OriGene technologies, Rockville MD for ACTH). The assays were conducted according to the kit instructions and using a Biotech Synergy plate reader; all samples within an experiment were measured on the same day. Assay sensitivity was 0.05 ng/mL for CORT and 150 pg/mL for ACTH.

Statistical analyses

Statistical analyses were performed using SPSS (version 23) software and consisted of between-group (Age and Hormone Treatment) factor analysis of variances (ANOVAs). Post hoc analyses consisted of t-tests. An alpha level of $p < 0.05$ was used to determine significance.

Results

Weight Gain 5 Days after Surgery.

I measured the weight gain of testosterone-treated and empty-treated (CTL) P45 and P75 male rats from the time of surgery to 5 days later as a measure of the efficacy of the testosterone implants; testosterone typically increases weigh gain (Gentry & Wade, 1976)). An Age X Treatment ANOVA on weight gain indicated that those given testosterone gaining more weight than those given CTL implants ($p < 0.001$) (see Figure 3). These results show that rats treated with a testosterone implant gained more weight than rats treated with an empty implant.

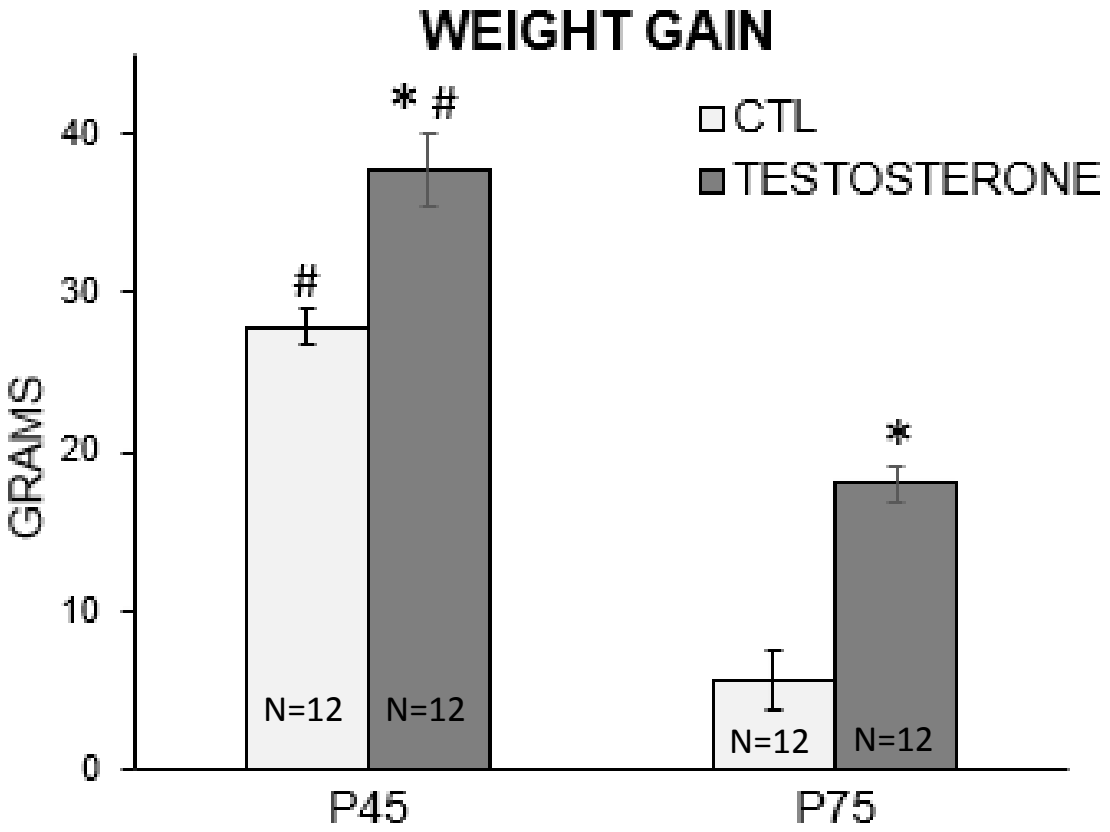

Fig. 3. The higher weight gain in testosterone-treated rats than in control rats shows that the implants used were effective. Mean (±S.E.M.) weight gain 5 days after surgery in P45 and P75 male rats. *Indicates drug effect within age ($p < 0.05$); # Indicates significant age difference within drug group (i.e. P45 v P75 for CTL and P45 v P75 for testosterone) ($p < 0.05$).

Plasma CORT Levels after Restraint.

I measured plasma CORT levels to investigate how testosterone treatment affects CORT levels in P45 and P75 male rats after 30 min of restraint stress. The interaction of Age X Treatment on plasma CORT concentrations immediately after 30 min of restraint was significant ($F_{1,35} = 12.51$, $p = 0.001$). At P75, testosterone-treated rats had lower CORT than CTL rats ($p = 0.004$), whereas in P45 rats, an opposite trend was observed; CORT levels were lower in CTL rats than those given testosterone ($p = 0.065$). Among CTL rats, P75 had higher CORT concentrations than P45 rats ($p = 0.049$), whereas among those given testosterone, P75 had lower CORT concentrations than P45 rats ($p = 0.009$) (see Figure 4). The results show that testosterone increases (albeit non-significantly) plasma CORT levels at P45, but decreases plasma CORT levels at P75.

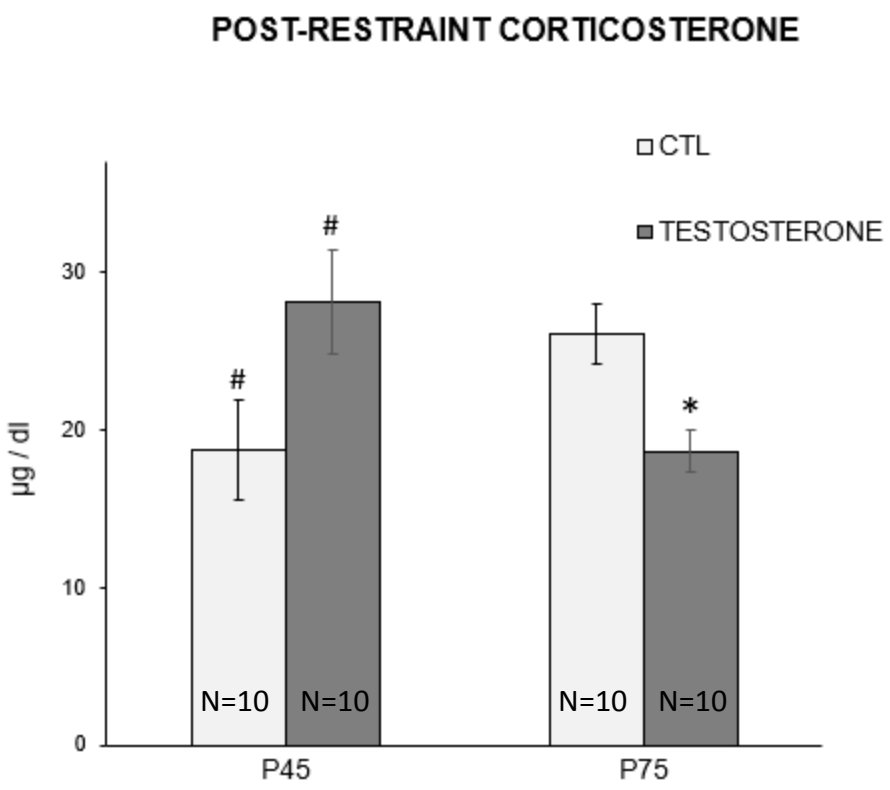

Fig. 4. Plasma CORT levels after restraint show an opposite effect of testosterone at P45 than at P75. Mean (±S.E.M.) plasma hormone concentrations of corticosterone immediately after 30 min of restraint stress in P45 and P75 male rats. *Indicates significant drug effect within age ($p < 0.05$); # Indicates significant age difference within drug group (i.e. P45 v P75 for CTL and P45 v P75 for testosterone) ($p < 0.05$).

Plasma ACTH and CORT Levels, 60 Mins after Injection with ACTH.

I measured plasma ACTH and CORT levels after an ACTH injection to examine testosterone's effect on adrenal sensitivity to ACTH in P45 and P75 male rats. 60 min after injection of ACTH, there was no main effect or interaction of age and treatment on plasma ACTH concentrations (all $p > 0.15$) (see Figure 5). CORT concentrations were highly variable, yet somewhat low (i.e., baseline levels), and the lower CORT levels in testosterone-treated rats than in CTL rats did not meet statistical significance ($F_{1,44} = 3.279$, $p = 0.077$). There was no effect of age ($p = 0.698$) or interaction with treatment ($p = 0.629$) (see Figure 5). I also examined the association between ACTH and CORT to confirm that the measures I obtain above were likely valid. ACTH and CORT concentrations were highly correlated in all four groups (P75-CTL, $r_{10} = 0.64$, $p = 0.034$; P45-CTL, $r_{11} = 0.88$, $p < 0.001$; P75-T, $r_{10} = 0.55$, $p = 0.079$; P45-T, $r_9 = 0.58$, $p = 0.08$ with two outliers removed and with outliers in, $r_{11} = 0.04$, $p = 0.895$) (see Figure 6). We found that higher plasma CORT levels were associated with higher plasma ACTH levels, indicating that ACTH injections were effective.

