ROLE OF THE ANDROGEN RECEPTOR IN TESTOSTERONE'S EFFECTS ON ANXIETY-RELATED BEHAVIOR AND CORTICOSTERONE RESPONSE IN MICE

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ABSTRACT

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Anxiety disorders affect more women than men. Because gonadal hormones like testosterone (T) play a key role in establishing many sex differences, T may underlie the sex differences in human anxiety. Indeed, androgens act as anxiolytics in humans and other species. In mice, this effect normally requires functional androgen receptors (AR) since T has no such anxiolytic effects in genetic males carrying a dysfunctional allele for *AR*. Cre-LoxP technology was used to recombine *AR*, producing a dysfunctional allele that resulted in androgen insensitivity in XY mice, a phenotype referred to as *induced* testicular feminization mutation (*i*Tfm). These *i*Tfm males show more anxiety related behavior than their wild type (WT) brothers in several tests, including the novel object test and elevated plus maze. When the mice were tested during the animals' resting phase (lights on period), *i*Tfms did not differ from WT males in the light/dark box (L/D) test, replicating earlier results in our lab. However, when tested in the active phase, *i*Tfm males appeared more anxious in the light-dark box than their WT brothers, consonant with the differences between these mice in other tests of anxiety. WT males castrated as adults and treated with T have a reduced HPA response to mild stress, as measured by corticosterone release, than untreated WT castrates. In contrast, *i*Tfm males given T displayed a greater and more prolonged HPA response than WT males, indicating that AR activation normally attenuates the HPA axis. To determine whether

ARs affect anxiety-related behaviors and HPA response by acting within the central nervous system (CNS), mice carrying a transgene utilizing the nestin promoter to drive expression of Cre recombinase were crossed with mice carrying a floxed allele of AR. Offspring carrying both constructs were designated NesARKO. Contradicting expectations, the NesARKO mice displayed only a partial knockout (KO) of AR expression in the brain: while there was a full KO in the hippocampus, medial prefrontal cortex (mPFC), bed nucleus of the stria terminalis (BNST), and periaqueductal grey (PAG), AR immunoreactivity was still seen in many cells in the amygdala and hypothalamus. No differences in anxiety-related behaviors or HPA function were seen between NesARKOs and WT males, demonstrating that full KO of AR in the mPFC and hippocampus had no effect on these behaviors. Consequently, those regions cannot be sites at which AR acts to modulate these behaviors in WT males. A subsequent study monitoring neuronal activity as reflected in cFos expression indicated that WT males with T have more cells respond in the basolateral amygdala (blAMY) and fewer cells respond in the suprachiasmatic nucleus of the hypothalamus (SCN) than either *i*Tfms with T, or WTs without T. In WT males treated with T, approximately 65% of cells in the blAMY and SCN are AR positive, therefore AR may act directly on these cells to affect their response to mild stress. Together, these results demonstrate that T requires functional AR to modulate anxiety-related behavior and HPA function in mice, but does not act in the mPFC, hippocampus, BNST, or PAG to do so. T also acts through AR to affect the response of cells in the blAMY and SCN following mild stress, so these brain regions remain potential sites of action for T's anxiolytic effects in mice.

This dissertation is dedicated to my Papa, my Mama, Hao, Hui and Byron for their unconditional love, continuous support and encouragement.

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CHAPTER 1: INTRODUCTION

Anxiety disorders are the second most prevalent mood disorder. Each year, approximately 40 million American adults aged 18 and older, or about 18 percent of individuals in this age group, are diagnosed with an anxiety disorder (Kessler et al., 2005). This prevalence translates to more than $42 billion dollars in annual cost (Greenberg et al., 1999), approximately one-third of the cost for all mental health treatment (Health Central, web info). Anxiety disorders are unevenly distributed among the sexes in the United states and worldwide (Seedat et al., 2009); subcategories such as phobias, generalized anxiety disorder, panic disorder, and post-traumatic stress disorder are more prevalent in women than men (Weinstock, 1999; DSM IV, 2005). In addition, women have a higher adolescent onset of these disorders (Zahn-Waxler et al., 2008), and report higher anxiety levels during times of hormonal flux, such as puberty, menopause, and premenstrual and post-partum periods (Ahokas et al., 2001; Parker and Brotchie, 2004; Douma et al., 2005; Solomon and Herman, 2009). The marked sex differences in prevalence, and fluctuations of anxiety with varying hormone levels in women, suggest a role for gonadal hormones, which are known to play a unique role in establishing and triggering many sex differences in the brain and behavior.

Gonadal hormones and anxiety in humans

Clinical data indicate that gonadal hormones have a significant impact on anxiety in humans. Women are more prone to an anxiety episode during the premenstrual and postpartum periods, when estrogen (E) levels are low (Douma et al., 2005; Dean et al.,

1981). Similarly, women show more indices of anxiety disorder as they approach menopause, when E levels start to decline (Tangen et al., 2008), and E replacement reduces anxiety levels (Yazici et al., 2003). In addition, E treatment ameliorates anxiety in postmenopausal women (Frye, 2009; Yazici et al., 2003), and in breast cancer survivors regardless of age (Decker et al., 2003). These findings support an anxiolytic effect of E in women. Similarly, T has also been shown to correlate with anxiety levels. Hypogonadal men exhibit a higher prevalence of anxiety disorders compared to those with normal levels of androgens (Shores et al., 2004; Zarrouf et al., 2009), an effect reversed by T administration (Rabkin et al., 2000; Pope et al., 2003). Congruently, the decline in salivary testosterone throughout the day due to circadian rhythms is correlated with an increase in anxiety in adolescent males (Granger et al., 2003). Likewise, as men age, their T levels naturally decline, often accompanied by increased levels of anxiety (Amore et al., 2009; Sternbach, 1998). Additionally, androgen blockade therapy for prostate cancer treatment is accompanied by an increase in anxiety (DiBlasio et al., 2008), which was alleviated with treatment termination (Almeida et al., 2004). In women, the diagnosis of an anxiety disorder, including generalized anxiety or agoraphobia, correlates with lower levels of salivary T compared to that found in control women (Giltay et al., 2012). Another study in women found that a single administration of T reduced anxiety in the fear-potentiated startle response compared to placebo-treated controls (Hermans et al., 2006). In accordance, T treatment ameliorates anxiety in older men (Wang et al., 1996) and women with bilateral oophorectomy (Shifren et al., 2000), while a combination treatment of E and T decreases anxiety in menopausal women (Montgomery et al., 1987).

Gonadal hormones and anxiety in rodents

Similar to humans, sex differences in anxiety-related behavior are also present in rodents. Although the direction of these differences varies by species and strain, E and androgens such as T and dihydrotestosterone (DHT), have also been correlated with anxiety-related behavior in rodents. Rodents are nocturnal prey species that are averse to open and brightly lit areas; therefore, tests like the open field (OF), novel object, elevated plus maze (EPM) and light/dark (L/D) box, which subject animals to open or lit spaces, are useful to infer anxiety levels in rodents (Nestler and Hyman, 2010). These tests indicate that E acts as an anxiolytic in rodents. Specifically, females in proestrous, when circulating E levels are high, spend more time in the open arms of the EPM compared to females in diestrous, when E levels are low (Marcondes et al., 2001; Frye et al., 2006). Likewise, E treated female mice enter the center area of an OF more often and spend more time in the open arms of an EPM than do untreated females (Walf et al., 2008). Similarly, aged female mice that have decreased levels of E enter the center area of an OF more often and spend more time in the light side of the L/D box when treated with E than do oil treated females (Walf et al., 2010). Like estrogen, androgens also affect anxiety-related behavior in rodents. Gonadectomized adult male rodents show increased indices of anxiety compared to sham operated controls (Slob et al., 1981; Adler et al., 1999; Frye and Seliga, 2001; Fernandez-Guasti and Martinez-Mota, 2003, 2005; Morsink et al., 2007), an effect reversed by testosterone replacement (Slob et al., 1981; Adler et al., 1999; Frye and Seliga, 2001; Fernandez-Guasti and Martinez-Mota, 2005). Similarly, T treatment has anxiolytic effects on gonadectomized males in

the OF, EPM and L/D box tests (Frye et al., 2008). In aged male mice with lower T, administration of this hormone reduced anxiety-related behavior in the OF and L/D box tests (Frye et al., 2008). DHT, a reduced metabolite of testosterone (via the enzyme 5α-reductase) also acts as an anxiolytic in male and female rats on measures of the OF and EPM tests (Frye and Walf, 2009; Edinger et al., 2006; Edinger et al., 2004). Although T can reduce anxiety indices in females, there have been cases where T protected against the development of anxiety-like behaviors in males but did not do so in females (Carrier and Kabbaj, 2012).

Role of estrogen and androgen receptors in anxiety-related behavior

Estrogen and T play a crucial role in establishing and triggering sex differences in brain and behavior in non-human mammals, and most of these effects are achieved through "organizational" or "activational" effects of estrogen receptor (ER) or androgen receptor (AR) activation. During early development, different levels of gonadal hormone production set the internal environment for sexual differentiation of brain and body. In males, the SRY gene on the Y chromosome triggers testes differentiation and consequent T production. T, capable of crossing the brain-blood-barrier, is locally aromatized to estrogen, which activates ER to masculinize and defeminize the male brain in rats and mice (Tsukahara, 2009; Johansen et al., 2004). Recently, using the *s*Tfm (*spontaneous* testicular feminization mutation) androgen insensitive model, our laboratory discovered that AR also plays a crucial role in the full masculinization of the male brain and behavior (Zuloaga et al., 2008, 2011). *s*Tfm males are universally androgen insensitive since they carry a dysfunctional *AR* gene that resulted from a

spontaneous mutation. This dysfunction not only affects animals' external appearance (short anogenital distance, presence of nipples, small undescended testes), but also prevents full masculinization of certain brain regions and male typical behaviors (Zuloaga et al., 2008, 2011). The "organizational" period responsible for sexual determination early in life has recently been extended to further encompass the pubertal period, where gonadal hormones also have the capability to "organize" the brain for adult behavior (Schulz et al., 2009). Given the different routes that may lead to anxiety-related behavior, it is important to decipher the factors that contribute to the expression of this behavior. In other words, it is important to understand not only which gonadal hormones play a role in anxiety, but also which *receptors* the hormones are activating, and whether their activity acts through an organizational or activational effect.

Recent advancements in genetic, pharmacolocial and molecular techniques offer a way to shed light on the receptors that mediate the anxiolytic effects of gonadal hormones. Estrogens have high affinity for both estrogen receptors (ER) α and β. While the anxiolytic effects of ERβ activation have been demonstrated, it seems that ERα does not have a role on anxiety-related behavior. Specifically, ERβ knockout (KO) male and female mice show increased anxiety levels in the OF and EPM compared to wild type mice (Imwalle et al., 2005; Krezel et al., 2001). Furthermore, the ERβ agonist diarylpropionitrile has anxiolytic effects on gonadectomized males (Hughes et al., 2008) but does not reduce anxiety-related behavior on ERβ KO mice (Rocha et al., 2005; Walf et al., 2008; Walf et al., 2009). Unlike ERβ, ERα does not seem to affect anxiety-related behavior. ERα KO mice show no differences from WT counterparts in anxiety levels

(Krezel et al., 2001) and ERα agonist propylpyrazoletriol does not affect indices of anxiety in ovariectomized female rats (Walf et al., 2005; Lund et al., 2004).

While the role of ER has been widely studied, a role of AR has been neglected until recently. T and DHT both activate AR, however, they are also capable of activating ER via their by-products. T can be aromatized to E, and DHT can be reduced to 3β-diol (Handa et al., 2008; Oliveira et al., 2007), both of which have an affinity for ERβ (Handa et al., 2008; Pak et al., 2005). This capacity of androgens to activate both ER and AR leaves the question of the role of AR in anxiety-related behavior unanswered. Our laboratory recently found an anxiolytic effect for T through AR activation using the *s*Tfm model. Specifically, T treatment had anxiolytic effects on measures of anxiety-related behavior only in WT but not *s*Tfm males, suggesting that T exerts its anxiolytic effects through AR activation (Zuloaga et al., 2008, 2011).