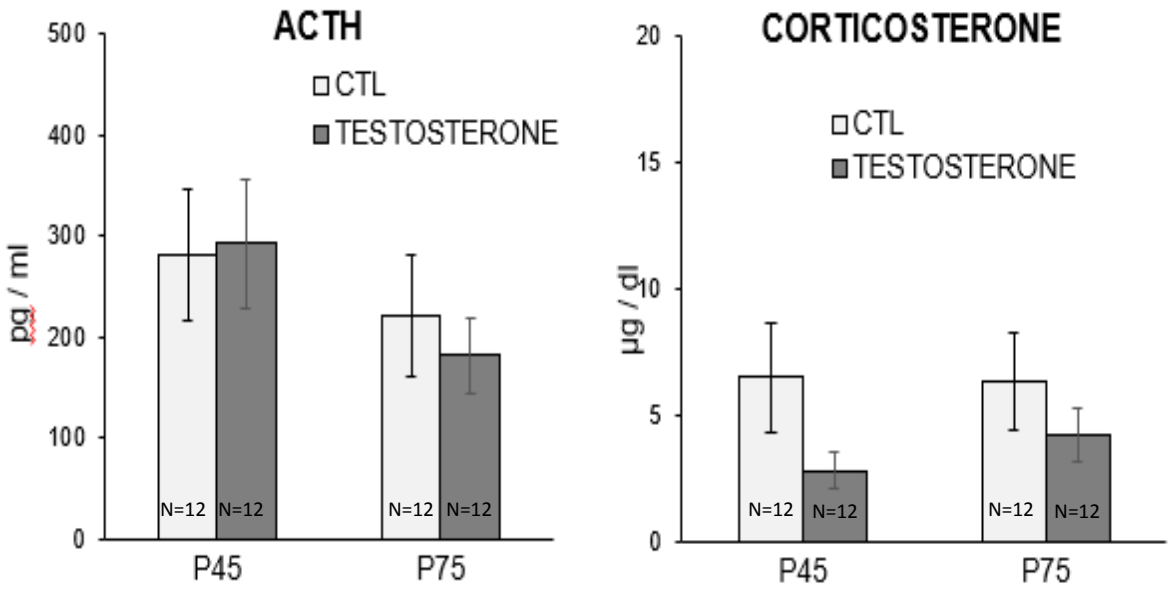

Fig. 5. Plasma ACTH and CORT levels were not affected by testosterone at either age.

Mean (±S.E.M.) plasma hormone concentrations of ACTH and corticosterone 60 mins after an ACTH injection in P45 and P75 male rats.

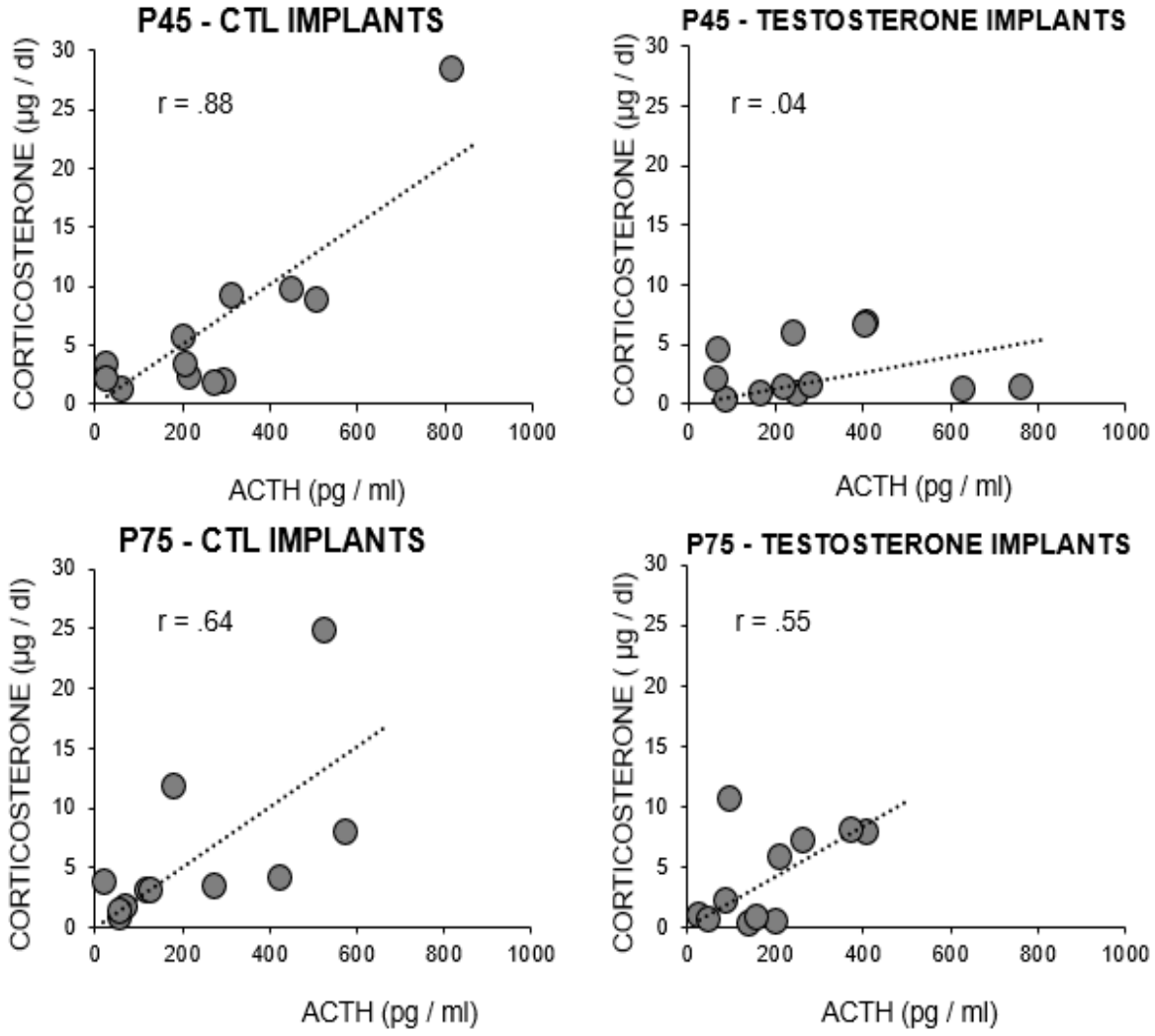

Fig. 6. A positive correlation between plasma ACTH and corticosterone levels, 60 min after an ACTH injection. Correlation between plasma ACTH and corticosterone 60 mins after an ACTH injection in P45 and P75 male rats. P75-CTL ($r_{10} = 0.64$), P45-CTL ($r_{11} = 0.88$), P75-T ($r_{10} = 0.55$), P45-T ($r_9 = 0.04$). (No outliers removed in figures).

Discussion

The weight gain (from surgery to 5 days later) data shows that irrespective of age, rats that were given testosterone gained more weight than those given CTL implants. An increase in weight gain is expected in testosterone-treated rats because testosterone increases food intake (Gentry & Wade, 1976). Thus, the weight gain results suggest that the implants were successfully delivering testosterone.

Consistent with the findings in Green (2017), Experiment 1 indicated that the CORT release in response to restraint stress in P45 males is different from that of P75. P45 treated with testosterone seemed to have greater CORT release compared with their controls, whereas in P75 rats, testosterone dampened the release of CORT, when compared to their controls. In contrast, among CTL rats, P75 had higher CORT concentrations than P45 rats, which highlights the importance of testosterone in dampening the stress response in adult males. These results suggest that there are developmental shifts in the stress system that occur in the time around puberty and which depend on the presence or absence of testosterone. In the current experiment, the age difference in CORT release after testosterone treatment was not explained by adrenal sensitivity to ACTH. This experiment, however, was limited by the relatively low bloodstream concentrations of ACTH after injection, such that although CORT concentrations were elevated, they did not result in "stress-level" concentrations. The strong association, however, between plasma ACTH and CORT concentrations indicates that the ACTH injections and the measurement of ACTH and of CORT were valid. Age differences in CORT levels may only be found under conditions of stress and/or higher concentrations of ACTH.

There are other factors that may explain this age difference in CORT release. For example, one hypothesis is that there is more conversion of testosterone to estradiol at the level

of the brain (PVN of the hypothalamus) in P45 rats. Estradiol is known to increase the HPA activity in adult rats (Handa & Weiser, 2014). Further, testosterone replacement delivered directly to the hypothalamus rather than systemically, dampened the HPA response to stressors in adult male rats (Viau & Meaney 1991; McCormick et al., 2002). This highlights the significance of the hypothalamus as an important brain area for the regulation of HPA axis by testosterone. Thus, differences between adolescents and adults in testosterone's regulation of the HPA axis may occur, in part, at the level of the hypothalamus.

Experiment 2

Introduction

The results obtained from Experiment 1 of age difference in CORT release between P45 and P75 rats suggest that there may be greater conversion of testosterone to estradiol in adolescents than in adults happening in the brain or other tissues involved in HPA regulation. If this is true, then treatment with DHT, an androgen that cannot be metabolized to estradiol, might attenuate the age difference in CORT release to stress. DHT is a selective, and more potent, agonist for androgen receptors and is known to dampen CORT release in response to stress in adults (McCormick et al., 2002; Handa & Weiser, 2014). Therefore, in Experiment 2 I test the hypothesis that providing GDX adolescents with the non-aromatizable androgen, DHT, would attenuate age differences in HPA function. I used Experiment 2 to take new measurements such as plasma progesterone levels because of its importance in steroidogenesis (e.g., a precursor for CORT production). I also examined how androgens might affect CRH and AVP producing cells in the PVN because these two neuropeptides are responsible for initiating ACTH release, which results in CORT release. In addition, I examined aromatase producing cells within the PVN because I suspected that age differences in aromatase production might be a reason for the age differences in CORT release evident in Experiment 1. For Lephart & Ojeda, (1990) showed that aromatase activity within the hypothalamus is twice higher in post-puberty adolescents (P48) than in adult rats (P68). Consequently, I predicted that in P75 rats, both testosterone and DHT treatment would suppress stress-induced CORT and progesterone release, and decrease AVP, CRH, and aromatase immune-reactive cells in the paraventricular nucleus (PVN). I also predicted that in P45 rats, DHT would suppress stress-induced CORT and progesterone release, and decrease AVP, CRH, and aromatase immune-reactive cells in the PVN, but the opposite

effects would be found in testosterone-treated rats. I predicted that testosterone-treated and DHT-treated P35 rats would not differ from control, vehicle-treated P35 rats on any measure based on evidence that corticosterone release in P35 rats is insensitive to androgens (Romeo et al., 2004; Green, 2017). However, whether the hypothalamus is sensitive to androgens at P35 is unknown.

Methods

Animals

For Experiment 2, male Long-Evans rats were obtained at P26 (n = 25; 50–85 g), P36 (n = 25; 90–115 grams), and P66 (n = 25; 280–320 g) from Charles River (St. Constant, Quebec) and housed in same-aged pairs. Rats were given free access to food and water and kept on a 12 h light-dark cycle (lights on at 09:00). All procedures were approved by the Brock University Animal Care and Use Committee and were in keeping with the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) as well as the Canadian Council on Animal Care guidelines.

Surgery

Four days after the arrival of the animals to the facility, all rats were weighed and then underwent GDX surgery under isoflurane anesthetic. Rats from each age group were implanted subcutaneously with silastic tubing (0.015 mm i.d., 0.031 mm o.d.) containing either crystalline testosterone propionate (TP: Sigma, T-1875; 2.0 cm long tubes; n= 8 per age as in McCormick et al., 1998) or crystalline 5α dihydrotestosterone (DHT: Sigma, A-8380; 2.0 cm long tubes; n= 8 per age as in Handa et al., 1994), or left empty (blank tubes were 2.0 cm long; n= 9 per age). Silastic tubes were sealed with medical grade silastic glue

Acute stressor procedure and sample collection

The acute stressor procedure was conducted in subgroups across three days, with the first stressor procedure five days after surgery. Rats were thus (±1 day) P35, P45, or P75 at collection. On each collection day, all age and treatment groups were represented. Within ~2–5 h after lights on, tail blood was collected immediately after 30 min of restraint stress (post- restraint) in Plexiglas® restrainers. This range in time of day was chosen for the experiments so that samples were collected during the phase of the light cycle when basal plasma CORT concentrations are low. Tail blood was collected within 2 minutes into 2ml microcentrifuge tubes containing EDTA and centrifuged at 3000 RCF for 10 min. Plasma was collected and stored at -20^{o}C until hormone assays were conducted.

Hormone assays

Steroids were extracted from plasma using diethyl ether for the measurement of CORT and progesterone. All extracted samples were reconstituted in buffer provided in the enzyme-linked immunosorbent assay kits (Neogen, Lansing MI) and the assays were conducted according to the kit instructions and using a Biotech Synergy plate reader. All samples within an experiment were measured on the same day. Assay sensitivity was 0.05 ng/mL for CORT, and 0.4 ng/mL for progesterone.

Immunohistochemistry

Immediately after the blood sampling, rats were deeply anaesthetized by an overdose of sodium pentobarbital (150 mg/kg) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4). Brains were removed from the skulls and post-fixed in a 30% sucrose and 4% paraformaldehyde solution until equilibrated.

Coronal sections (30 µm) were collected throughout the PVN of the hypothalamus and were stored in cryoprotectant at -20^{o}C until the time of assay.

Free-floating coronal sections were washed thoroughly in 0.1 M PBS, then in PBS-X (0.1 M PBS with 3% Triton X-100), and incubated at room temperature in a 0.3% H2O2 in 0.1 M PBS-X solution for 30 min. Sections were then washed in PBS-X, blocked at room temperature in 10% goat serum (Sigma) solution for 1h, and incubated at 4◦C overnight in primary anti-body (1:10,000 CRH rabbit mAb, A12789; 1:30,000 AVP rabbit mAb, T-4563; Peninsula laboratories; 1:2,000 aromatase rabbit mAb, ab18995 abcam) in PBS-X. The next day, sections were washed in PBS-X and then incubated for 2 h at room temperature in secondary antibody (biotinylated goat anti-rabbit IgG; 1:400; Vector Laboratories, Inc.). After another series of washes in PBS-X, sections were incubated in an avidin–biotin horseradish peroxidase complex (Vector Laboratories, Inc.) for 1.5 h at room temperature. Horseradish peroxidase was visualized with 3,3-diaminobenzidine (DAB) in a 3 M sodium acetate buffer containing 0.05% H2O2 (Vector Laboratories, Inc.). After a final series of washes in PBS-X, sections were mounted on Superfrost Plus slides (Fisher Scientific, Inc.), dried, dehydrated in increasing concentrations of ethanol (70%, 95%, 100%), placed in xylenes, and cover slipped using Permount mounting medium (Fisher Scientific, Inc.).

Microscopy and cell counting

Immunostained sections were analyzed using a Nikon Eclipse 80i microscope equipped with a digital camera (Nikon DXM1200F) and Nikon ACT-1 software. Immunoreactive (ir) cell counts were conducted blind to experimental condition and at 100× magnification in a 250 µm^2 area in each hemisphere of the (PVN). Within the PVN, AVP-expressing cells were counted separately in the magnocellular area and in the parvocellular area. This is done because it is the

parvocellular area that plays a role in HPA function (Martinez et al., 2002). So, I expect changes in the parvocellular area, and not the magnocellular area. The PVN region was identified according to the atlas of Paxinos and Watson (Paxinos and Watson, 2005): PVN sections used for counting were within the coordinates from bregma−1.44 and −1.72. The mean number of ir-cells per hemisphere, per brain region, per rat, was used for analysis for the PVN region of interest.

Statistical analyses

Statistical analyses were performed using SPSS (version 23) software and consisted of between group factor analysis of variances (ANOVAs) and post hocs that were independent groups t-tests. An alpha level of $p < 0.05$ was used to determine significance.

Results

Weight Gain 5 Days after Surgery.

I measured how much weight the testosterone-treated, DHT-treated, and empty-treated P35, P45, and P75 male rats gained from surgery to 5 days after, to test whether the testosterone and DHT implants I provided were effective. An Age X Treatment ANOVA on weight gain found the interaction significant ($F_{4,66} = 7.18$, $p < 0.001$). There was no effect of treatment on weight gain for P35 rats ($p = 0.63$). For P45, CTL rats gained less weight than testosterone-treated rats ($p = 0.003$) and DHT-treated rats ($p = 0.031$), which did not differ from each other ($p = 0.339$). For P75, CTL rats lost weight whereas testosterone-treated rats ($p = 0.001$) and DHT-treated rats ($p < 0.001$) rats gained weight. The higher weight gain in DHT-treated rats than in testosterone-treated rats did not reach significance ($p = 0.063$) (see Figure 7). These results show that P45 and P75 rats treated with testosterone or DHT implants gained more weight than rats treated with an empty implant.

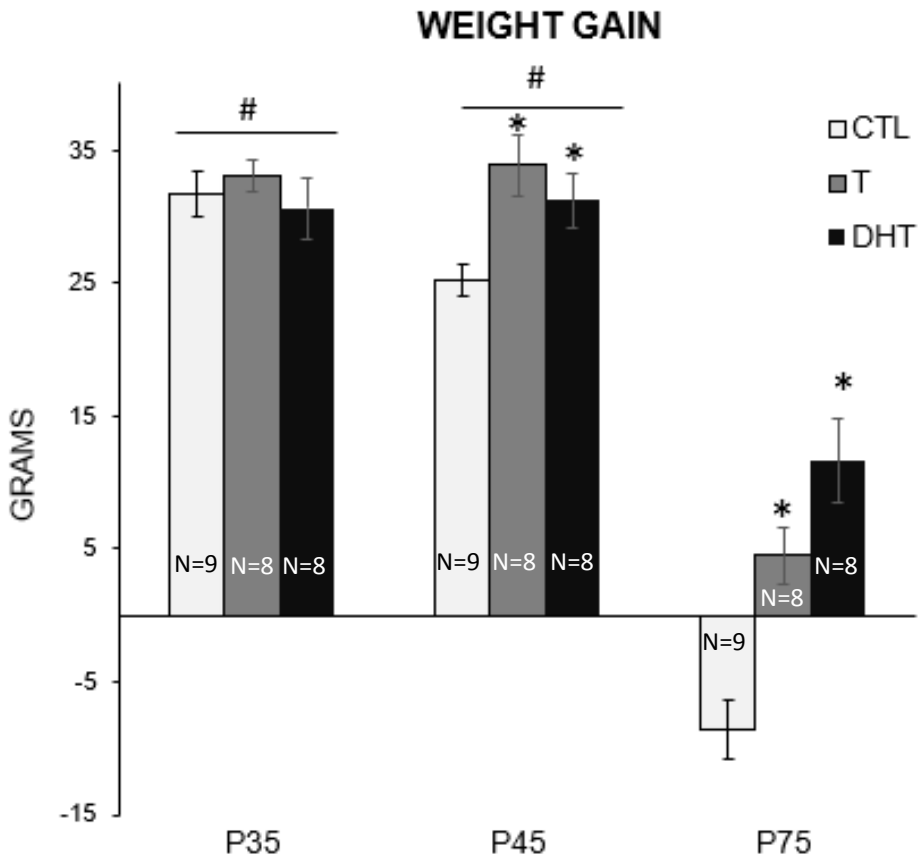

Fig. 7. More weight gain in testosterone-treated and DHT-treated rats than in controls indicates that implants used were effective. Mean (±S.E.M.) weight gain 5 days after surgery in P35, P45, and P75 male rats. *Indicates significant drug effect within age ($p < 0.05$). # Indicates significant increase compared to adults (main effect of age) ($p < 0.05$).

Plasma CORT and Progesterone Levels after Restraint

I measured plasma CORT to investigate how testosterone and DHT affect CORT levels (stress response) in P35, P45, and P75 male rats after 30 min of restraint stress. I also measured progesterone because it acts as a precursor for CORT production and because progesterone is also released from the adrenal cortex during stress**.** An Age X Treatment ANOVA revealed no main effect for CORT levels or significant interaction (all $p > 0.40$) (see Figure 8). For progesterone levels, the effect of age was significant ($F_{2,48} = 3.29$, $p = 0.046$), with P75 rats having lower progesterone levels than P45 rats ($p = 0.015$) or P35 rats ($p = 0.068$), which did not differ from each other (P35 and P45) ($p = 0.905$). The effect of treatment also was significant ($F_{2,48} = 5.97$, $p = 0.005$), whereby CTL rats had higher progesterone levels than DHT-treated rats ($p = 0.002$) and testosterone-treated rats ($p = 0.004$) rats, which did not differ from each other ($p = 0.905$). The interaction of age and treatment was not significant ($p = 0.939$) (see Figure 9). In summary, these results showed no effect of either androgen on plasma CORT concentration following stress at all ages; however, both androgens decreased plasma progesterone levels at all ages, and it appears that P75 rats have less progesterone than P35 and P45 rats.