Testosterone, anxiety and the hypothalamic-pituitary-adrenal (HPA) axis

Normally, the HPA axis responds to a stressor by releasing corticotrophin-releasing hormone (CRH) from hypophysiotropic neurons located in the medial parvocellular subdivision of the paraventricular nucleus of the hypothalamus (PVN). Released CRF passes through the hypophysial portal vessels to access receptors in the anterior pituitary gland. This, in turn, stimulates pituitary cells to release adreno-corticotrophin hormone (ACTH) into the circulatory system. Circulating ACTH principally targets the adrenal cortex and stimulates glucocorticoid synthesis and secretion from the zona fasciculata (Henry, 1992; Papadimitriou and Priftis, 2009). These glucocorticoids, principally cortisol in humans and corticosterone in rodents, affect targets throughout

the brain and periphery, and their main function is to suppress immune activity and maintain or increase glucose in the blood, both adaptive and necessary for survival. In humans, most anxiety disorders are linked to abnormal HPA function (Kallen et al., 2008; Faravelli et al., 2012). Similarly, CRH is often dysregulated in those with anxiety disorder (Arborelius et al., 1999; Reul and Holsboer, 2002). Interestingly, men that have gonadal suppression have a greater HPA response (Roca et al., 2005; Rubinow et al., 2005), and women treated with T show an attenuated stress response compared to untreated controls (Hermans et al., 2007). Congruent with these findings in humans, chronic stress in animal models has been shown to alter brain regions implicated in anxiety such as the hippocampus, amygdala and prefrontal cortex (McEwen, 2005; Rodrigues et al., 2009). In addition, male rats have lower levels of CRH than females, which is reversed by gonadectomy and reinstated by DHT treatment (Haas and George, 1988; Bingaman et al., 1994; Lund et al., 2004). The mode of action of T, however, has not been previously studied.

The experiments in this dissertation seek to further the understanding of the role of T and AR in anxiety-related behavior and HPA function in mice, and the ways in which AR modulates the effects of T on anxiety and HPA function.

Preview of the chapters that follow:

Chapter 2: These experiments seek to validate a conditional AR knock out mouse model by replicating previous findings by Zuloaga et al., 2008. Specifically, initial studies

determined that T exerts its anxiolytic effects through functional ARs in male rats and mice by comparing anxiety-related behavior in T or blank (B)-treated castrated adult wild type (WT) and *i*Tfm mice (*induced* Tfm, obtained using the Cre-LoxP technology). Further experiments tested whether time of testing (circadian time) had an effect on the anxiolytic effects of T acting through AR. Finally, the role of T and AR on HPA function were investigated by exposing WT and *i*Tfm mice to a mild anxiogenic stimulus and analyzing corticosterone levels at baseline and subsequent time points.

Chapter 3: These studies sought to determine the role of central nervous system (CNS) AR on the anxiolytic effects of T. Using the Cre-LoxP technology, I sought to knock out CNS AR. While this approach fully knocked out AR in some brain regions, it only partially eliminated AR in other brain regions relevant to anxiety-related behavior. The effects of this partial AR KO on anxiety-related behavior and HPA function were investigated.

Chapter 4: The brain regions where AR mediates cell response to a mild anxiogenic stimulus were studied by exposing WT and *i*Tfm mice to the anxiogenic L/D box and analyzing brain expression of the immediate early gene cFos immunoreactivity in response. Brain regions associated with anxiety-related behaviors were analyzed (basolateral, centromedial and centrolateral amygdala, hippocampus, medial prefrontal cortex, PVN, oval nucleus and anterodorsal bed nucleus of the stria terminalis, dorsal periaqueductal grey). In those areas where cFos expression differed between WT and

*i*Tfm animals, the percentage of AR positive cells were analyzed to determine the likelihood that AR might directly modulate cell activity.

Chapter 5: A final discussion is presented based on findings in this dissertation, their place in the existing literature, and their implications.

CHAPTER 2: NEW KNOCKOUT MODEL CONFIRMS A ROLE FOR ANDROGEN RECEPTORS IN REGULATING ANXIETY-LIKE BEHAVIORS AND HPA RESPONSE IN MICE

ABSTRACT

Men are less likely than women to suffer from anxiety disorders. Because gonadal hormones play a crucial role in many behavioral sex differences, they may underlie sex differences in human anxiety. In rodents, testosterone (T) exerts anxiolytic effects via the androgen receptor (AR): we found that male mice with a naturally-occurring mutation rendering the AR dysfunctional, referred to as *spontaneous* testicular feminization mutation (*s*Tfm), showed more anxiety-like behaviors than wildtype (WT) males. Here, we used CreLox recombination technology to create another dysfunctional allele for AR. These *induced* Tfm (*i*Tfm) animals also displayed more anxiety-like behaviors than WTs. We further found that AR-modulation of these behaviors interacts with circadian phase. When tested in the resting phase, *i*Tfms appeared more anxious than WTs in the open field, novel object and elevated plus maze tests, but not the light/dark box. However, when tested during the active phase (lights off), *i*Tfms showed more anxiety-related behavior than WTs in all four tests. Finally, we confirmed a role of T acting via AR in regulating HPA axis activity, as WT males with T showed a lower baseline and overall corticosterone response, and a faster return to baseline following mild stress than did WT males without T or *i*Tfms. These findings demonstrate that this

recombined AR allele is a valuable model for studying androgenic modulation of anxiety, that the anxiolytic effects of AR in mice are more prominent in the active phase, and that HPA axis modulation by T is AR dependent.

INTRODUCTION

Anxiety disorders are the second most prevalent mood disorder. Each year, of approximately 40 million American adults, about 18 percent are diagnosed with an anxiety disorder (Kessler et al., 2005), resulting in more than $42 billion in annual costs (Greenberg et al., 1999). Interestingly, anxiety disorders are unevenly distributed among the sexes; subcategories such as agoraphobia, specific phobias, generalized anxiety disorder, panic disorder, and post-traumatic stress disorder are more prevalent in women than in men (McLean et al., 2011), a sex difference that emerges in adolescence (Zahn-Waxler et al., 2008). The onset at puberty and marked sex differences in prevalence suggest a role for gonadal hormones in anxiety disorders (Menger et al., 2010; Wu and Shah, 2011).

Clinical data indicate that gonadal hormones affect anxiety in humans. Women are more prone to episodes of anxiety during the premenstrual and postpartum periods, when estrogen (E) levels are low (Dean and Kendell, 1981; Douma et al., 2005; Freeman, 2002), and as they approach menopause, when E levels start to decline (Llaneza et al., 2012; Tangen and Mykletun, 2008). In addition, E treatment ameliorates

anxiety in some postmenopausal women (Frye, 2009; Yazici et al., 2003), and in breast cancer survivors regardless of age (Decker et al., 2003). Like E, testosterone (T) has also been linked to anxiety levels in women, with low levels correlating with high anxiety levels (Giltay et al., 2012). Accordingly, treating women with T following ovariectomy decreases anxiety (Shifren et al., 2000). In aging men, anxiety levels increase with decreasing levels of T (Amore et al., 2009; Sternbach, 1998), and T treatment ameliorates anxiety in such men (Wang et al., 1996). Similarly, androgen blockade therapy for prostate cancer treatment has been reported to increase anxiety, which was alleviated when treatment ended (Almeida et al., 2004). Although the nature of the relationship between these steroid hormones and anxiety level is not always consistent (Demetrio et al., 2011; Kiesner, 2011; Thomson and Oswald, 1977), they appear to exert anxiolytic effects in a number of different circumstances.

Sex differences in anxiety-related behavior are also present in other mammals, including laboratory rodents. Although the direction of these differences varies by species and strain, gonadal hormones also have anxiolytic effects in rodents. Laboratory rodents are nocturnal prey species that are averse to open and brightly lit areas; therefore, behavioral tests like the open field (OF), novel object (NO), elevated plus maze (EPM) and light/dark (L/D) box, which subject animals to open or lit spaces, are used to infer anxiety levels. Results from these tests suggest that E acts as an anxiolytic. Specifically, when circulating E levels are high during proestrous, female rodents spend more time in the open arms of the EPM than when E levels are low during diestrous (Marcondes et al., 2001; Walf et al., 2009; Zuloaga et al., 2011a).

Likewise, E-treated females enter the center area of an OF more often and spend more time in the open arms of an EPM than do untreated females (Walf et al., 2008), while females given inhibitors of estrogen biosynthesis show more anxiety-related behavior in the OF and EPM (Meng et al., 2011; however, Morgan and Pfaff, 2002). Similarly, aged females enter the center area of an OF more often and spend more time in the light side of the L/D box when treated with E than do oil treated females (Walf and Frye, 2010). These effects of E appear to be mediated through ERβ (Hughes et al., 2008; Imwalle et al., 2005; Krezel et al., 2001; Rocha et al., 2005; Walf et al., 2009; Walf et al., 2008) and not ERα (Krezel et al., 2001; Lund et al., 2005; Walf and Frye, 2005).

Androgen treatment can also have anxiolytic effects in rodents. T treatment reduces anxiety-like behaviors in gonadectomized male mice in the OF, EPM and L/D box tests (Aikey et al., 2002; Frye et al., 2008). T also has anxiolytic effects on females, as measured by their increased number of entries into the center area of an OF and more time spent in the open arms of an EPM (Frye and Lacey, 2001). DHT, a 5α-reduced form of testosterone, also acts as an anxiolytic in male and female rats on measures of the OF and EPM tests (Aikey et al., 2002; Edinger and Frye, 2004, 2006; Frye and Lacey, 2001). However, it is not clear from these studies whether these androgens are acting on AR or ER. T and DHT both activate AR, but they are also capable of activating ER via their by-products. T can be aromatized into E, and DHT can be reduced to 3β-diol (Handa et al., 2008; Oliveira et al., 2007), which has an affinity for ERβ, the estrogen receptor that has been implicated in anxiolysis. While the role of ER

has been widely studied, a role of androgen receptors (AR) has been neglected until recently.

Our laboratory recently found a role for ARs in anxiety-related behavior in rats (Zuloaga et al., 2011b) and in mice (Zuloaga et al., 2008a). Such studies were conducted during the resting phase (lights on) of mice with a naturally-occurring mutation rendering the AR dysfunctional, which we refer to as *spontaneous* testicular feminization mutation (*s*Tfm). Specifically, T treatment had anxiolytic effects on measures of anxiety-related behavior in wild type males, but not *s*Tfm males. Here, we replicate the anxiolytic effects of T mediated through AR using an allele for AR that has been altered by recombination using CreLox technology, thus demonstrating that this recombination indeed interferes with AR's anxiolytic action. We also expand these findings and demonstrate an effect of the circadian phase on AR's anxiolytic influence and confirm a role of AR in regulating the HPA axis response to mild stress.

MATERIALS AND METHODS

Experimental Animals

We used the CreLox system in mice in an attempt to recapitulate the effect of a spontaneous mutation in the AR gene, namely the *s*Tfm. Mice carrying a conserved lox sequence of 34 base pairs at the two ends of exon 2 of the AR gene ("floxed" AR, a generous gift from De Gendt et al., 2004) were crossed with transgenic animals carrying

the cyclization recombination (Cre) transgene under the control of the universal adenovirus Ella promoter ("deleter" mice, Jax stock 003724). The presence of both genotypes in the same cell should result in the excision of the targeted sequence in AR exon 2, which encodes the first zinc finger of the DNA-binding domain, essential for the recognition of androgen response elements. This deletion should cause a frame-shift mutation resulting in the premature termination of AR transcription, rendering AR dysfunctional (De Gendt et al., 2004). Female offspring carrying such a recombined AR allele were identified by PCR, as described below, then bred to wildtype (WT) C57BL/6 (Jax) males; the recombined AR allele was transmitted to some female progeny without the Cre transgene, as confirmed by PCR. These females were used to found a colony of mice perpetuating the recombined AR allele. We confirmed that breeding these females with WT males produces genetic male offspring that recapitulate the phenotype of XY mice carrying the *s*Tfm mutant of AR: small, abdominal testes, short anogenital distance, feminine external genitalia, and nipples (Figure 1). Hence, we refer to these mice as *induced* Tfm, or *i*Tfm mice, to distinguish them from the *s*Tfm mice. As with our *s*Tfm colony, these females also produced male offspring carrying the WT AR allele to serve as controls. AR immunocytochemistry (methods described below) confirmed full knock out of AR in experimental animals, as we found no AR immunoreactivity in the brains of *i*Tfm males (Figures 2B,D), while their WT brothers showed AR expression in typical AR positive regions (Figures 2A,C). Circulating T measures revealed that *i*Tfm males have lower adult circulating T levels than their WT counterparts (*i*Tfm: M=4.2 $\pm$ 0.23 (SEM) nmol/l; WT: M=29.8 $\pm$ 4.9 nmol/l; methods detailed below), further confirming the disruption of AR in these animals since *s*Tfm mice also have lower T

levels than WTs (*s*Tfm: *M*=3.26 ± 0.52 nmol/l; WT: *M*=19.75 ± 4.39 nmol/l; Zuloaga et al., 2008).

Mice born in this *i*Tfm colony were housed in plastic cages (29x18x18cm) at approximately 27^0C in a 12:12 LD cycle, and provided *ad libitum* tap water and rodent chow (Harlan Teklad 8640 Rodent Diet [Madison, WI]). Mice were ear punched for genotyping and weaned at postnatal (PN) day 23 and group housed with other phenotypically similar males or females. For experiments 1 and 3, WT and *i*Tfm males were castrated at postnatal (PN) day 60 and subcutaneously implanted either with testosterone-filled (T) or blank (B) capsules (1.6 mm-inner and 3.2 mm-outer diameter, 1.6 cm effective length). Behavior testing and blood collection took place on PN90-120. All housing conditions and experiments were performed in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the MSU Institutional Animal Care and Use Committee.

Polymerase chain reaction (PCR) identification of genotype

PCR was run to detect the recombined AR allele and the *Sry* gene. To discriminate the WT and recombined AR alleles, primers targeted and amplified the sequence that includes the lox sites and exon 2 of the AR gene. The primers used were AGC CTG TAT ACT CAG TTG GGG and AAT GCA TCA CAT TAA GTT GAT ACC. The resulting products were 860bp for the wild type AR, and 400bp for the recombined AR allele. Animals that were positive for both *Sry* and WT AR were classified as WT males

whereas mice positive for both *Sry* and a recombined AR were classified as *i*Tfm males. The genotype determined by PCR was also verified based on the phenotype of each mouse as described below (experiments 1 and 3), and/or at sacrifice (experiment 2).

AR Immunocytochemistry (ICC)

To facilitate detecting AR, all mice in these studies were injected with 1mg of testosterone propionate in 0.1ml sesame oil sc two hours before being sacrificed. Animals were then injected with an overdose of sodium pentobarbital i.p. and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered Saline (PBS, pH 7.4). Brains were collected and post-fixed for 2 hours in 4% paraformaldehyde, at 4^{o}C. Tissues were then transferred into a 20% sucrose solution and kept at 4^{o}C until they sunk to the bottom (approx. 2 days). Once sunk, brains were microtome sectioned in the coronal plane at 35µm. All reactions for the ICC were performed at room temperature and on a rotomixer unless otherwise indicated. Tissues were rinsed in four 5 minute washes in a phosphate-buffered gelatin Triton solution (PBS-GT; 0.1% gelatin, 0.3% Triton X-100, in PBS, pH 7.4), followed by 0.5% sodium borohydride in PBS-GT for 15 minutes, and three 5 minute washes in PBS-GT. Sections were then incubated in 10% NGS in PBS-GT for 1 hour to block non-specific binding of the secondary antibody. Two 5 minute washes in PBS-GT, and later a 10 minute incubation in avidin block followed. This was succeeded by two 5 minute PBS-GT washes and then incubation for 10 minutes in biotin block (Avidin/Biotin Blocking Kit, Vector, cat#SP-2001). Two 5 minute PBS-GT washes took place before the tissue was incubated for 36 hours at 4°C in 1% NGS in PBS-GT with AR primary

antisera at 1:5000 concentration (rabbit monoclonal - Abcam, code#ab52615, clone ID EP670Y). Following incubation in the primary antibody, the tissue was rinsed in PBS-GT, and incubated 1 hour in 1% NGS in PBS-GT with biotinylated goat anti-rabbit secondary antisera at 1:1000 concentration (Jackson Immunoresearch, lot # 88762, code # 711-065-152). Brain sections were rinsed again in PBS-GT, followed by a 1 hour incubation in PBS-GT with an Avidin-Biotin complex solution made 30 minutes before use (1 drop of each solution A and B per 10ml of PBS-GT; ABC Elite Kit [standard], Vector Laboratories, catalogue # PK6100). The tissue was again washed in PBS-GT before being stained with NiCl-enhanced diaminobenzidine (DAB, Sigma, St. Louis, MO) in a 0.05 M Tris Buffer, pH 7.2. Following staining, the tissue was first washed in PBS-GT and then in mounting solution before being mounted and coverslipped after dehydration through graded alcohols and xylene.

Experiment 1: Validation of the *i*Tfm model

To examine whether disabling AR via the Cre-LoxP system produces effects on anxiety-like behavior comparable to that seen in *s*Tfm mice (Zuloaga et al., 2008a), castrated WT and *i*Tfm male mice with T or B capsules were tested for anxiety-related behavior on PN 90-120. Mice were tested on the OF, the NO, the EPM, and the L/D box tests, with 2 days between tests. The order of the last two tests (the EPM and L/D box) was counterbalanced to assess any test-order effect (detailed information below). Mice were taken in their home cages to the behavior testing room to habituate an hour before testing. Mice were tested during their resting phase, 2 hrs after lights on.

Experiment 2: Effects of photoperiodicity on anxiolytic effects of T and AR activity

Additional groups of behaviorally naïve WT and *i*Tfm male mice were tested on the same behavioral assays as above, except the tests were conducted during their active phase, 2 hrs after lights off. Animals were taken to the behavior testing room covered with a dark cloth to minimize exposure to ambient light and remained covered until testing. Mice were only exposed to light during behavioral tests.

Experiment 3: Corticosterone response to mild stress in T-treated WT and *i*Tfm males

Based on findings in experiments 1 and 2, and the relation between anxiety-related behavior and HPA axis activity, experiment 3 was conducted to determine whether disabling AR through recombination alters HPA response to mild stress. Terminal blood samples for corticosterone (CORT) levels were collected on PN90 from behaviorally naïve T-treated WT and *i*Tfm mice at baseline, and at 20, 40 60, and 120 min after a 10-min exposure to the L/D box. Castrated WT males implanted with blank capsules provided an additional control group. Exposure to the L/D box and blood collection took place during the animals' active phase, 2 hours after lights off, parameters chosen based on the robust group differences seen in Experiment 2 (detailed below).

Castration and Silastic capsule implant for androgen treatment

At PN60 before any testing began, mice in experiments 1 and 3 were anesthetized with isoflurane and their backs shaved for capsule implants. Castrations in WT mice were

performed through a 5-mm long incision along the midline of the scrotal sac. Testes were visualized and silk suture was used to tie the vas deferens, blood vessels and associated fat pad before removing the testes. The incision was closed using surgical staples. In *i*Tfm mice, the testes were undescended and small as expected (De Gendt et al., 2004; Figure 1D); thus, an abdominal incision was made through which the testes were visualized on each side of the bladder, tied and removed as described above. The abdominal muscle wall was closed with suture and the overlying skin closed with clips.

During the same period of anesthesia, animals also received subcutaneous implants of Silastic capsules containing either testosterone (T) or left blank (B) (1.6mm inner diameter, 3.2mm outer diameter; 6mm effective release length). Such capsules produce T levels at near-normal circulating levels in mice (Zuloaga et al., 2008a) and were implanted on the dorsal surface just caudal to the interscapular fat pad. The incision over the capsule was closed with surgical staples, and mice were injected with 0.05 ml of Ketofen (100mg/ml, sc) for analgesia.

Anxiety-related behavior testing

All testing chambers were cleaned with 70% EtOH and dried between subjects.

Open field and novel object tests: The OF was a rectangular unlidded Plexiglas box, 40.6 x 40.6 cm with walls 30.5 cm high. Testing took place under fluorescent lights projecting 460 lux throughout the arena. Mice were placed in the testing chamber facing a corner and allowed to move freely for 5 min. Animals were returned to their home cage for 15 min, then placed back into the chamber in which a novel object (a clear cylindrical petri dish with color tape, 5 cm in diameter) had been placed in the center of

the field for another 5 min. Locomotor activity was tracked based on the interruption of laser beams and collected by computer software (Versamax). For the OF portion of the test, measures included the latency to enter the middle portion of the arena (a 20.3 x 20.3 cm area located in the center of the field), the number of entries into the center area, the total time spent in the center area, and the number of rearings. For the NO portion of the test, measures included the latency to visit the novel object, the number of visits paid to the object, the total time spent near the object, and the number of rearings performed anywhere in the chamber.

Elevated plus maze test: The EPM consisted of four arms, 25.4 cm in length and 5.7 cm in width, stretched out from a center square at 90 degree intervals. Two of the opposing arms had walls of 13.3 cm in height. The maze rested 43.8 cm from the floor. Testing took place with an overhead lamp that provided 50 lux lighting in the open arms and 25 lux in the closed arms. Animals were placed into the maze facing a closed arm and allowed 10 minutes to move freely. An overhead camera recorded their activity. Measures taken from video recordings included the number of open arm entries (4 paws in an open arm), the number of stretch attends into open arms (2 paws in an open arm), the total percentage of time spent in open arms (time in open arm/[time spent in closed arms + time spent in open arms]), and the number of head dippings while in an open arm.

Light/dark box test: The L/D box consisted of a rectangular Plexiglas arena divided at the center by a wall to yield two areas measuring 24.1 x 18.4 cm each. The “light” part of the box was open-topped and had 3 transparent walls, while the “dark” part was a fully-enclosed black box. The dividing black wall between the light and dark sides of the

box had a 10.2x5.1cm opening, which allowed mice to move freely between the sides. Testing took place with an overhead lamp providing 500 lux in the light area and 2 lux in the dark area. Animals were placed on the light side of the apparatus facing the connecting doorway and allowed 10 minutes to move freely. Behavior coding started after animals first enter the dark side. Measures made from video recordings included number of stretch attends towards the light chamber (2 paws in light side), number of entries into the light chamber (4 paws in light side), total time spent in the light chamber, and number of rearings made during time spent in the light chamber (number of rears/time in light side).

Blood collection for CORT sampling

To study CORT hormone response, we exposed mice to the L/D box two hours into their active phase. Mice were anesthetized with isoflurane and decapitated for blood collection. Blood was collected into heparinized tubes, which were centrifuged at 8^{o} C for 20 minutes at 3000 rcf. Plasma samples were stored at -80^{o} C until analyses. Samples were assayed for corticosterone using Coat-a-Count Corticosterone kits (Diagnostics Products Corporation, Los Angeles, CA. USA) at the Diagnostic Center for Population and Animals Health at MSU. All samples were run in duplicate and the average of the two samples from each mouse was used for analysis.

Blood samples for basal T level analysis were taken from intact adult WT and *i*Tfm animals. All procedures were the same as mentioned above, and Coat-a-Count Total Testosterone kits were used for T assays (Diagnostics Products Corporation, Los Angeles, CA. USA).

Statistics

Experiment 1: Analyses for the OF and NO tests consisted of two-way ANOVAs with genotype (WT versus *i*Tfm) and hormone treatment (T versus B) as independent factors. Analysis for the EPM and LD box tests initially consisted of three-way ANOVAs run to determine a potential test order effect with genotype (WT versus *i*Tfm), hormone treatment (T versus B) and test order (EPM/LD versus LD/EPM) as independent factors. As these analyses indicated no effect of test order, subsequent two-way ANOVAs were conducted with genotype and hormone treatment alone. Analyses were followed up by Post-hoc LSD tests.

Experiment 2: Analysis for the OF and NO tests consisted of one-way ANOVAs with genotype (WT versus *i*Tfm) as the independent factor. For the EPM and LD box tests, an initial two-way ANOVA was run with genotype (WT versus *i*Tfm) and test order (EPM/LD versus LD/EPM) as independent factors. As these did not reveal a test order effect, one-way ANOVAs were conducted with genotype as the independent factor.

Experiment 3: Analysis consisted of a two-way ANOVA with group (WT+T, *i*Tfm+T, WT+B) and blood collection time (baseline, 20, 40, 60, 120 minutes after exposure to the L/D box) as independent factors, followed by a post-hoc LSD test.

Differences were considered significant when $p<0.05$. For two-way ANOVAs and pairwise comparisons that showed significant differences, eta squared and Cohen's d effect sizes were calculated, respectively.

RESULTS

Experiment 1: Validation of the *i*Tfm model in the resting phase

In the resting phase (lights on), *i*Tfm males showed evidence of greater anxiety than wildtype males in several behavioral indices, confirming findings with *s*Tfms (Zuloaga et al., 2008a). For the OF test, there was a significant main effect of genotype on the number of rears ($p < .005$; $d = .806$) since WT males reared more than *i*Tfm mice, regardless of hormone treatment. However, there was no main effect of hormone or interaction on number of rears. Post-hoc comparisons confirmed that WT+T mice reared more than either *i*Tfm+T or *i*Tfm+B (all p's<.05, unless specified; $d = 1.083$ and $d = .944$, respectively; Figure 3A). There was also a main effect of hormone on the number of center area entries ($\eta^2 = .009$), where animals treated with T entered the area more than those given blank capsules ($d = .554$), but there was no main effect of genotype or interaction on this measure (data not shown). No main effects or interactions were found for latency to enter or time spent in the center area.