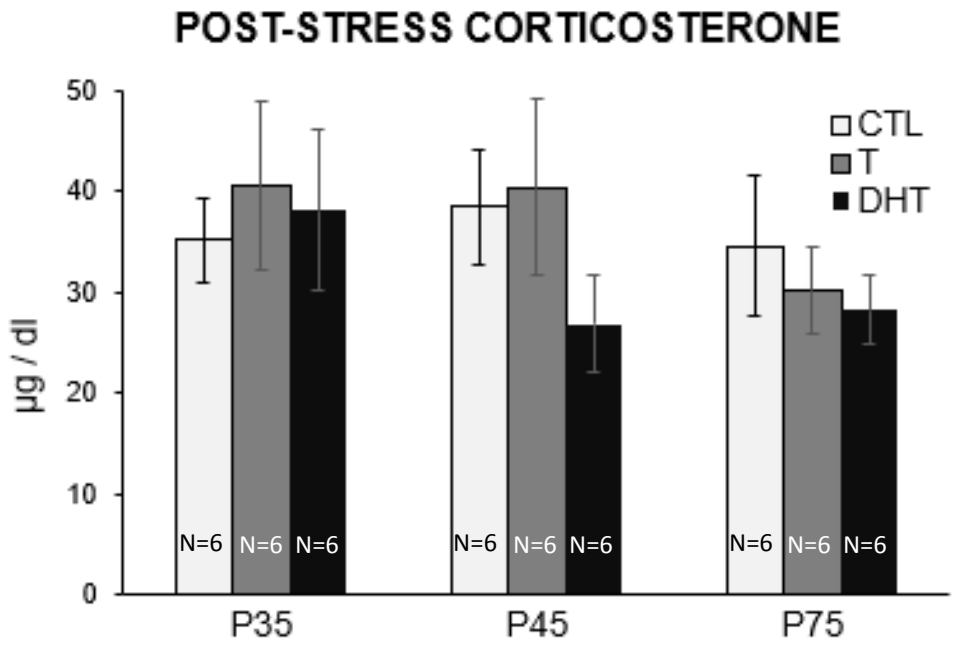

Fig. 8. Testosterone and DHT did not affect plasma CORT levels at any age. Mean (±S.E.M.) plasma hormone concentrations of corticosterone immediately after 30 min of restraint stress in P35, P45, and P75 male rats.

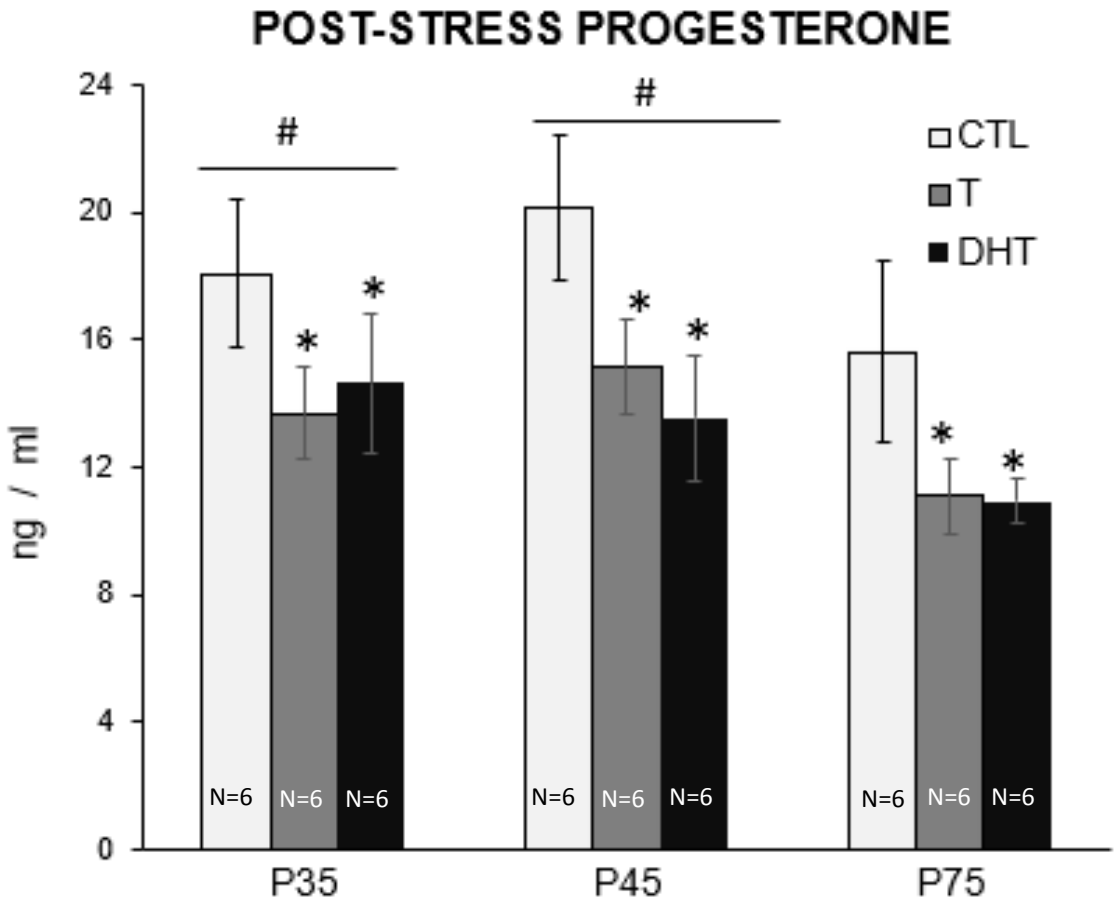

Fig. 9. Testosterone and DHT reduced plasma progesterone levels at all ages and adolescent rats (P35 and P45) exhibit higher progesterone levels than P75 rats. Mean (±S.E.M.) plasma hormone concentrations of progesterone immediately after 30 min of restraint stress in P35, P45, and P75 male rats. *Indicates significantly lower than CTL ($p < 0.05$). # Indicates significantly higher compared to adults ($p < 0.05$).

Immunoreactive (ir) Cell Counts of CRH, AVP, and Aromatase, 45 Mins after Restraint.

I counted AVP-ir cells within the parvocellular PVN at all ages to see how androgens affect such cells that produce this neuropeptide because AVP production from the parvocellular PVN is known to cause the release of ACTH from the anterior pituitary and is regulated by androgens in adults. I also counted AVP-ir cells within the magnocellular PVN in which neurons in that area also secrete AVP, but have no impact on initiating the stress response, so AVP-ir cells within the magnocellular PVN served as a control. For AVP-ir cell counts in the parvocellular PVN, the Age X Treatment ANOVA found only an effect of treatment ($F_{2,50}$ = 8.86, p = 0.001) There was neither an age effect (p > 0.30), nor an interaction between age and treatment (p > 0.30). CTL rats had higher AVP-ir cell counts than DHT-treated rats (p = 0.002) and testosterone-treated rats (p = 0.004), which did not differ from each other (p = 0.905). There was no effect of age, treatment, or interaction for AVP-ir cells in the magnocellular PVN (all p >0.23) (see Figure 10).

I also counted CRH-ir cells within the parvocellular PVN, because CRH is also produced and secreted from neurons within the parvocellular PVN and is involved in initiating the stress response. The Age X Treatment ANOVA found only an effect of treatment ($F_{2,40}$ = 64.7, p < 0.0001). There was neither an age effect (p > 0.15), nor an interaction between age and treatment (p > 0.15). CTL rats had higher CRH-ir cell counts than DHT-treated rats (p < 0.001) and testosterone-treated rats (p < 0.001), which did not differ (p = 0.416) (see Figure 10).

Finally, I counted aromatase-ir cell counts in the parvocellular PVN to test whether the age difference in aromatase production might be a reason for having an age difference in CORT release seen in Experiment 1 (Figure 4). The Age X Treatment ANOVA was marginal ($F_{2,49}$ = 2.63, p = 0.082; age effect and interaction, both p >0.68) (see Figure 10).

In summary, both testosterone and DHT reduced AVP, and CRH-ir cells within the parvocellular PVN of the hypothalamus in all age groups, but had no effect on aromatase-ir cell numbers.

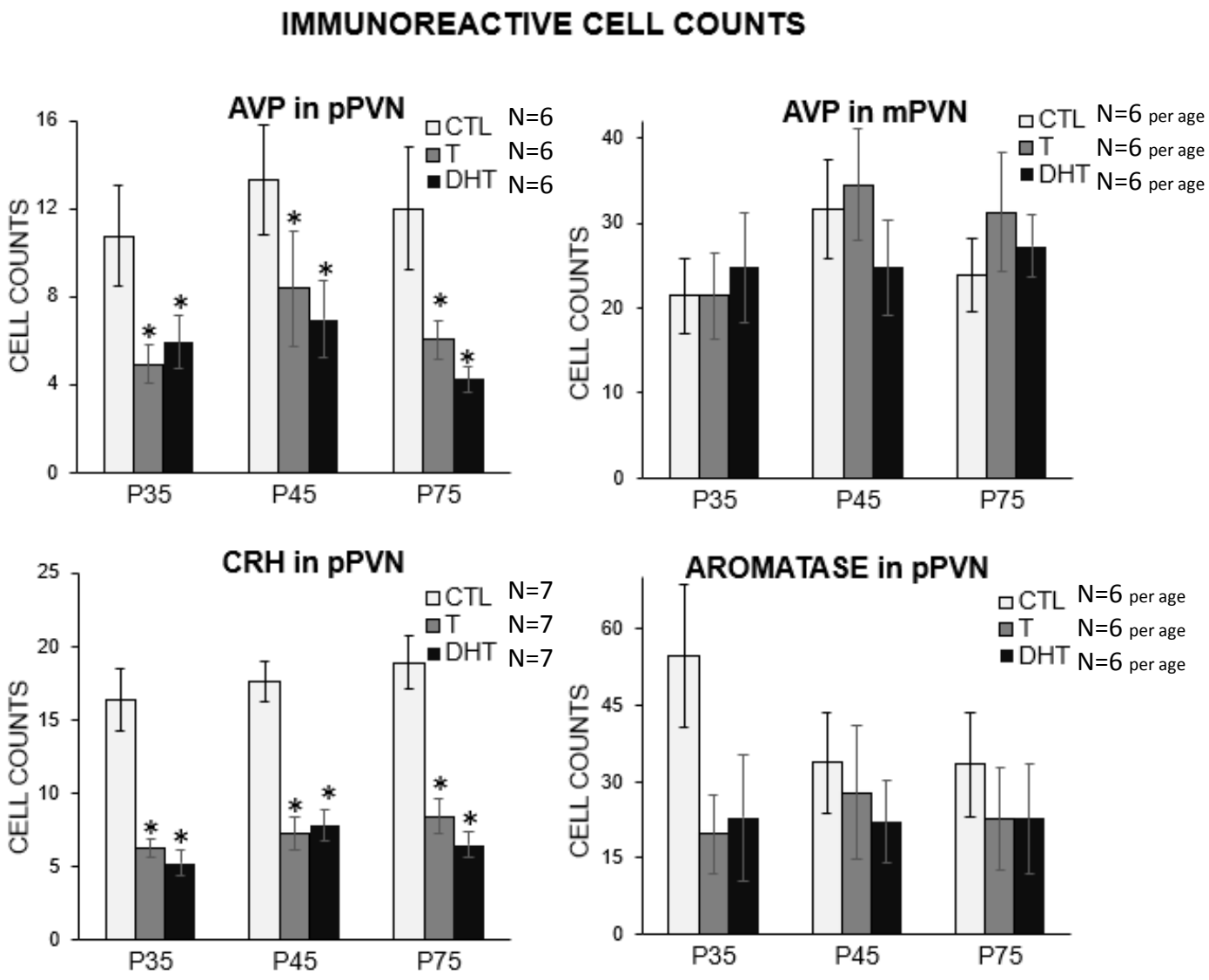

Fig. 10. Testosterone and DHT decreased immunoreactive (ir) cell counts of CRH and AVP at all ages, but not aromatase in the parvocellular PVN of the hypothalamus. Mean (±S.E.M.) number of cells expressing AVP in the parvocellular PVN and magnocellular PVN, CRH and Aromatase in the parvocellular PVN, 45min after a 30min restraint stress in P35, P45, and P75 male rats. *Indicates significantly lower than CTL ($p < 0.05$).

Discussion

Consistent with the results in Experiment 1, the weight gain data show that the implants were delivering androgens, as both DHT-treated and testosterone-treated rats gained more weight than their controls in P45 and in P75 rats. In P35, there was no difference in weight gain between the testosterone and DHT-treated groups compared to their controls, which suggests that P35 rats are insensitive to androgen actions on body weight. One reason for this insensitivity could be that the body of P35 rats continues to develop and is not fully developed to respond to testosterone the same way a fully developed/mature adult would. In other words, under normal conditions P35 rats exhibit very low plasma testosterone levels compared to P75 rats (Green et al., 2016). Although why P35 rats did not respond to androgens on this measure is unknown, one possibility is that there is such a significant rate of growth at this age, no further growth can be promoted (a ceiling effect). The same increase of about 35 grams in P35 and P45 rats is a higher percentage increase in body weight in P35 than in P45.

In the current study, I measured progesterone levels because of its significant role in steroidogenesis (e.g., a precursor for CORT production), and because progesterone is also released from the adrenal cortex during stress (Hueston & Deak, 2014). Therefore, the effects of androgens are not only evident on CORT levels, but also on progesterone levels. There was a decrease in plasma progesterone levels in testosterone and DHT-treated rats compared to CTL rats at all three ages (Figure 9). Thus, androgens can dampen progesterone release at all 3 ages, but do not eliminate differences in progesterone secretion. Thus, the age differences in HPA responses to stress are not solely the result of immature gonadal status in adolescents relative to adults. Further, because I had found an increase in CORT release in testosterone-treated rats at P45 in Experiment 1 (Figure 4), I expected that progesterone levels might increase in P45 rats after testosterone treatment in Experiment 2. However, this prediction was not supported. My

results are consistent with the results of Green (2017), who also found that CTL rats had higher progesterone concentrations than both DHT-treated and testosterone-treated rats and that P75 rats had lower progesterone concentrations than P45 and P35 rats. Those results are also consistent with what was found in pre-pubertal adolescents compared with adults in another study (Romeo et al., 2004, 2005).

In contrast to the results in Experiment 1 and the initial results of Green (2017), there was no dampening effect of either androgen in P75 rats (Figure 8). Further, at P45 we expected that testosterone treatment would increase CORT levels, as in Experiment 1 and in Green, (2017). I also expected that DHT treatment would decrease CORT levels because DHT cannot be metabolized to estradiol and increase CORT release. Both expectations were not supported by my results in Experiment 2 (Figure 8). The inability to detect a difference maybe because of the reduced sample size and the variabilities within the groups.

I also investigated androgens' effect on the HPA axis at the level of the hypothalamus. Treatment with both androgens resulted in a decrease of CRH and AVP-ir cell counts in the parvocellular PVN in P35, P45, and P75 rats (Figure 10). Such results indicate that the PVN of the hypothalamus appears responsive to androgens at all ages. This result contradicts my hypothesis, as I predicted that in P35 rats, androgen treatment would not affect AVP and CRH-ir cell counts. This expectation is based on previous studies showing that HPA function at the level of the pituitary and the adrenal in P35 rats is not responsive to the effects of testosterone treatment (Romeo et al., 2004). My results indicate that androgen treatment in P35 rats decreased AVP and CRH-ir cell counts compared to CTL rats (Figure 10). Further, I predicted that in P45, testosterone treatment would increase stress-induced AVP and CRH-ir cell counts because having greater number of AVP and CRH secreting cells would explain the greater CORT release

seen in Experiment 1 (Figure 4). Instead, testosterone treatment decreased AVP and CRH-ir cell counts compared to CTL rats (Figure 10). I also expected that testosterone treatment would increase stress-induced aromatase-ir cell counts in the parvocellular PVN because I proposed that an age difference in aromatase production might be a reason for having an age difference in CORT release seen in Experiment 1 (Figure 4). However, my results show that testosterone treatment had no effect on aromatase-ir cell counts compared to CTL rats (Figure 10).

Consequently, there could be two explanations for these results. The first possibility is that treatment with both androgens can dampen adolescent HPA function, as evident by the decrease in progesterone levels and AVP and CRH cell count in the parvocellular PVN of testosterone and DHT-treated rats. But such results leave the CORT data from Experiment 1 (Figure 4) and initial results of Green (2017) unexplained. The second possibility is that testosterone's differential effects in adolescence and adults occur at the level of the adrenal gland, but that the present experiment resulted in too much variation in CORT levels (Figure 8) thereby preventing the effect of androgens to be evident on CORT levels across the three age groups (P35, P45, P75). That the variability in CORT levels (Figure 8) masked effects is a likely probability, since the majority of the studies in adults show that testosterone and DHT usually (Viau & Meaney, 1991; Handa et al., 1994; Romeo et al, 2004; Green et al, 2016; Lund et al., 2004, 2006; Handa et al., 2011), but not always (Leśniewska et al., 1989; Buckingham, 1982), significantly dampen stress-induced CORT. Further, my results examining CORT levels are not consistent with the vast number of studies showing greater CORT release in adolescents than in adults, in response to stress (Klein & Romeo., 2013; Green & McCormick 2016; Green et al., 2016; Lui et al., 2012, Romeo et al., 2014). Moreover, if possibility 2 is indeed the case, the results suggest that at the level of the adrenal gland, testosterone's age-dependent actions on

CORT levels might happen downstream of progesterone, such as altering the enzymes (CYP21 and CYP11B2) that convert progesterone to CORT.

Testosterone might have age-dependent actions to regulate HPA function at neural sites other than the PVN, such as the medial preoptic area (mPOA), which is an important site for androgen's effects. The mPOA is rich in ARs and has functional connections to the PVN. Studies involving androgen replacement limited to the mPOA decreased AVP hnRNA in the PVN (Williamson et al., 2010) and dampened corticosterone release (McCormick et al., 2002). A study by Viau & Meaney, (1996) on adult male rats showed that the mPOA is a critical site for the inhibitory effects of androgens on the HPA. They showed that CORT receptor binding in the mPOA was increased in GDX males with high testosterone replacement levels. This result suggests that testosterone may inhibit HPA responses to stress by enhancing glucocorticoid feedback at the level of the mPOA. The same study showed that in mPOA-lesioned rats, high peripheral testosterone replacement levels failed to inhibit CORT release during restraint stress, which also favor the mPOA as a critical site for the effects of testosterone on HPA response to stress. Progesterone and CORT are associated, for the former acts as a precursor for the latter and ACTH acts as a secretagogue of both hormones (Hueston & Deak, 2014). However, progesterone can act as a precursor for other steroids such as testosterone; therefore, we might not expect the same effects of androgens on progesterone levels compared to CORT.

Experiment 3

Introduction

The results from Experiment 1 and 2 indicate that androgens can alter aspects of the HPA axis such as CRH and AVP cells within the PVN in both prepubertal and post-pubertal adolescents, but does not resolve the basis of the age differences in HPA responses to stressors. Thus, in Experiment 3, I changed the research strategy from the comparison of GDX and GDX + replacement rats at each age to the investigation of non-operated rats. This change was done to remove the additional variation that might have resulted from surgical stress (Morales & Spear, 2012). Another rationale for this approach is based on the findings of age differences in stress-induced CORT release in post-pubertal adolescents compared with adults, despite similar concentrations of testosterone (Green et al., 2016). The primary hypothesis for Experiment 3 is that age differences in stress-induced CORT levels are the result of a differential metabolism of testosterone at P45 and P75. Specifically, the hypothesis is that at P45 there is greater conversion of testosterone to estradiol, and less conversion of testosterone to DHT, whereas at P75 there is less conversion of testosterone to estradiol, and greater conversion of testosterone to DHT. To test this hypothesis, I used a vehicle control and three different drugs: a 5α-reductase inhibitor (finasteride) that blocks DHT production from testosterone, an aromatase inhibitor (fadrozole) that blocks estradiol production from testosterone, and an androgen receptor blocker (flutamide) to show that androgens cannot impose their effect on the stress system, if their receptors are blocked. I predicted that inhibition of aromatase (enzyme that converts testosterone to estradiol) would lead to a decrease of stress-induced CORT levels at P45 and P75 compared with vehicle-treated rats, but this decrease in CORT levels would be greater at P45 than at P75. I also predicted that either inhibition of 5α-reductase or androgen receptor blockade would lead to

increased CORT release at P45 and P75 compared with vehicle-treated rats, but this increase in CORT levels would be greater at P75 than at P45.

Methods

Animals

For Experiment 3, male Long-Evans rats were obtained at P34 (n = 48; 90–115 grams), and P63 (n = 48; 280–320 g) from Charles River (St. Constant, Quebec) and housed in same-aged pairs. Rats were given free access to food and water and kept on a 12 h light-dark cycle (lights on at 09:00). All procedures were approved by the Brock University Animal Care and Use Committee and were in keeping with the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) as well as the Canadian Council on Animal Care guidelines.

Injections

Five days after the arrival of the rats to the facility, they were weighed and then injected daily for three days with either a 5α reductase inhibitor (finasteride, 25mg/kg as in George 1997, n = 12 per age group) dissolved in 5% β (beta)-cyclodextrin in saline, or an aromatase inhibitor (fadrozole, 1mg/kg as in Browyn & Milad, 2014, n = 12 per age group) dissolved in 0.9% saline, or an androgen receptor blocker (flutamide suspended in sesame oil, 25 mg/kg as in Shin et al., 2002, n = 12 per age group), or 0.9% saline (control group, n = 12). All injections were done within one hour of lights on.