For the NO test, there was a significant main effect of genotype on the number of rears ($\eta^2 = .015$) because WT males reared more than *i*Tfm mice ($d = .72$). There was no main effect of hormone or interaction, paralleling what we found in the OF. Post-hoc comparisons confirmed that WT+T mice reared more than T-treated *i*Tfm males ($d = .755$; Figure 3B). T treatment also reduced the latency to approach the novel object, but only in WT animals; a post-hoc LSD test showed that WT+T animals approached the object faster than WT+B mice ($d = .887$; Figure 3C). *i*Tfm males in both groups were

intermediate on this measure, not differing from either WT group. No main effect of genotype or hormone, and no interactions were found for number of object visits and total time spent with the object.

For the EPM, a three-way ANOVA did not indicate a main effect of test order or any interaction of that factor with others. Therefore, data were collapsed across test order for the rest of the analysis. Subsequent two-way ANOVA revealed a significant main effect of genotype on the number of open arm entries (η^2=.028), where WT males entered the open arms more often than *i*Tfms (*d*=.525). No main effect of hormone or interaction was found. Post-hoc LSD revealed that T significantly increased the number of open arm entries (*d*=.74; Figure 3D), and the time spent in open arms (*d*=1.203; Figure 3E) only in WT and not in *i*Tfm males. *i*Tfm males, irrespective of hormone treatment condition, were like WTs without T, spending less time in the open arms.

We found no difference in anxiety-like behavior in *i*Tfm males compared to WT males in the LD box during the resting phase (Figure 3F-G). To determine whether circadian time affected these results, we performed follow-up tests on a separate cohort of mice during the active phase (lights off), described next.

Experiment 2: Effects of circadian phase on anxiolytic effects of T and AR activity

As in the previous tests during the resting phase, we found a difference between WT and *i*Tfm animals in the number of rears in the OF test in the active phase (*d*=.941;

Figure 4A), but no significant differences for time spent in the center, latency to enter the center, or total time spent in the center.

For the NO test, WT mice again reared significantly more than *i*Tfms (*d*=1.093; Figure 4B), with a trend toward approaching the novel object sooner than *i*Tfm animals (*p*=.079, two-tailed, Figure 4C). No significant differences were found for latency to visit the novel object, number of object visits, or total time spent with the object.

For the EPM, two-way ANOVAs again indicated no effect of test order. Collapsing across test order, one-way ANOVAs revealed that WT males performed more stretch attends than *i*Tfm mice (*d*=1.288; Figure 4D), but there were no significant differences for other measures (number of open arm entries, total time spent in open arms, and number of head dips) during the lights off phase.

For the LD box, a two-way ANOVA first confirmed that there was no effect of test order for any of the parameters. Subsequent one-way ANOVAs revealed that WT males reared more often (*d*=1.418; Figure 4E) and spent more time in the light side (*d*=1.135; Figure 4F) than did *i*Tfm males when tested in the active phase. There were no significant differences for any other measure in this test.

Experiment 3: Corticosterone response to mild stress in T-treated WT and *i*Tfm males

Testosterone had a clear modulatory effect on both the basal levels of corticosterone (CORT) and the time course of recovery after exposure to a mild stressor in WT male mice (Figure 5). Moreover, both baseline and recovery of CORT were affected by disruption of AR. Two-way ANOVA, with treatment group and time of blood collection as factors, indicated significant main effects of group (η^2=.036) and time (η^2=.055), but no significant interaction. Post-hoc tests revealed that WT+T mice showed consistently lower CORT throughout the HPA response than either *i*Tfm+T (*d*=1.335) or WT+B (*d*=1.182) mice. Pairwise comparisons across time also indicated that CORT levels returned to baseline sooner in T-treated WTs compared to the other two groups (*i*Tfm+T and WT+B), since CORT levels at 60 minutes were comparable to baseline levels in WT+T mice (p>0.118) whereas CORT levels remained significantly higher than baseline for both *i*Tfm males and blank-treated WT males at this time point.

DISCUSSION

ARs appear to play a role in mediating the anxiolytic effects of testosterone in mice, as measured by anxiety-related behaviors and HPA activity in both the resting and active phases of the circadian cycle. Using Cre-LoxP technology to recapitulate the spontaneous Tfm model (Zuloaga et al., 2008a), we replicated the role of ARs in the anxiolytic effects of T in a so-called “induced” Tfm (*i*Tfm) model and also expanded on these findings. Specifically, T treatment reduced some indices of anxiety only in WT males, not *i*Tfm males. Compared to *i*Tfm+T male mice, WT+T males entered the open

arms of the EPM more times, took less time to approach a novel object in the NO test, and showed less exploration in the OF and NO tests, as indicated by the number of rearings. Testing conditions in this first experiment replicated the phenomenon found earlier (Zuloaga et al., 2008a) where *s*Tfm and WT male mice were tested for anxiety in their resting phase (lights on). Both studies indicated an anxiolytic role of AR, although there were some differences between the two Tfm models in terms of which parameters displayed statistically significant differences (Zuloaga et al., 2008a). This may be due to minor differences in how the tests were conducted or scored, as the testing staff was different in the two studies. In *s*Tfms, the natural mutation is caused by a single base deletion in the coding region of the n-terminus, which creates a change in the reading frame, resulting in a truncated AR that lacks both DNA and steroid binding domains (Charest et al., 1991). In *i*Tfms, exon 2 of the AR gene is excised (De Gendt et al., 2004). Exon 2 codes for the 1st zinc finger of the AR DNA-binding domain, and, though this also creates a premature termination of transcription, the resulting transcripts would be different, but as no functional protein is produced in either model, it is difficult to see how the different transcripts would affect behavior.

The role of functional AR was also examined in a different cohort of mice during their active phase (lights off) to assess the possibility that anxiety-related behavior and the apparent role of AR might be affected by circadian phase. To a large extent, we found the same pattern of differences in both phases: WT males reared more in the OF and NO tests, and performed more stretch attends in the EPM, a measure of higher anxiety (McLean et al., 2011). However, differences in anxiety-like behavior between WT and

*i*Tfm males in the LD box were revealed only in the active phase, with *i*Tfm males rearing significantly less and spending significantly less time on the light side of the box than WT controls, suggesting an effect of photoperiodicity on the anxiolytic effects of activated AR in this particular test. Two potential mechanisms may underlie this interaction. First, T might work through functional AR in WT mice to dampen the effects of rising CORT levels that occur during the active phase (Halberg et al., 1959; Zuloaga et al., 2011b), which in turn may reduce anxiety-like behavior in the LD box. The effect that we saw of T on CORT levels in WT males in the active phase (Figure 5) is consistent with this view. Second, T and AR may reduce the anxiogenic effects that bright light has on animals during their active phase through a mechanism that is independent of CORT. Since the core of the suprachiasmatic nucleus of the hypothalamus (SCN) responds to photic inputs to the retina, and core cells contain AR (Karatsoreos and Silver, 2007), T may act directly on SCN neurons to regulate their response to light.

Results based on the OF, NO, EPM and LD box tests during the resting and active phases leave questions still unanswered. First, it is not apparent why the anxiolytic effects of AR affect some measures of anxiety-related behavior in these tests and not others, considering that they are all exploration-based anxiety tests. Other studies (Juntti et al., 2010; Raskin et al., 2009) also suggest that T signaling through AR regulates some, but not all, behaviors, including anxiety-related behaviors. Second, since *i*Tfm mice lack AR throughout development and AR is crucial for differentiation of several brain regions and behaviors (Bodo and Rissman, 2007; Dugger et al., 2007;

Durazzo et al., 2007; Garcia-Falgueras et al., 2005; Jones and Watson, 2005; Meaney et al., 1983; Morris et al., 2005; Olsen and Whalen, 1981; Rizk et al., 2005; Zuloaga et al., 2008b), it is unclear whether the effects seen are solely due to the adult activational effects of T.

Here, we also demonstrate for the first time that the capacity for T to reduce HPA activity requires a functional AR. WT mice treated with T show lower basal CORT levels than WT+B or *i*Tfm+T males. When exposed to the LD box, all animals showed a CORT increase from their respective baseline levels, which tapers down with time; however, only WT+T males return to baseline levels by 60 minutes after exposure, while WT+B and *i*Tfm+T take longer, indicating a capacity for T to curtail HPA activity through functional AR. Neither the presence of T alone nor functional AR alone was sufficient to curtail HPA activity, as CORT levels in *i*Tfm+T and WT+B mice remained above baseline after 60 minutes. That intact WT male mice have lower *baseline* CORT compared to mice that lack functional AR has previously been shown in our lab based on *s*Tfm mice (Zuloaga et al., 2008a), but here we additionally demonstrate that T acts through functional AR to lower HPA response and recovery. Since previous work was conducted in the resting phase (Zuloaga et al., 2008a), the present finding also extends the importance of T and AR in regulating HPA activity to the active phase. Our results also agree with Evuarherhe and colleagues' (2009) finding that T-treated castrated adult rats show lower basal corticosterone levels than castrated controls. In addition, castration increases corticotropin releasing hormone and parvocellular arginine vasopressin mRNA in the paraventricular nucleus of the hypothalamus, a phenomenon

that is abolished with T restoration (Evuarherhe et al., 2009; Zhou et al., 1994). It is possible that AR mediates these modulatory effects of T on mRNA levels.

These findings also validate the *i*Tfm mouse model as one suited for testing anxiety mechanisms in mice, which offers possibilities for using the Cre-LoxP system to knock out AR selectively, in different regions and/or at different time points. Although it is clear that AR has a role in anxiety-like behavior, the mechanism by which AR activation reduces anxiety is yet to be established. One possibility is that AR stimulation may activate GABAergic drive, known to reduce anxiety (Lydiard, 2003; Rago et al., 1988; Reynolds, 2008). In fact, androgenized female mice show greater GABAergic postsynaptic current frequency and larger hypothalamic cells than control females, an effect blocked by the AR antagonist flutamide (Sullivan and Moenter, 2004), suggesting a capacity of AR to modulate GABA function. Similarly, chronic exposure to anabolic androgenic steroids increases selective GABA(A) receptor subunit mRNAs and GABAergic synaptic current decay in the medial preoptic area in WT male mice, an effect that *s*Tfm mice do not show (Penatti et al., 2009). Since chronic, but not single, exposure to androgens can lower anxiety-related behavior (Fernandez-Guasti and Martinez-Mota, 2005; Penatti et al., 2009) and is blocked by flutamide (Fernandez-Guasti and Martinez-Mota, 2005), it is very likely that prolonged androgen exposure acting on ARs triggers a downstream cascade of events that in time results in altered GABA function, leading to anxiolytic effects.

The present results in mice complement our previous findings in rats, where again Tfm animals show more anxiety-related behaviors than WT males in a variety of tests (Zuloaga et al., 2011b). Thus it seems likely that T also acts through functional AR to reduce anxiety in men. This effect could contribute to sex differences in the prevalence of anxiety disorders. To date, much is known about sex differences in prevalence and course of the disorder and, though little is known about treatment, a few studies show a sex difference in response to treatment (Bekker and van Mens-Verhulst, 2007). Understanding the downstream actions of T and AR might shed light on potential common pathways present in both sexes, potentially leading to treatments for both men and women.

CHAPTER 3: DOWN, BUT NOT OUT: PARTIAL ELIMINATION OF ANDROGEN RECETORS IN THE MALE MOUSE BRAIN FAILS TO AFFECT ANXIETY AND HPA ACTIVITY

ABSTRACT

We previously found that androgen receptor (AR) activity mediates two effects of testosterone (T) in adult male mice: reduction of anxiety-like behaviors and dampening the hypothalamic-pituitary-adrenal (HPA) response to stress. To determine whether ARs in the central nervous system (CNS) mediate these effects, we used the Cre-LoxP technology seeking to knockout (KO) AR. Female mice carrying the floxed AR allele (ARlox) were crossed with males carrying Cre recombinase transgene controlled by the *nestin* promoter (Nes-Cre), producing Cre in neurons and glia. Four genotypes from this cross resulted: males carrying ARlox and Nes-Cre (NesARKO), and three control groups (males carrying neither transgene [WT], Nes-Cre males, and ARlox males). Reporter mice indicated ubiquitous Cre expression in the CNS. However, careful evaluation of AR expression using immunocytochemistry in NesARKO mice revealed efficient KO of AR in some brain regions, including the hippocampus and medial prefrontal cortex [mPFC], but not others. Substantial AR protein was seen in the amygdala and hypothalamus, among other regions. This selective KO allowed for testing the role of AR in hippocampus and mPFC. Animals were castrated and implanted with T at postnatal (PN) day 60, before testing on PN90-100. In contrast to

males with a universal knockout of AR, there was no evidence of increased anxiety-related behavior or HPA activity in NesARKO males. These results leave open the possibility that AR acting in the CNS mediates these effects of T, but demonstrate that hippocampal and mPFC AR is not necessary for T's effects on anxiety or HPA response.