Acute stressor procedure and sample collection

The acute stressor procedure was conducted within ~3 h after the third injection. Tail blood was collected from rats either directly from the home cage (baseline), or immediately after

30 min of restraint stress in Plexiglas® restrainers (post-restraint) into 2 ml microcentrifuge tubes containing EDTA. After 20 min of recovery from restraint stress, all rats were decapitated and trunk blood was collected. This range in time of day was chosen for the experiments so that samples were collected during the phase of the light cycle when basal plasma corticosterone concentrations are low. Blood samples were centrifuged at 3000 RCF for 10 min (for tail blood) and 3000 RCF for 15 min (for trunk blood). Plasma was collected and stored at -20^{o}C until hormone assays were conducted. Brains were quickly extracted, sliced into 1 mm thick sections on ice, and frozen on dry ice before stored at -80^{o}C until further processed. Adrenals were also extracted and collected into microcentrifuge tubes, fast-frozen on dry ice, and then stored at -80^{o}C until further processed.

Hormone assays

Steroids were extracted from plasma using diethyl ether for the measurement of CORT, progesterone, and testosterone. All extracted samples were reconstituted in buffer provided in the enzyme-linked immunosorbent assay kits (Neogen, Lansing MI) and the assays were conducted according to the kit instructions and using a Biotech Synergy plate reader; all samples within an experiment were measured on the same day. Assay sensitivity was 0.05 ng/mL for CORT, 0.4 ng/mL for progesterone, and 0.002 ng/mL for testosterone (the Elisa kit for testosterone had a 100% cross reactivity with dihydrotestosterone).

Statistical analyses

Statistical analyses were performed using SPSS (version 23) software and consisted of mixed (first and last day of treatment as a within factor for weight) and between group (Age, Drug) factor analysis of variances (ANOVAs). Post hoc analyses consisted of F tests for simple

effects and Fisher's Protected Least Square Differences (LSD), and t-tests where appropriate. An alpha level of $p < 0.05$ was used to determine significance.

Results

Weight Gain 2 Days after First Injection.

I measured the weight gain in P45 and P75 male rats from the first day of drug (VEH, flutamide, finasteride, and fadrozole) injection to third day of injection to confirm that the drugs were effective. An Age X Treatment X Time (first injection day, third injection day) on weight found an interaction of age and time ($F_{3,88}$= 9.38, p = 0.003), an interaction of treatment and time (p = 0.001),but a three-way interaction was not significant (p = 0.055). To better explore treatment effects, an ANOVA was conducted on weight gain in adolescents and adults separately. In adolescents, the effect of treatment was significant ($F_{3,44}$ = 5.39, p = 0.003), with flutamide-treated rats gaining less weight than the three other groups (all p < or = to 0.01), which did not differ from each other (all p > 0.45). In adults, the effect of treatment was significant ($F_{3,44}$ = 2.83, p = 0.045), with both flutamide-treated (p = 0.034) and finasteride-treated rats (p = 0.024) gaining less weight than the VEH-treated rats. All other comparisons were not significant (all p >0.06) (see Figure 11). These weight data show that the. drug injections carried out were effective.

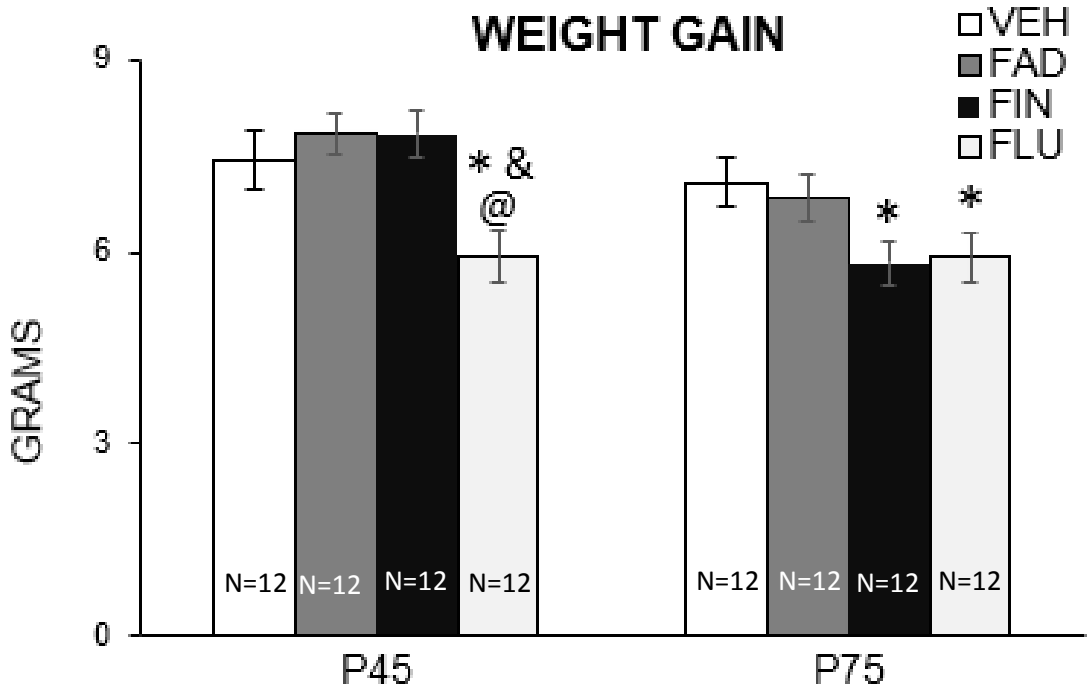

Fig. 11. Weight gain data indicates androgen receptor blockade decreases weight gain at both ages, and that blockade of testosterone conversion to DHT decreases weight gain only at P75. Mean (±S.E.M.) weight gain from first day to third day of injections in P45 and P75 male rats. VEH –=vehicle; FAD = fadrozole, aromatase inhibitor; FIN = finasteride, 5a-reductase inhibitor; FLU = flutamide, androgen receptor (AR) antagonist. *Indicates a significant difference from VEH ($p < 0.05$); & Indicates significant decrease from FAD ($p < 0.05$); @ Indicates significant decrease from FIN ($p < 0.05$).

Plasma Testosterone Levels before Restraint (Baseline Levels).

I measured plasma testosterone levels to see how each drug would affect baseline testosterone concentrations in P45 and P75 male rats because age difference in testosterone levels might explain why CORT release is typically higher in P45 than in P75 rats, since testosterone typically dampens CORT release. An Age X Treatment ANOVA on baseline testosterone concentrations found a significant interaction ($F_{3,66} = 4.38$, $p = 0.007$). In P45 rats, testosterone concentrations were higher in flutamide-treated rats than in the other groups (VEH, $p = 0.029$; fadrozole, $p = 0.002$; finasteride, $p = 0.02$; all other comparisons, $p > 0.21$). In P75 rats, testosterone concentrations were higher in flutamide-treated rats than in the other groups (all $p < 0.001$; all other comparisons, $p > 0.14$). P45 rats had higher concentrations of testosterone than P75 rats in the finasteride group ($p = 0.004$), and P75 rats had higher concentrations of testosterone than P45 rats in the flutamide group ($p = 0.024$) (other age comparisons, $p > 0.42$) (see Figure 12). In summary, the main conclusion from these results is that under normal conditions (VEH group) P45 and P75 have the same baseline plasma testosterone. The higher testosterone in flutamide treated rats is likely a result of negative feedback, with androgen receptor blockade promoting an increase in testosterone production.

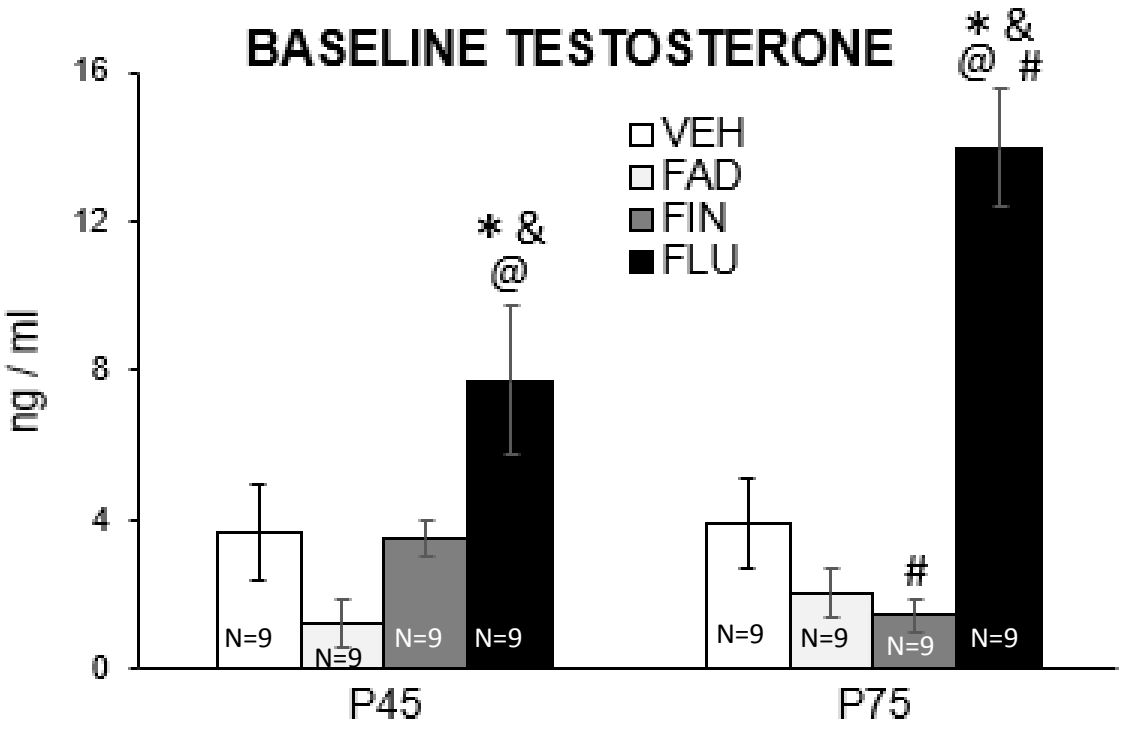

Fig. 12. Baseline testosterone levels show that in VEH group, P45 and P75 have the same concentration of plasma testosterone. Mean (±S.E.M.) plasma hormone concentrations of testosterone at baseline in P45 and P75 male rats. VEH = vehicle; FAD = fadrozole, aromatase inhibitor; FIN = finasteride, 5a-reductase inhibitor; FLU = flutamide, androgen receptor (AR) antagonist. *Indicates a significant difference from VEH ($p < 0.05$); & Indicates significant increase from FAD ($p < 0.05$); @ Indicates significant increase from than FIN ($p < 0.05$). # Indicates significant age difference for that drug group ($p < 0.05$).