INTRODUCTION

Anxiety disorders are the second most prevalent mood disorder. Each year, about 18 percent of adults in the United States are diagnosed with an anxiety disorder (Kessler et al., 2005), resulting in more than $42 billion in annual costs (Greenberg et al., 1999). Interestingly, these disorders are more prevalent in women than in men (McLean at al., 2011), a sex difference that emerges in adolescence (Zahn-Waxler et al., 2008). The onset at puberty and marked sex differences in prevalence suggest a role for gonadal hormones, such as testosterone (T), in anxiety disorders (Menger et al., 2010; Wu and Shah, 2011).

Circulating T modulates anxiety levels in humans. In aging men, anxiety levels increase with declining levels of T (Amore et al., 2009; Sternbach, 1998), and decrease with T treatment (Wang et al., 1996). Similarly, androgen blockade therapy for prostate cancer treatment increases anxiety, which is alleviated when treatment ends (Almeida et al., 2004). T has also been linked to anxiety in women, as female patients experiencing

generalized anxiety disorder have lower salivary testosterone levels than female controls (Giltay et al., 2012).

The capacity for T to modulate anxiety levels also extends to several other species including rats and mice, as indicated by exploration-based anxiety-related behavioral tests. Male mice given T spend more time in the open arms of the elevated plus maze (EPM) compared to control males (Aikey et al., 2002; Frye et al., 2008). In females, T treatment increases the number of entries into the center area of an OF and the time spent in the open arms of an EPM (Frye and Lacey, 2001). Dihydrotestosterone, a 5α-reduced form of T, also has anxiolytic effects on the OF and EPM tests in male and female rats (Aikey et al., 2002; Edinger and Frye, 2004, 2006; Frye and Lacey, 2001).

We recently found this anxiolytic effect of T is modulated by activation of androgen receptors (AR; Chen et al., under review; Zuloaga et al, 2008). In castrated male mice, T treatment reduced anxiety-related behavior in wild type (WT) males, but had no effect on males with universally disabled AR, whether AR dysfunction was caused by a natural mutation or a conditional knock out (Zuloaga et al., 2008; Chen et al., under review, respectively).

Anxiety levels also fluctuate along with hypothalamic-pituitary-adrenal (HPA) function in humans (Kallen et al., 2008; Faravelli et al., 2012). Like anxiety, HPA function is also modulated by T (Rubinow et al., 2005; Papadopoulos and Wardlaw, 2000; Chen et al.,

submitted to Hormones and Behavior) acting on functional ARs (Zuloaga et al., 2008; Chen et al., submitted to Hormones and Behavior).

Here, we sought to determine whether central nervous system (CNS) AR mediates the anxiolytic effects of T. To disable AR in the CNS, we crossed mice carrying the floxed AR allele with mice carrying the Cre recombinase transgene controlled by the *nestin* promoter, producing Cre in neurons and glia throughout the CNS. Interestingly, this cross fully eliminated AR only in some brain areas, including the hippocampus and medial prefrontal cortex (mPFC), but left abundant AR protein in others (e.g., amygdala and hypothalamus). This selective KO allowed us to determine the role of AR in the hippocampus and mPFC. Compared to WT mice, animals with this selective KO showed no differences in anxiety-related behavior or HPA response, indicating that T does not act on AR in those two regions to modulate these behaviors.

MATERIALS AND METHODS

Experimental Animals

All the mice in this study were born in our colony housed in plastic cages (29x18x18cm) at approximately 27^0C in a 12:12 LD cycle (lights on at 11:00), and provided *ad libitum* tap water and rodent chow (Harlan Teklad 8640 Rodent Diet [Madison, WI]). All housing conditions and experiments were performed in compliance with guidelines established

by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the MSU Institutional Animal Care and Use Committee.

To use the CreLox system to disable the *AR* gene in the CNS, female mice carrying the conserved lox sequence of 34 base pairs at two ends of exon 2 of the *AR* gene (ARlox, a generous gift from De Gendt et al., 2004) were crossed with males carrying a Cre recombinase transgene under the control of the *nestin* promoter (Nes-Cre; Jax stock 003771), expressed in neurons and glia. The presence of both genotypes in a cell should result in the excision of the targeted sequence in AR exon 2, which encodes the first zinc finger of the DNA-binding domain, essential for the recognition of androgen response elements, throughout the CNS. This deletion causes a frame-shift mutation resulting in the formation of a premature termination codon, which renders AR dysfunctional. In a previous report, we confirmed that this recombination of the ARlox gene, when accomplished universally in mice, recapitulates the phenotype seen in spontaneous mutations disabling the AR gene, including increased anxiety-like behaviors and accentuated HPA response to stress (Chen et al., under review).

For the present study, the cross of ARlox animals with Nes-Cre mice yielded four genotypes of interest: experimental males that carry both Nes-Cre and ARlox (NesARKO), and three control groups: males carrying neither ARlox nor Nes-Cre (wildtype [WT] controls), Nes-Cre males, and ARlox males. Mice were weaned at post-natal (PN) day 23, ear punched for PCR genotyping and group housed. On PN 60, animals were castrated and implanted with T capsules before testing on PN90-100. To ascertain the efficacy of the CNS AR KO using the chosen transgenic mouse lines, Cre expression was characterized using a reporter mouse (Jax 003504), and AR expression

was analyzed using immunocytochemistry, in separate cohorts detailed in experiment 1 below.

Experiment 1: Determination of AR KO efficacy

To ascertain the efficacy of the CNS expression of Cre, Nes-Cre male mice were crossed with female reporter mice (Jax 003504) to obtain offspring for Cre expression characterization using X-gal staining (detailed methodology below). To determine the deletion of AR protein in a separate cohort of NesARKO mice, AR expression was probed using immunocytochemistry. Tissue was also collected from males from our colony of *i*Tfm mice (*induced* testicular feminization mutation, a universal conditional AR KO model obtained using the Cre-LoxP technology [Chen et al., submitted to Hormones and Behavior) and WT males as negative and positive controls, respectively (details below).

Experiment 2: Role of CNS AR on the anxiolytic effects of testosterone

Universal AR dysfunction blocks T's anxiolytic effects in mice (Chen et al., submitted to Hormones and Behavior; Zuloaga et al., 2008). To determine whether hippocampal or mPFC AR plays a role in these anxiolytic effects of T, castrated and T-treated NesARKO, WT, ARlox and Nes-Cre males were tested on anxiety-related behavior between PN90-100. Mice were tested on the open field (OF), the novel object (NO), the elevated plus maze (EPM), and the light/dark box (LD) tests, with 2 days between tests. The order of the EPM and L/D box was reversed for half the mice to look for a test-order effect (detailed methodology below). Mice were transported in their home cages with

shielding from light to the behavior testing room an hour before testing. Tests were performed during their active phase, starting 2 hours after lights off.

Experiment 3: Difference in HPA response between WT and NesARKO mice

T lowers basal and overall HPA response in WT castrated male mice, a phenomenon not seen in mice with universal AR dysfunction (Chen et al., submitted to Hormones and Behavior). To compare HPA response in WT versus NesARKO animals, mice were exposed to the L/D box for 10 min, then blood was collected for corticosterone measurement 20, 40, 60 and 120 minutes after initial exposure to the test. Baseline measures were taken from animals that were not exposed to the box. ARlox and Nes-Cre males served as additional controls. Mice were separated and single housed in individual cages the day before testing. On the day of testing, animals were taken in their cages and protected from light to the behavior room an hour before exposure to the L/D box. Tests were performed during their active phase, starting 2 hours after lights off.

PCR identification of genotype

PCR was run with ear punches taken from pups at weaning to detect mice carrying the Cre gene, and the WT or floxed AR allele. To discriminate between the two AR alleles, PCR primers used were AGC CTG TAT ACT CAG TTG GGG and AAT GCA TCA CAT TAA GTT GAT ACC. The resulting bands were 930bp for the floxed AR allele and 860bp for the WT allele. Therefore, PCR results for animals with ear punches that were positive for Cre and showed a band for the WT floxed AR were considered NesARKO

males; animals that were negative for Cre and had a WT AR band were considered WT males; animals that were positive for Cre and had a WT AR band were considered Nes-Cre males; and animals that were negative for Cre and had a band for WT floxed AR were considered ARlox males.

X-gal Staining

Ear punches were taken to determine genotype of adult male mice. Animals expressing both the lacZ reporter and Nes-Cre transgenes were experimental animals, while those expressing lacZ without Nes-Cre were negative controls. Animals were overdosed with sodium pentobarbital i.p. and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). Brains, pituitaries, adrenal glands and testes were collected and post-fixed for 2 hours in 4% paraformaldehyde, at 4^{o}C. Tissues were then transferred into a 20% sucrose solution at 4^{o}C until they sank (approx. 2 days). Then brains were frozen sectioned in the coronal plane at 35μm, while pituitaries, adrenals and testes were left whole. Free floating brain sections and whole tissues were rinsed three times in PBS for 5 minutes each before incubating overnight in X-gal solution at room temperature and protected from light. Brain tissues were rinsed three times in PBS for 5 minutes, mounted onto slides, and coverslipped after dehydration through graded alcohols and citrisolv.

AR Immunocytochemistry (ICC)

A separate cohort of animals derived from the Nes-Cre x loxAR cross were overdosed with sodium pentobarbital i.p. and intracardially perfused with 0.9% saline followed by

4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). Brains were collected and post-fixed 2 hours in 4% paraformaldehyde, at 4^{o}C. Tissues were transferred into a 20% sucrose solution at 4^{o}C approx. 2 days, then frozen sectioned in the coronal plane at 35µm. Pituitaries, adrenals, and testes were fresh harvested from animals overdosed with pentobarbital, and were cryostat sectioned at 14µm. All ICC reactions were performed at room temperature on a rotomixer unless otherwise indicated. Brains were stained as free-floating sections and the remaining tissue stained on slides. Brain sections were rinsed in four 5 minute washes in a phosphate-buffered gelatin Triton solution (PBS-GT; 0.1% gelatin, 0.3% Triton X-100, in PBS, pH 7.4), followed by 0.5% sodium borohydride in PBS-GT for 15 minutes, and three 5 minute washes in PBS-GT. Sections were incubated in 10% NGS in PBS-GT for 1 hour to block non-specific binding of the secondary antibody. Two 5 minute washes in PBS-GT, and later a 10 minute incubation in avidin block followed. After two 5 minute PBS-GT washes, they were incubated 10 minutes in biotin block (Avidin/Biotin Blocking Kit, Vector, cat#SP-2001). Two 5 minute PBS-GT washes took place before the tissue was incubated for 36 hours at 4°C in 1% NGS in PBS-GT with AR primary antisera at 1:5000 concentration (rabbit monoclonal - Abcam, code#ab52615, clone ID EP670Y). Then tissue was rinsed in PBS-GT, and incubated 1 hour in 1% NGS in PBS-GT with biotinylated goat anti-rabbit secondary antisera at 1:1000 concentration (Jackson Immunoresearch, lot # 88762, code # 711-065-152). Brain sections were rinsed again in PBS-GT, followed by 1 hour incubation in PBS-GT with an Avidin-Biotin complex solution made 30 minutes before use (1 drop of each solution A and B per 10ml of PBS-GT; ABC Elite Kit [standard], Vector Laboratories, catalogue # PK6100). The tissue was

again washed in PBS-GT before staining with NiCl-enhanced diaminobenzidine (DAB, Sigma, St. Louis, MO) in a 0.05 M Tris Buffer, pH 7.2. Then tissue was washed in PBS-GT and in mounting solution before being mounted and coverslipped after dehydration through graded alcohols and xylene. Pituitary, adrenal and testis slides were treated the same as for the brain sections, except that a phosphate-buffered solution without gelatin and Triton was used, sodium borohydride was not used, and slides were subjected to 5 minute fix in 4% paraformaldehyde between initial washes and NGS block.

Castration and Silastic capsule implant for androgen treatment

At PN60, mice were anesthetized with isoflurane, and their scrotal sacs and backs shaved for castration and capsule implants. Castrations were performed through a 5mm incision along the midline of the scrotal sac. Testes were visualized and silk suture was used to tie the vas deferens, blood vessels and associated fat pad before removing the testes. The testes of NesARKO mice were significantly smaller than those from the other genotypes (p<.001; NesARKO: M=.12g+0.01 (standard error of the mean); WT: M=.18g+0.01; Nes-Cre: M=.18g+0.01; LoxAR: M=.19g+0.01). To equilibrate circulating androgens across groups, animals also received subcutaneous implants on the dorsal surface of Silastic capsules containing T (1.6mm inner diameter, 3.2mm outer diameter; 6mm effective release length). T capsules of these dimensions produce T levels approximating those of WT males (Zuloaga et al., 2008). Incisions were closed with surgical staples. Mice were injected with .05ml Ketofen (100mg/ml, sc) analgesia for two consecutive days after surgery.