Plasma CORT Levels immediately after Restraint and 20 Mins after Restraint.

I measured plasma CORT to investigate how each drug affects CORT levels in P45 and P75 male rats immediately after restraint and 20 min after restraint. An Age X Treatment X Timepoint ANOVA on CORT concentration found all main effects and interactions significant (all $p < 0.016$) except for the interaction of Age X Treatment ($p = 0.835$) and the three-way interaction ($p = 0.944$). Thus, separate analyses were conducted for each timepoint.

Immediately after restraint, P45 rats had higher CORT levels than P75 rats ($F_{1,68} = 4.17$, $p = 0.045$), and there was an effect of treatment ($F_{3,68} = 9.61$, $p < 0.0401$; interaction, $p = 0.352$), whereby VEH-treated rats had lower CORT concentrations than flutamide-treated rats ($p = 0.021$). Further, At P45 VEH-treated rats had higher CORT concentrations than did fadrozole-treated rats ($p = 0.011$) VEH-treated rats did not differ from finasteride-treated rats ($p = 0.087$). Fadrozole-treated rats had lower CORT concentrations than finasteride-treated rats ($p < 0.001$) and flutamide-treated rats ($p < 0.001$). Although, the interaction was not significant, the pattern of means suggested treatment differences for P45 rats and P75 rats that were consistent with my prior predictions, and thus separate analyses were conducted at the two ages. In P45 rats, fadrozole-treated rats had lower CORT concentrations than in the other three groups (all $p < 0.012$), and no other comparison was significant ($p > 0.28$). In P75 rats, fadrozole-treated rats had lower CORT than finasteride-treated rats ($p = 0.002$) and flutamide-treated rats ($p = 0.007$), which did not differ ($p = 0.726$), and fadrozole did not differ from VEH ($p = 0.279$). Also, in P75 rats, VEH had lower CORT than finasteride-treated rats ($p = 0.03$) and was not significantly different from flutamide-treated rats ($p = 0.076$). In VEH rats, P45 rats had higher CORT concentrations than did P75 rats ($p = 0.003$), and no other age difference was significant (all $p > 0.09$) (see Figure 13).

I also measured CORT levels at 20 min after restraint to see if there were treatment differences in CORT levels during the recovery period after a stressful situation. At 20 min after restraint, P45 rats had higher CORT levels than P75 rats ($F_{1,79} = 17.45$, $p < 0.001$). There was an effect of treatment ($F_{3,79} = 5.126$, $p = 0.003$; interaction, $p = 0.819$), whereby fadrozole-treated rats had lower CORT levels than both finasteride-treated rats ($p = 0.014$) and flutamide-treated rats ($p < 0.001$). The lower CORT levels in fadrozole-treated rats relative to VEH-treated rats did not meet statistical significance ($p = 0.053$). Also, the lower CORT levels in VEH-treated rats relative to flutamide-treated rats, did not meet statistical significance ($p = 0.075$; both other comparisons $p > 0.16$) (see Figure 13).

In summary, blocking aromatase action using Fadrozole injection in P45 eliminated the age difference in CORT release seen in VEH groups in P45 and P75. Blocking 5α-reductase using finasteride injection increases CORT release when compared to VEH, but only in P75. These results support the hypothesis than age differences in CORT release after stress involves a lower conversion of testosterone to DHT and a higher conversion of testosterone to estradiol in P45 rats compared with P75 rats.

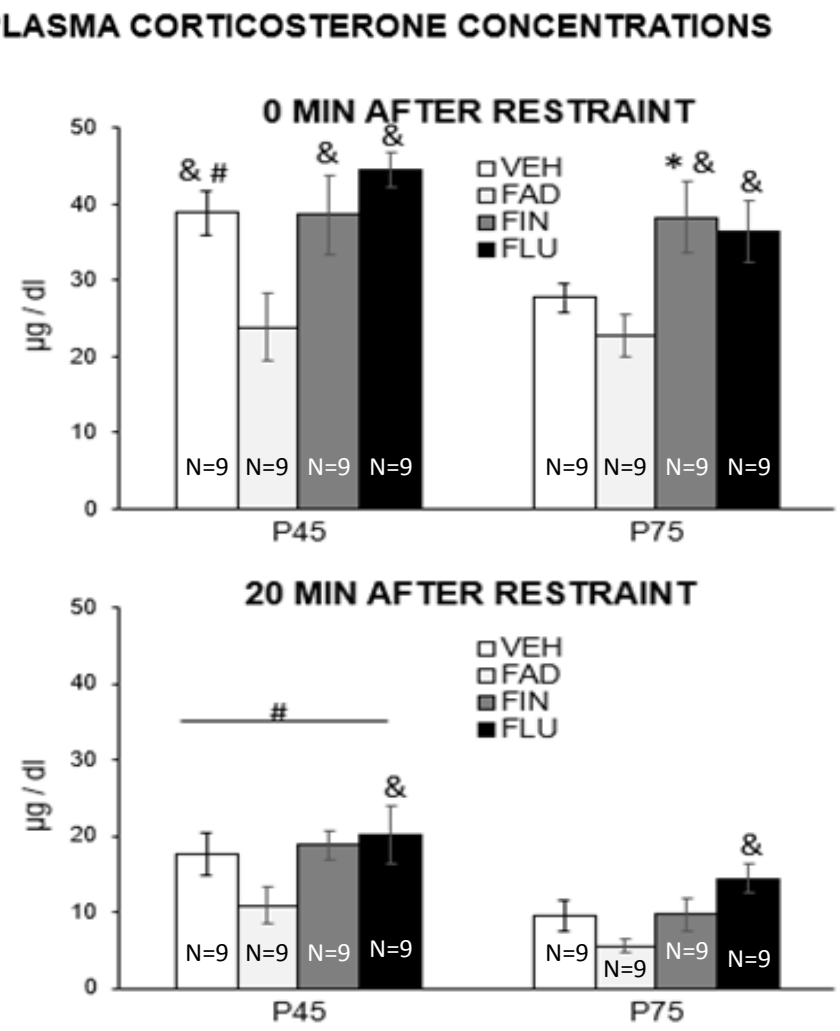

Fig. 13. **Blocking testosterone's conversion to estradiol attenuates the age difference in CORT concentrations immediately after restraint.** Mean (±S.E.M.) plasma hormone concentrations of corticosterone 0 min after restraint stress and 20 min after restraint stress in P45 and P75 male rats. VEH = vehicle; FAD = fadrozole, aromatase inhibitor; FIN = finasteride, 5a-reductase inhibitor; FLU = flutamide, androgen receptor (AR) antagonist. *Indicates significant difference from VEH ($p < 0.05$); & Indicates significant increase from FAD ($p < 0.05$). # Indicates significant age difference for that drug group ($p < 0.05$).

Plasma Progesterone Levels, 20 Mins after Restraint.

As mentioned previously, I measured plasma progesterone because of its importance in steroidogenesis (e.g., a precursor for CORT production) and thus provided an additional measure of androgen's effects on stress responses. An Age X Treatment ANOVA found that 20 min after restraint, P45 rats did not differ in progesterone concentrations from those of P75 rats ($F_{1,72}$ = 1.80, p = 0.184), and there was an effect of treatment ($F_{3,72}$ = 10.99, p < 0.001; interaction, p = 0.499). Flutamide-treated rats had higher progesterone levels than the other three groups (all p < 0.04). Finasteride-treated rats had higher progesterone levels than fadrozole-treated rats (p = 0.001) and higher progesterone levels compared to VEH-treated rats did not meet statistical significance (p = 0.056). VEH-treated rats and fadrozole-treated rats did not differ significantly (p = 0.154). The means suggested similar effects of treatment in both ages, thus I only tested for age differences within each group based on my previous finding of higher progesterone levels in P45 than in P75 rats. In VEH-treated rats, P45 rats had higher progesterone concentrations than P75 rats (p = 0.034), and no other age difference was significant (all p > 0.20) (see Figure 14). These results show that under normal conditions (VEH group) P45 rats have higher progesterone levels than P75 rats in response to stress. Further, blocking ARs with flutamide injection increases progesterone levels at both ages when compared to VEH-treated rats.

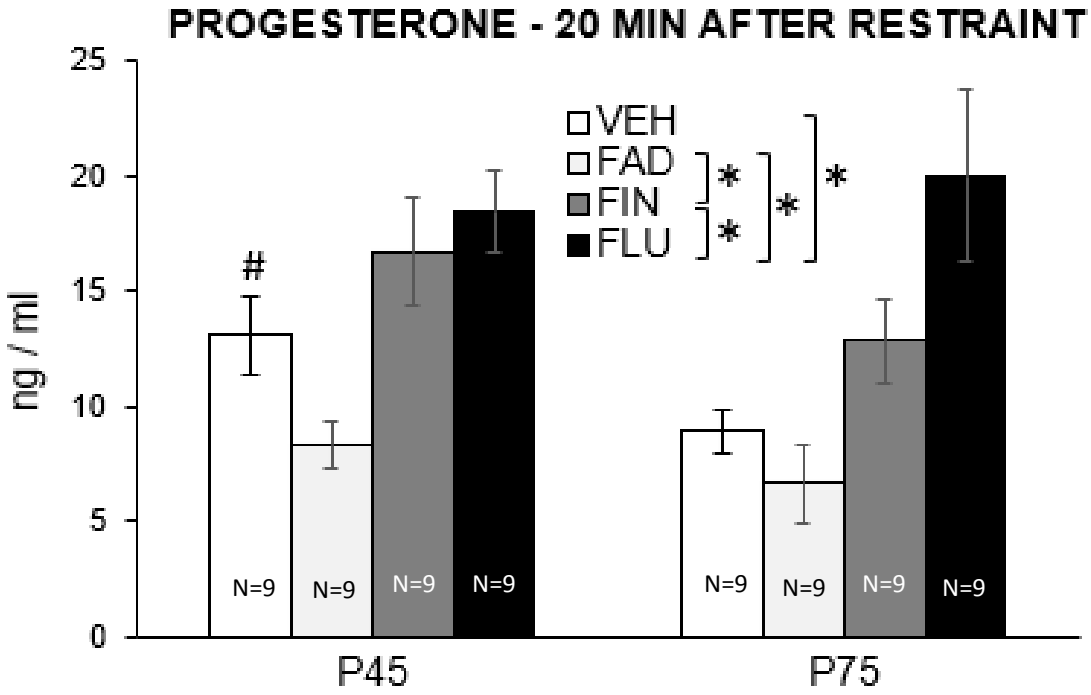

Fig. 14. Plasma progesterone levels 20 min after a restraint stress indicates that in VEH-treated rats, P45 have higher progesterone levels than P75 rats, and the age difference is attenuated by treatment with fadrozole. Mean (±S.E.M.) plasma hormone concentrations of progesterone 20 min after restraint stress in P45 and P75 male rats. VEH = vehicle; FAD = fadrozole, aromatase inhibitor; FIN = finasteride, 5a-reductase inhibitor; FLU = flutamide, androgen receptor (AR) antagonist. *Indicates significant difference among injection groups ($p < 0.05$). # Indicates significant age difference for that drug group ($p < 0.05$).