Anxiety-related behavior testing

All testing chambers were cleaned with 70% ethanol and dried between subjects.

Open field (OF) and novel object (NO) tests: The OF was a rectangular unlidded Plexiglas box (40.6 x 40.6 cm with walls 30.5 cm high). Testing took place under fluorescent lights projecting 460 lux throughout the arena. Mice were placed in the testing chamber facing a corner and allowed to move freely for 5 min. Animals were returned to their home cage for 15 min, then placed back into the chamber in which a novel object (a clear cylindrical petri dish with color tape, 5 cm in diameter) had been placed in the center of the field for another 5 min. Locomotor activity was tracked based on the interruption of photobeams and collected by computer software (Versamax). For the OF portion of the test, measures included latency to enter the middle portion of the arena (a 20.3 x 20.3 cm area in the center), number of entries into the center area, total time spent in the center area, and number of rearings. For the NO portion of the test, measures included latency to visit the novel object, number of visits paid to the object, total time spent near the object, and number of rearings performed anywhere in the chamber.

Elevated plus maze test: The EPM consisted of four black arms, 25.4 cm long and 5.7 cm wide, stretched out from a center square at 90 degree intervals. Two of the opposing arms had black walls of 13.3 cm tall. The maze rested 43.8 cm from the floor. Testing took place with an overhead lamp that provided 50 lux lighting in the open arms and 25 lux in the closed arms. Animals were placed into the maze facing a closed arm and allowed 10 minutes to move freely. An overhead camera recorded their activity. Measures taken from video recordings included number of open arm entries (4 paws in

an open arm), number of stretch attends into open arms (2 paws in an open arm), total percentage of time spent in open arms (time in open arm/[time spent in closed arms + time spent in open arms]), and number of head dips over the edge of the arm while in an open arm.

Light/dark box test: The L/D box was a rectangular Plexiglas arena divided at the center by a wall to yield two areas 24.1 x 18.4 cm each. The "light" part of the box was open-topped and had 3 transparent walls, while the "dark" part was a fully-enclosed black box. The dividing black wall between the light and dark sides of the box had a 10.2x5.1cm opening, allowing mice to move freely between the sides. Testing took place with an overhead lamp providing 500 lux in the light area and 2 lux in the dark area. Animals were placed on the light side of the apparatus facing the connecting doorway and allowed 10 minutes to move freely. Behavior coding started after animals first enter the dark side. Measures made from video recordings included number of stretch attends towards the light chamber (2 paws in light side), number of entries into the light chamber (4 paws in light side), total time spent in the light chamber, and number of rearings made during time spent in the light chamber (number of rears/time in light side).

Exposure to L/D Box and Blood collection for CORT sampling

A separate cohort of castrated, T-treated mice of each genotype were used to study CORT hormone response to the L/D box in the active phase. Animals were separated and individually housed the day before L/D box exposure and blood collection. Mice were transported in their home cages protected from light to the behavior room and

allowed to acclimate for an hour before exposure to the L/D box. Starting two hours after lights off, mice were exposed to the anxiety provoking L/D box chamber for 10 minutes before sacrifice for blood collection. At time of blood collection (20, 40, 60 and 120 minutes after initial exposure), mice were heavily anesthetized with isoflurane and decapitated. Baseline CORT was also collected from animals not exposed to the L/D box. Blood was collected into heparinized tubes, spun in a centrifuge at 8^{o}C for 20 minutes at 3000 rcf. Separated plasma samples were stored at -80^{o}C until analyses. Samples were assayed for CORT using Coat-a-Count kits (Diagnostics Products Corporation, Los Angeles, CA. USA) at the Diagnostic Center for Population and Animals Health at MSU. All samples were run in duplicate and averages were used for analysis.

Statistics

Experiment 2: analyses for the OF and NO tests consisted of one-way ANOVAs with genotype (WT, NesARKO, ARlox, Nes-Cre) as an independent factor. Analysis for the EPM and LD box tests initially consisted of two-way ANOVAs to determine a potential test order effect with genotype (WT, NesARKO, ARlox, Nes-Cre) and test order (EPM/LD versus LD/EPM) as independent factors. If this analysis did not reveal an effect of test order, subsequent one-way ANOVAs were conducted with genotype alone as independent factor. Analyses were followed up by post-hoc LSD tests. If the two-way ANOVA found a test order effect, data was subsequently analyzed separately for each test order using one-way ANOVAs with genotype as the independent factor.

Experiment 3: analysis consisted of a two-way ANOVA with group (NesARKO, WT males, Nes-Cre, ARlox) and blood collection time (baseline, 20, 40, 60, 120mins) as independent factors, followed by post-hoc LSD tests.

RESULTS

Experiment 1: Validation studies

Cre expression: Examination of lacZ expression in the mice carrying both Nes-Cre and the reporter construct, where presence of Cre should remove a stop codon, allowing constitutively expressed beta-galactosidase, indicated widespread expression of Cre in the CNS of Nes-Cre mice (Figure 6A-C). No staining was seen in brain tissue from animals carrying the lacZ construct without the Nes-Cre transgene. Mice carrying both transgenes also showed scattered X-gal staining in whole pituitary, adrenals and testes.

AR immunoreactivity (-ir): Probing for AR protein in mice carrying both Nes-Cre and loxAR revealed maintained AR protein in some brain regions but not others. Eight sets of animals were run, each consisting of a NesARKO experimental animal, a WT positive control, and an *i*Tfm male (in which the loxAR allele is recombined in all tissues) as negative control. In all sets, WT males showed normal AR-ir in all predicted brain areas including those mentioned above (Figures 6D-F), while *i*Tfm mice showed no AR-ir (Figures 1J-L). NesARKO mice showed no AR+ cells in the hippocampal formation (Figure 6H), mPFC (Figure 6I), bed nucleus of the stria terminalis (BNST), and dorsal periaqueductal grey (dPAG), all of which have been implicated in regulating anxiety-

related behavior and the HPA axis. However, abundant AR protein was seen throughout the amygdala (Figure 6G), and hypothalamus, including the paraventricular nucleus of the hypothalamus (PVN). The pattern of AR-ir staining was similar in all NesARKO animals. AR-ir in pituitary, adrenal and testes was present and comparable in NesARKO and WT mice, while absent in *i*Tfm animals.

The absence of AR expression in particular brain regions of the NesARKO mice, including the hippocampus and mPFC, offered an opportunity to see whether AR in those brain regions mediate testosterone's anxiolytic effects on behavior and moderating effects on HPA response. We therefore tested these processes in NesARKO and control males.

Experiment 2: Anxiety-related behaviors

Deletion of AR in the hippocampus and mPFC had no effect on anxiety-related behavior, as NesARKO mice did not differ from WT controls. The only difference across genotypes seen was an unexpected one, specifically that the Nes-Cre transgene slightly affected anxiety-like behavior. In the OF test, one-way ANOVA revealed a significant effect of genotype on rearing ($p<.05$), which post-hoc LSD comparisons confirmed was due to WT mice rearing less than Nes-Cre males (Figure 7A). No significant effects of genotype were found on time spent in the center area (Figure 7B), latency to center area, or number of entries to the center area.

For the NO test, we saw no effects of genotype on time spent with novel object (Figure 2C), number of visits to the object (Figure 7D), latency to visit object, or number of rears.

For the EPM, two-way ANOVAs indicated no main effect of test order. Consequently, one-way ANOVA revealed an effect of genotype on number of head dips ($p<.05$). Post-hoc LSD comparisons confirmed that WT mice performed more head dips compared to either of the groups carrying the Nes-Cre transgene: NesARKO and Nes-Cre animals (Figure 7E), suggesting that the presence of Nes-Cre alone affects this behavior. No significant effects of genotype were found on time spent in open arms (Figure 7F), number of open arm entries, or number of stretch attends.

For the L/D box, two-way ANOVA found a main effect of test order. Mice that ran the L/D box after the EPM test entered the light side fewer times, performed fewer rears, spent less time in the light side, and performed more stretch attends than animals tested before the EPM test. No interactions were found between genotype and test order. Due to these effects of test order, data were separated by test order (L/D before or after the EPM). One-way ANOVAs found no significant differences between groups in any parameters measured in either test order (Figure 7G).

Experiment 3: HPA activity

No group differences were found on either the basal levels of CORT, or the CORT response and recovery after exposure to a mild stressor (Figure 8). Neither the NesARKO manipulation nor the presence of Nes-Cre or ARlox transgenes seemed to affect basal CORT levels. Similarly, neither of the transgenes, either together or individually, altered HPA initial response or subsequent recovery.

DISCUSSION

We used the Cre-LoxP technology seeking to disable AR in the entire CNS to determine the role of neural AR on anxiety-related behavior and HPA activity. Surprisingly, this cross generated full KO only in some brain areas and incomplete KO in others, despite evidence that Cre was ubiquitously expressed in the brain. AR was fully knocked out in some anxiety-related areas like the hippocampus, mPFC, BNST and dPAG, but was only knocked down in others, like the amygdala and hypothalamus, including the PVN. However, this selective brain KO allowed us to probe the role of AR in those particular brain regions. We found that NesARKO animals did not differ from WT males in anxiety-related behaviors or HPA response, indicating that ARs in the hippocampus, mPFC, BNST and dPAG are not necessary for T's modulation of these behaviors and response.

The single behavioral difference found between genotypes confirms the importance of control groups for studies using Cre-LoxP technology. NesARKO mice performed fewer head dips than their WT counterparts in the EPM. However, this is apparently due to the presence of Cre, not to excision of AR, because Nes-Cre controls also showed fewer head dips than WT mice, and did not differ from NesARKO animals. The apparent influence of Cre on anxiety-related behavior was reinforced when Nes-Cre animals performed fewer rearings than WT males in the OF. Although our animals appeared healthy, studies have reported that Cre recombinase enzyme alters the phenotype of cells (Xiao et al., 2012) and, in high levels, can cause defects in brain development

(Forni et al., 2006). It is not clear whether behavioral differences seen in our study are due to the *Cre* gene or the recombinase enzyme. Nevertheless, these findings reinforce the need to examine all three control groups accompanying Cre-LoxP studies.

The NesARKO manipulation also had no effect on HPA activity. We previously found that mice with ubiquitously disabled AR show higher basal CORT levels, and a heightened and longer HPA response after exposure to the L/D box, than WT males. This effect of AR on HPA activity, especially on negative feedback, is not likely to be mediated by AR in the hippocampus or mPFC, despite their implication in the negative feedback aspect of the HPA function (Jankord and Herman, 2008; Smith and Vale, 2006), because NesARKO males that lack AR in those areas did not differ from WT males in CORT response. However, since AR was only partially knocked out in the PVN and amygdala, which have also been implicated in HPA function (Smith and Vale, 2006; Jankord and Herman, 2008), it is possible that AR in these regions is responsible for the modulation of CORT seen in universal AR KO animals. Consonant with this idea, Carrasco and colleagues (2008) showed that CORT levels were similar, while CRF expression in PVN and amygdala differed, between mouse lines bred for high versus low anxiety. Another possibility is that intact *peripheral* AR in NesARKO, specifically in the pituitary and adrenal glands, plays a role in regulating HPA function, resulting in normal responses. In fact, rats bred for high anxiety show differences in the adrenals and pituitary compared to those bred for lower anxiety (Salome et al., 2006).

It is also important to note that testicular AR-ir in NesARKO was similar to WT, yet the testes were smaller in NesARKO males. This difference might be due to scattered expression of Cre in testes, which might have excised some AR. Indeed, ubiquitous AR KO results in dramatically smaller testes (Verhoeven et al., 2010). In spite of smaller testes, NesARKO mice have slightly, but not significantly, higher circulating T levels than WT animals (Juntti et al., 2010). It is not clear how similar T levels could be produced by different size testes in the two genotypes.

The present study reveals that AR in the hippocampus, mPFC, BNST, and dPAG are not necessary for the anxiolytic effects of T or for the modulation of HPA function. This work also suggests a potential role of AR in the amygdala and hypothalamus, specially the PVN, on anxiety-related behavior and HPA activity. From the present results, a role for peripheral AR, especially in adrenals and pituitary, cannot be ruled out. Further studies looking at cFos expression in response to an anxiogenic stimulus in WT versus *i*Tfm mice could determine the brain areas that differ in cell activity to pinpoint regions where AR might be modulating these behaviors and response.

CHAPTER 4: TESTOSTERONE WORKS THROUGH ANDROGEN RECETORS TO MODULATE NEURONAL RESPONSE TO AN ANXIOGENIC STIMULUS

ABSTRACT

In rodents, testosterone (T) exerts anxiolytic effects through functional androgen receptors (AR). Treatment of castrated adult mice with T reduces indices of anxiety in wild-type (WT) males, but not males with a spontaneous mutation of AR that renders it dysfunctional. We used Cre-LoxP technology to create males carrying another dysfunctional AR allele, referred to as *i*Tfm (*induced* testicular feminization mutation). Adult WT and *i*Tfm mice were castrated and treated with T. Thirty days later, they were exposed to the anxiogenic light/dark box, sacrificed 50 minutes later and their brains processed for cFos immunoreactivity. Castrated WTs given a blank capsule (WT+B) were used as additional controls. Qualitative assessment indicated that the same brain regions responded to the stimulus in all groups. Quantitative cFos analyses showed that the basolateral amygdala (blAMY) of WT+T males contained more cFos expressing cells than did *i*Tfm+T or WT+B mice. In contrast, WT+T males displayed fewer cFos+ cells than *i*Tfm+T or WT+B groups in the suprachiasmatic nucleus of the hypothalamus (SCN). No effects of genotype or hormone were seen in cFos expression in the hippocampus, medial prefrontal cortex, periventricular nucleus of the hypothalamus, oval and anterodorsal bed nucleus of the stria terminalis or dorsal periaqueductal grey.

AR immunohistochemistry indicated that ~65% of cells in the blAMY and SCN are AR+, therefore functional AR may act directly on cells in those regions in WT males to modulate response to anxiogenic stimuli.

INTRODUCTION

Anxiety disorders are the second most prevalent mood disorder with an estimated lifetime prevalence of 28.8% in the United States (Kessler et al., 2005). Interestingly, these disorders are more prevalent in women than in men (McLean at al., 2011), a sex difference that arises in adolescence (Zahn-Waxler et al., 2008). The sex difference and onset at puberty suggest a role for gonadal hormones, including testosterone (T), in anxiety disorders (Menger et al., 2010; Wu and Shah, 2011).

Endogenous testosterone (T) appears to reduce anxiety in men and women. In aging men, as T declines, anxiety level rises (Amore et al., 2009; Sternbach, 1998) and is reduced with T treatment (Wang et al., 1996). Congruently, anxiety level increases with androgen receptor (AR) blockade therapy for prostate cancer treatment and is alleviated when treatment ends (Almeida et al., 2004). T has also been linked to anxiety in women, as females experiencing generalized anxiety disorder had lower salivary testosterone levels than control women (Giltay et al., 2012).

T also has the capacity to modulate anxiety in other species including rodents, as revealed by tests of anxiety-related behaviors. Compared to intact males, mice given T spend more time in the open arms of the elevated plus maze (EPM; Aikey et al., 2002; Frye et al., 2008). T treatment in female rats increases the time spent in the open arms of an EPM and the number of entries into the center area of an OF (Frye and Lacey, 2001). The 5α-reduced form of T, dihydrotestosterone, also has anxiolytic effects on the OF and EPM tests in male and female rats (Aikey et al., 2002; Edinger and Frye, 2004, 2006; Frye and Lacey, 2001).

We previously found that the anxiolytic effect of T is modulated by activation of ARs (Chen et al., submitted to Hormones and Behavior; Zuloaga et al, 2008). In adult castrated male mice, T treatment reduces anxiety-related behavior in wild type (WT) males, but has no effect on males with universally disabled AR, whether AR dysfunction is due to a spontaneous mutation of the allele (Zuloaga et al., 2008) or an induced knock out (KO; Chen et al., submitted to Hormones and Behavior). In subsequent studies we achieved conditional KO of the AR allele in several brain regions, including the hippocampus, medial prefrontal cortex (mPFC), periventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BNST), and dorsal periaqueductal grey (dPAG). Despite the loss of AR in those brain regions, T treatment still had an anxiolytic effect (Chen et al., in preparation), indicating that those brain regions are not the site of androgen action for reducing anxiety. Because the animals in this study retained AR expression in several other brain regions, including the amygdala and hypothalamus, those areas remain possible sites of androgen's anxyiolytic action.

Here, we sought to determine which brain nuclei in mice respond to anxiety-provoking stimuli and whether AR is normally expressed in those regions. To do this, we compared cFos response in WT male mice with genetic males carrying an induced KO of the AR allele, an *induced* testicular feminization mutation (*i*Tfm; described in Chen et al., submitted to Hormones and Behavior, and below). cFos immunohistochemistry revealed that, given T, adult WT mice show more cFos expressing cells in the basolateral amygdala (blAMY) and fewer such cells in the suprachiasmatic nucleus (SCN) than *i*Tfm mice after exposure to the anxiogenic light/dark box. AR immunohistochemistry in WT brains revealed that 60-65% of cells in these two regions are AR positive, suggesting a potential for AR to directly modulate the response of these cells to anxiogenic stimuli.

MATERIALS AND METHODS

Experimental animals

We used the CreLox system in mice to create animals with dysfunctional AR, the *induced* testicular feminization mutation mice (*i*Tfm), as described previously (Chen et al., in preparation). Briefly, mice carrying a conserved lox sequence of 34 base pairs at both ends of exon 2 of the AR gene ("floxed" AR, a generous gift from De Gendt et al., 2004) were crossed with animals expressing the cyclization recombination enzyme (Cre) universally ("deleter" mice, Jax stock 003724). When both transgenes are present,

exon 2 of the AR gene is excised, creating a frame-shift mutation that renders AR dysfunctional. Female offspring carrying this recombined AR allele were bred to wildtype (WT) C57B16 (Jax) males, and the recombined AR allele was transmitted to some female progeny without the *Cre* transgene, as confirmed by PCR. These females were used to found the colony of *i*Tfm mice perpetuating the disrupted AR allele. We confirmed that breeding these females with WT males produced genetic male offspring with dysfunctional AR and consequently a recapitulation of the phenotype in mice with the spontaneous mutation (sTfm): small, abdominal testes, short anogenital distance, feminine external genitalia, external nipples, and low circulating T in adulthood. Female carrier dams also produced male offspring carrying the WT AR allele to serve as controls.

All mice were born in our laboratory, housed in plastic cages (29x18x18cm) at approximately 27^0C in a 12:12 LD cycle, and provided *ad libitum* tap water and rodent chow (Harlan Teklad 8640 Rodent Diet [Madison, WI]). Mice were weaned at postnatal (PN) day 23 and group housed. On the day of weaning, animals were ear punched for genotyping with polymerase chain reaction (PCR) as described below. WT and *i*Tfm males were castrated at postnatal (PN) day 60 and implanted either with testosterone (T) or blank (B) Silastic capsules as described previously (Chen et al., submitted to Hormones and Behavior). Exposure to the light/dark box (L/D box) and subsequent brain collection for immunohistochemistry took place on PN90. All housing conditions and experiments were performed in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the MSU Institutional Animal Care and Use Committee.

Experiment 1: Differences in brain activity pattern in response to exposure to the L/D box between WT and *i*Tfm mice

T reduces anxiety-related behavior in adult WT male mice but not *i*Tfms with dysfunctional AR, indicating a role for AR in anxiety in mice (Chen et al., submitted to Hormones and Behavior). Here, we looked at the immediate early gene cFos in castrated and T-treated WT male (WT+T) and *i*Tfm (*i*Tfm+T) mice to determine whether there are any differences in the pattern of cFos activation in the brain after exposure to the L/D box that parallel differences in their behavior. Gonadectomized WT males given blank capsules (WT+B) were used as an extra control to confirm that T reduces anxiety in WT males. An initial qualitative examination was conducted to determine any differences in activity pattern between the groups. A follow up quantitative analysis was run to determine cFos expression differences between the groups on areas previously shown to be related to anxiety (areas and their circuitry in Figure 9).

Experiment 2. Localization of AR in anxiety-related brain regions

Experiment 1 revealed brain areas where cFos expression differed between WT+T and *i*Tfm+T mice after exposure to the anxiogenic L/D box, indicating an effect of T and AR on neuronal activity. To determine the likelihood that AR positive cells were activated by the stimulus exposure, the percentage of AR positive cells was estimated in the brain regions of interest in another cohort of WT+T mice. Adult WT C57B16 males were castrated and implanted with T capsules on PN60 and sacrificed on PN90 for AR ICC. Every other brain section was collected for AR immunohistochemistry while the

intervening sections were used for neutral red Nissl counterstain to determine total cell number in each region.

PCR identification of genotype

PCR was used to detect the recombined AR allele and the *Sry* gene. To discriminate the WT and recombined AR alleles, primers targeted and amplified the sequence that includes the lox sites and exon 2 of the AR gene. The primers used were AGC CTG TAT ACT CAG TTG GGG and AAT GCA TCA CAT TAA GTT GAT ACC. The resulting products were 860bp for the wild type AR, and 400bp for the recombined AR allele. Animals with PCR results positive for *Sry* and WT AR were classified as WT males; mice positive for *Sry* and a recombined AR were classified as *i*Tfm males. The genotype indicated by PCR was verified by the phenotype revealed during castration as described below.

Castration and Silastic capsule implant for androgen treatment

At PN60, mice were anesthetized with isoflurane and their backs shaved for capsule implants. Castrations in WT mice were performed through a 5mm incision along the midline of the scrotal sac. Testes were visualized and silk suture was used to tie the vas deferens, blood vessels and associated fat pad before removing the testes. The incision was closed using surgical staples. In *i*Tfm mice, the testes were undescended and small as expected (De Gendt et al., 2004); thus, an abdominal incision was made through which the testes were visualized on each side of the bladder, tied and removed as

described above. The abdominal muscle wall was closed with suture and the overlying skin closed with clips.

During the same bout of anesthesia, animals also received subcutaneous implants on their dorsal surface of Silastic capsules containing either testosterone (T) or left blank (B) (1.6mm inner diameter, 3.2mm outer diameter; 6mm effective release length). Capsules of this size produce T levels at near-normal circulating levels in mice (Zuloaga et al., 2008). The incision over the capsule was closed with surgical staples, and mice were injected with .05ml Ketofen (100mg/ml, sc) for analgesia.

Exposure to the L/D box

Mice were taken to the behavior room in their home cages under a dark cloth to prevent exposure to light. Mice were then left in the room to acclimate for an hour. Exposure took place during the animals' active phase, 2 hours after lights off.

The L/D box consisted of a rectangular Plexiglas arena divided at the center by a wall to yield two areas measuring 24.1x18.4cm each. The "light" part of the box was open-topped with 3 transparent walls, while the "dark" part was an enclosed black box. The dividing black wall between the light and dark sides of the box had a 10.2x5.1cm opening, which allowed mice to move freely between the sides. Testing took place with an overhead lamp providing 500 lux in the light area and 2 lux in the dark area. Animals were placed on the light side of the apparatus facing the connecting doorway and allowed 10 minutes to move freely. The testing chamber was cleaned with 70% EtOH between subjects.

Immediate Early Gene cFos Immunohistochemistry (ICC)

Pilot studies sacrificing mice at 20, 30, 40, 50, 60 and 70 minutes after exposure to the L/D box indicated that the most robust cFos-ir was observed 50 minutes after the initial exposure. Therefore, experimental mice were all exposed to the apparatus for ten minutes and sacrificed 50 minutes after the start of the behavior testing. Animals were injected with an intraperitoneal overdose of sodium pentobarbital and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). Brains were collected and post-fixed overnight in 4% paraformaldehyde, at 4^{o}C. Brains were then transferred into a 20% sucrose solution at 4^{o}C until they sank (1-2 days), then scored on the right cortex and microtome sectioned in the coronal plane at 35µm for cFos ICC the same day. Every other section was collected for cFos ICC. All ICC reactions were performed at room temperature and on a rotomixer unless otherwise indicated. Brain tissue was stained as free-floating sections. Sections were rinsed in three 5 minute washes of 0.1M phosphate buffer solution with 0.3% triton and 0.1% gelatin (PBS-GT) followed by a 25 minute incubation of 10% normal goat serum (NGS) in PBS-GT. Following the block, brain sections were rinsed in PBS-GT, followed by a 24 hour incubation at 4^{o}C off the rotomixer in 1% NGS in PBS-GT with cFos primary antisera at 1:20,000 concentration (cFos rabbit polyclonal primary antibody from Santa Cruz Biotechnology, catalogue # sc-52). Tissue was then washed in PBS-GT, succeeded by incubation for 1 hour in 1% NGS in PBS-GT with biotinylated goat-anti rabbit secondary antisera at 1:200 concentration (Jackson Immunoresearch, lot # 88762, code # 711-065-152). Brain sections were then washed in PBS-GT, and treated for 1 hour with an Avidin-Biotin complex solution made 30

minutes before use (1 drop of solution A and 1 drop of solution B per 10ml of TBS - ABC Elite Kit [standard], Vector Laboratories, catalogue # PK6100). The tissue was again washed in PBS-GT before being stained with SG peroxidase substrate solution for 2 minutes (Vector Laboratories, catalogue # SK4700). The tissue was then washed in PBS-GT and mounting solution before being mounted and air dried overnight. Slides were then counterstained with neutral red and coverslipped after dehydration through graded alcohols and xylene.

cFos Quantification Analysis

For each brain region analyzed, the Stereo Investigator software (MBF Bioscience, Williston, VT) was used to trace the perimeter, and a counting frame was set so that all cells within the circumscribed area were quantified. Cells expressing cFos were counted with a probe while cells counterstained with neutral red (but negative for cFos) were counted with another probe. Brain regions in the right versus the left hemisphere were also tracked. In sum, measures for each brain region on each side included regional area, number of cFos-ir cells, and total number of cells (cFos-ir cells + neutral red stained cells). All tracings and analyses were done by an experimenter "blind" to group membership. Brain areas analyzed were those previously shown to be related to anxiety-related behavior (areas and their circuitry in Figure 9).