Discussion

In both adolescents and adults, rats treated with the AR blocker, flutamide, gained less weight than VEH-treated rats. This result is consistent with evidence that androgen actions at AR promote weight gain (Gentry & Wade., 1976). In adults, but not in adolescents, finasteride-treated rats gained less weight than VEH-treated rats. These results show that blocking DHT production impacts weight gain only in adults. This finding may be because of less DHT being produced in adolescents, which is in keeping with my hypothesis of less conversion of testosterone to DHT in adolescence. Thus, blocking 5α-reductase wouldn't influence weight gain at P45. Fadrozole-treated rats did not differ from controls in weight gain at either P45, or P75. Both male and female rats treated with estradiol in adulthood are shown to gain less weight than their controls (McCormick et al., 2002). It may be that blocking estradiol production with fadrozole injection for only three days is not sufficient to affect weight gain.

For CORT measured immediately after restraint stress, among vehicle-treated rats, P45 rats had greater CORT release compared to adult P75 rats. These results are consistent with previous reports in the literature. For example, Green and colleagues (2016) found that P45 rats had a greater stress response than P75 rats. Also, for vehicle-treated rats, P45 rats had greater progesterone release compared to adult P75 rats. This age difference is not explained by the difference in gonadal status because our testosterone data (Figure 12) show that baseline testosterone concentration is the same for both age groups, which is also in line with previous results (Green et al., 2016). The results from this experiment suggest that the age difference in CORT release could be because of greater conversion of testosterone to estradiol by aromatase in P45 than in P75 rats; P45 rats that were given fadrozole (an aromatase inhibitor) displayed reduced CORT levels and progesterone levels compared to their VEH control group and

eliminated the age difference that was evident in the vehicle group. Fadrozole in P75 rats did not influence either CORT or progesterone release when compared with vehicle-treated P75 rats. These results show that in P45 rats, the presence of aromatase and the conversion of testosterone to estradiol might be important for the heightened stress response, relative to adults. It is well-established that estradiol increases HPA function (Burgess & Handa, 1992; Handa et al., 1994; Suzuki et al., 2001; Weiser & Handa, 2009). My results also suggest that the aromatization of testosterone to estradiol does not play a significant role in modulating HPA activity in adults. Other research has indicated that aromatase activity within the preoptic area is lowest in adulthood than other stages in the developing male rat (Lauber, 1994). This result is relevant to my findings because it suggests that in the adult brain (preoptic area, a main site for androgen regulation of the HPA axis) there isn't much conversion of testosterone to estradiol that would increase the stress response, whereas in the adolescents there is more conversion of testosterone to estradiol.

In contrast, P75 rats were responsive to finasteride (5α-reductase inhibitor) whereas adolescents were not. Rats given finasteride at P75 showed increased CORT and progesterone release compared to vehicle-treated rats. These results show that regulation of the stress response in adults involves conversion of testosterone to DHT, which is consistent with evidence in Handa et al., (2013) that shows that blocking DHT production from testosterone increases the stress response in the adult rat. Such results highlight the importance of DHT production as a means to reduce stress responses in the adult rat. Unlike P75 rats, P45 rats treated with finasteride did not show a change in CORT and progesterone release when compared to their VEH-treated rats. Therefore, blocking DHT production at this age doesn't seem to affect HPA function, which is in keeping with my hypothesis that there is little conversion of testosterone to DHT in adolescence

either because of reduced 5α-reductase or increased aromatase at that age. A study by Melcangi et al., (1988) showed that within the hypothalamus, DHT formation was highest 6-7 days after birth and then decreased to be lowest in post-pubertal adolescence (40-45 PND), and then increased into adulthood.

The present results showed that blocking AR with flutamide increased CORT and progesterone release at P45. These results show there is some dampening effect of testosterone via ARs at this age as there is in adults (Handa & Weiser, 2014; Green & McCormick, 2016). In P75 rats, flutamide increased CORT release relative to vehicle, but this increase missed statistical significance ($p = 0.075$). However, flutamide was able to significantly increase progesterone release at P75, again showing the importance of ARs in modulating adrenal steroids. Blocking ARs also increased baseline plasma testosterone at both ages, but more so in P75 rats. This result could be because of a greater negative feedback response in P75 rats than in P45 rats and /or a greater capacity for testosterone production in adults.

In sum, the results from these experiments suggests that a greater conversion of testosterone to estradiol in adolescence than in adulthood is the basis for the greater release of CORT in response to stress in adolescents than in adults.

General Discussion

Adolescence is a transitional period between childhood and adult hood that is characterized by many psychosocial and physiological changes (McCormick et al., 2010; Romeo, 2013). One such change is how an individual responds to stressors. Specifically, adolescence is marked by significant shifts in HPA axis reactivity, resulting in heightened stress-induced hormonal responses (Romeo et al., 2005; Green et al., 2016). Adolescence is also a significant period of continued neural maturation, specifically within stress-sensitive limbic and cortical regions (Eiland & Romeo, 2013; Romeo, 2013). Therefore, the increases in stress-related dysfunctions during adolescence, such as anxiety, depression, schizophrenia, and drug abuse highlight the importance of a better understanding of the interaction between changes in stress reactivity and adolescent brain development. The experiments contained in this thesis are important because they can enhance our understanding of factors that determine adolescent development, and that allow for long-term organization of behavior by environmental stimuli during this time.

The overarching goal of this thesis was to investigate the basis of age differences in response to testosterone. In Experiment 1 I replicated the findings by Green, (2017), who found increased CORT release in response to testosterone at P45, in contrast to the dampening effect of testosterone at P75, after restraint stress. These findings reveal developmental shifts in HPA function and its regulation by gonadal hormones. In experiment 2 testosterone dampened AVP, CRH, and had no effect on aromatase within the PVN of the hypothalamus at both ages. These findings indicate that age-related differences in the effect of testosterone on stress-induced release of CORT do not involve differences in AVP, CRH, and aromatase production within the PVN. In Experiment 3, blocking aromatase eliminated the age difference in CORT release. which leads to the main conclusion that the basis for the age difference in CORT release is more

conversion of testosterone to estradiol at P45. However, the site where this conversion might be occurring is yet to be investigated. A study by Lephart & Ojeda, (1990) showed that aromatase activity within the hypothalamus is two times higher in post-puberty adolescents (P48) than in adult rats (P68). In addition, aromatase activity was up-regulated by androgens acting on AR in stress-related brain regions (e.g. BNST and mPOA) (Roselli & Resko, 1993), and the androgen-induced incearse was greater in post-puberty male rats compared with adults (Lephart & Ojeda, 1990). Therefore, a next step would be to examine immunoreactive cell counts of aromatase, ERα, and AR in the mPOA, which might help explain the age difference in HPA function, as studies have shown that the mPOA is a main site of aromatase activity (Roselli et al.,1985) and has a main role in regulating HPA function (Williamson et al., 2010; McCormick et al., 2002). Moreover, it would be important to examine the adrenals to measure gene expression of different proteins such as ERα, ERβ, AR, ACTH receptor, aromatase, and 5α-reductase, all of which are important to further elucidate the age difference in HPA function, and because of my evidence that some of the differential actions of testosterone in adolescents and adults may occur at the level of the adrenal gland.

Studies in this thesis have contributed to the field by providing evidence that there is a greater conversion of testosterone to estradiol in adolescents than in adults, which might be why adolescents have greater CORT release in response to stressors than adults; despite P45 and P75 rats having similar testosterone concentrations, testosterone does not have the dampening effect on CORT release at P45 that it does at P75. These results contribute to understanding the developmental shifts in HPA function during adolescence and adulthood. However, the mechanisms that direct some of the changes require additional future investigation. Another important question that remains to be investigated is why the adolescent HPA axis would be

differentially regulated by testosterone, and specifically, what is the potential adaptive value, if any, of a greater conversion of testosterone to estradiol in adolescence. One possibility is that more estradiol production is required in adolescence to ensure additional sexual differentiation of the brain at this point in development. In normal male development, the perinatal sexual differentiation of the brain primarily involves estradiol (Juraska et al., 2013; MacLusky et al., 1987). There is evidence that some additional sexual differentiation of the brain occurs in adolescence. For example, parts of hypothalamic regions such as the anteroventral periventricular nucleus (AVPV) and sexually dimorphic nucleus of the preoptic area (SDN) undergo sexual differentiation during adolescence. The AVPV and SDN of adult rats are sexually dimorphic, with the AVPV being larger in females than in males and the SDN being larger in males. This sexual difference is promoted by the presence or absence of testosterone during early postnatal development and adolescence (Arai et al., 1994, 1996; Juraska et al., 2013). Further, Cooke & Woolley (2009) showed that in pre-pubertal males, androgens acting on the medial amygdala (an important part of the mammalian social behavior system and sex-specific social behavior) promote the expression of sexually dimorphic behavior and promote the maintenance and development of new excitatory synapses in that brain rejoin. In early life, conversion of testosterone to estradiol via aromatase masculinizes the hypothalamus and adult reproductive behaviour and HPA function (Bingham et al., 2011; MacCarthy & Konkle, 2005). Thus, to allow proper development of the circuitry that allows for adult male sexual behavior may necessitate higher concentrations of estradiol in the brain.

Lastly, an answer to the above question, along with the results contained in this thesis, will improve our understanding of adolescent development and neuroendocrine function. Further, although the findings in rats are not always the same as in humans, they are important

for understanding the principles that govern neuroendocrine function in mammals and how it changes across development and provide a framework for studies in humans.

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