Basolateral (blAMY), centrolateral (clAMY), and centromedial (cmAMY) amygdala. Two sections were analyzed where these three regions were present. The first section was selected based on the presence of the rostral-most section where the arcuate nucleus first appears. The second selected was the following caudal section.

Suprachiasmatic Nucleus of the Hypothalamus (SCN). The SCN could be observed in a total of eight brain sections in each brain examined. The two middle sections that had the largest areas were used for analysis.

Hippocampal CA1 Area. The first hippocampal section used was where the caudal-most portion of the medial habenula was visible. The second hippocampal section analyzed was the next section rostral to the first.

Medial Prefrontal Cortex (mPFC). The first section used for analysis was the rostral-most section where the lateral ventricles first appear. The second analyzed was the following caudal section.

Periventricular Nucleus of the Hypothalamus (PVN). The PVN could be observed in a total of seven brain sections in each brain examined. The middle section was used for analysis.

Oval Nucleus (ovBNST) and anterodorsal (adBNST) Bed Nucleus of the Stria Terminalis. The section where the anterior commissure just comes together was used for analysis of these two nuclei.

Dorsal Periaqueductal Grey (dPAG). The dPAG section used was that in which the caudal-most portion of the Edinger-Westphal nucleus was found.

Cell size analysis in blAMY

The same blAMY sections used for cFos analysis were used to measure cell size. The perimeter was traced and Stereo Investigator counting frames set so that around 35-50 cells were measured per section. The two-dimensional profile of cFos positive and negative cells was measured. Measurements from each section were averaged so that

a single estimate of cell size, one for cFos positive and one for cFos negative, was collected per region analyzed.

AR ICC

A separate cohort of mice were perfused and brain sections obtained as for cFos ICC above, except that post-fix in 4% paraformaldehyde was for 2 hours. Brain sections were rinsed in four 5 minute washes in a phosphate-buffered gelatin Triton solution (PBS-GT; 0.1% gelatin, 0.3% Triton X-100, in PBS, pH 7.4), followed by 0.5% sodium borohydride in PBS-GT for 15 minutes, and three 5 minute washes in PBS-GT. Sections were incubated in 10% NGS in PBS-GT for 1 hour to block non-specific binding of the secondary antibody. Two 5 minute washes in PBS-GT, and later a 10 minute incubation in avidin block followed. After two 5 minute PBS-GT washes, sections were incubated 10 minutes in biotin block (Avidin/Biotin Blocking Kit, Vector, cat#SP-2001). Two 5 minute PBS-GT washes took place before the tissue was incubated 36 hours at 4°C in 1% NGS in PBS-GT with AR primary antisera at 1:5000 concentration (rabbit monoclonal - Abcam, code#ab52615, clone ID EP670Y). Then tissue was rinsed in PBS-GT, and incubated 1 hour in 1% NGS in PBS-GT with biotinylated goat anti-rabbit secondary antisera at 1:1000 concentration (Jackson Immunoresearch, lot # 88762, code # 711-065-152). Brain sections were rinsed again in PBS-GT, followed by 1 hour incubation in PBS-GT with an Avidin-Biotin complex solution made 30 minutes before use (1 drop of each solution A and B per 10ml of PBS-GT; ABC Elite Kit [standard], Vector Laboratories, catalogue # PK6100). The tissue was again washed in PBS-GT before reacting with NiCl-enhanced diaminobenzidine (DAB, Sigma, St. Louis, MO) in a

0.05 M Tris Buffer, pH 7.2. Then tissue was washed in PBS-GT and mounting solution before being mounted and coverslipped after dehydration through graded alcohols and xylene.

Neutral red Nissl staining

Intervening sections not used for the above AR ICC were mounted and air dried at least overnight. Sections were then rehydrated 6 minutes in water before being exposed to neutral red for 3 minutes. Slides were then dehydrated through graded alcohols and xylene before being coverslipped.

AR Quantification Analysis

As with the cFos quantification, Stereo Investigator software was used for AR analysis. Since AR-ir and Nissl stains were run in alternate brain sections, perimeters were traced for both, and compared to look for potential tracing differences that could affect cell count. The counting frame for AR-ir and neutral red stained sections were set so that all cells within the area were quantified. Brain regions in the right versus the left hemisphere were examined. In sum, measures for each brain region on each side included regional area, number of AR-ir cells, total number of cells (neutral red stained), and percentage of AR-ir cells (number of AR-ir cells x 100 / total number of cells). Regions analyzed were those where differences were found between WT+T and *i*Tfm+T mice in cFos response to the anxiogenic L/D box: blAMY, clAMY, and SCN.

Statistical Analysis

Experiment 1 - cFos ICC analysis. For the blAMY, clAMY, cmAMY, SCN, hippocampus and mPFC an initial repeated measures ANOVA was run with laterality (left vs. right) and section (1 vs. 2) as repeated measures, and genotype (WT+T, *i*Tfm+T and WT+B) as independent variable. When no main effect of laterality was found, right and left hemisphere counts were averaged. Section counts were also averaged. After the collapse of data across sides, a one-way ANOVA was run with genotype as independent variable, followed by post-hoc LSD tests. When an effect of laterality was found with the clAMY, a two-way ANOVA was run with laterality and genotype (WT+T and *i*Tfm+T) as independent variables. Matched paired t-tests were also run to compare left and right measurements with each group. For the PVN, ovBNST, adBNST and dPAG, an initial repeated measures ANOVA was run with laterality as repeated measure and genotype as independent variable. If no laterality differences were found, hemisphere data was averaged and a one-way ANOVA was run with genotype as independent variable, followed by post-hoc LSD tests.

For cFos positive and negative cells, separately, repeated measures ANOVAs were run with laterality and section as repeated measures, and genotype as independent variable. Since no laterality or section differences were found, both measures were collapsed so that each animal provided a single data point for cFos positive and cFos negative cell counts. A subsequent two-way ANOVA was run with cell type (cFos positive vs. negative) and genotype as independent variables, followed by post-hoc LSD tests.

Experiment 2 - AR ICC analysis. For the blAMY, clAMY and SCN, a one-way ANOVA was first run with area (obtained from AR-ir sections vs. Nissl sections) as independent

variables to determine potential influence of area measure on cells included for AR quantification analysis. Area measures obtained from AR-ir and Nissl stain were later averaged. Then, ANOVAs were run for each region with laterality and section as repeated measures. As with cFos analysis, if no differences in laterality were found, data from both hemispheres were averaged. Section data was also averaged. Descriptive statistics were run for area size, number of AR positive cells, total cell number and percent of AR positive cells.

RESULTS

Experiment 1. cFos Quantification

blAMY. Repeated measures ANOVA found no laterality differences between the groups, and subsequent one-way ANOVA with left and right sides collapsed indicated that WT+T males displayed more cFos expressing cells than either *i*Tfm+T or WT+B mice (Figure 10A). There were no group differences for area or total number of cells. Cell size was measured for cFos positive and negative cells in all groups to determine whether the differences seen was a true difference or could be attributed to cell size difference instead. A repeated measures ANOVA found no laterality or section differences. A subsequent 2-way ANOVA comparing the profile area of cFos negative versus cFos positive cells across the treatment groups found no differences in size between cell type (cFos positive and negative) or genotype (Figure 11). Because blAMY

cell size did not differ across groups, counts of cFos positive cells are not biased on account of cell size.

SCN. No laterality differences were found. One way ANOVA after collapsing data across sides indicated that the number of cFos expressing cells was lower in WT+T males than either *i*Tfm+T or WT+B males, which did not differ from each other (Figure 10B). No differences were seen in area size or total cell number in the SCN.

clAMY. Repeated measures ANOVA revealed a main effect of laterality on number of cFos positive cell counts (p=.036), as counts from the right hemisphere were greater than those from the left (Figure 10C). A laterality by genotype interaction was also found for number of cFos positive cells (p=.01). A two-way ANOVA comparing only T-treated animals (WT+T and *i*Tfm+T) revealed no main effect of laterality or genotype, and no interaction in these measures. However, visual examination of the data suggested a potential laterality difference in *i*Tfm+T mice, which a paired t-test run comparing the two hemispheres in these animals confirmed (p=.007). There was no such laterality difference in the number of cFos positive cells in WT+T animals (p=.66). There were no laterality or genotype differences found for the area or the total number of cells in clAMY.

For the hippocampus and mPFC, no laterality or group differences in cFos expression were found (Figures 10D, E, respectively). Nor were any laterality or group differences seen in the cmAMY, PVN, ovBNST, adBNST or dPAG (Table 1).

Experiment 2. AR Quantification

The area of the blAMY in sampled sections did not differ between tracings taken from AR ICC and tracings taken from Nissl-stained sections. A repeated measures ANOVA found no effects of laterality. More than 60% of cells were AR-ir in all three regions examined: blAMY, clAMY and SCN (Figure 12).

DISCUSSION

T acts on functional AR to attenuate anxiety-related behavior (Chen et al., submitted to Hormones and Behavior). Here, using cFos ICC, we sought to determine the brain areas where functional AR modulates cell activity to affect this behavior. The initial qualitative evaluation indicated that the same brain regions responded to the anxiogenic stimulus in all three groups tested. Quantitative cFos analysis in known anxiety-related areas revealed that AR modulates cell activity in the blAMY and SCN. AR ICC with WT brains indicated that AR in these areas might be directly modulating cell activity since 60-65% of cells were AR positive. Other regions analyzed showed no difference in cFos expression among the groups.

In the blAMY, while area size did not differ among the groups, WT+T males showed more cFos expression in response to the anxiogenic L/D box than did *i*Tfm+T or WT+B mice. This group difference was not due to differences in cell size since cFos positive and negative cells did not differ in size across the groups. Activation of glutamatergic projections from the blAMY to the clAMY has been found to decrease anxiety-related

behavior (Figure 9; Tye et al., 2011). Therefore, it is possible that AR, present in about 65% of cells in the blAMY, is modulating activity of glutamatergic cells that project to the clAMY, which in turn reduce anxiety-related behavior. In fact, treatment with anabolic-androgenic steroids has been shown to increase phosphate activated glutaminase, the rate-limiting enzyme in the synthesis of glutamate (Carrillo et al., 2009).

AR activation by T also altered cell activity in the SCN since WT+T males showed less cFos expression in this area than did *i*Tfm+T and WT+B mice. Again, no area differences among the groups were found. The core of the SCN, rich in AR (Karatsoreos and Silver, 2007), usually shows cell activation in response to photic inputs to the retina. However, our data suggest that this activity is reduced by AR activation by T. Interestingly, cFos expression is lower even though WT+T animals spend more time exposed to light in the light side of the L/D box compared to *i*Tfm+T males (Chen et al., submitted to Hormones and Behavior). This decreased neural response to the anxiogenic light might encourage WT+T males to explore more by reducing anxiety.

Activity of AR in the clAMY did not affect cFos expression there, since no group differences were found in cFos activation. However, an effect of laterality was seen only in *i*Tfm+T mice where there was more cFos expression on the right hemisphere compared to the left. Related to the anxiety circuitry, the clAMY receives input from the blAMY and projects to the cmAMY (Figure 9; Pitkanen et al., 2000; Pare et al., 2004; Ciocchi et al., 2010; Tye et al., 2011), both of which showed no laterality in cFos

expression after exposure to the L/D box. These results suggest that the clAMY might communicate with regions relevant to anxiety other than the blAMY and cmAMY. The functional significance of the laterality difference seen in *i*Tfm+T mice is unclear.

Examination of cFos expression in other anxiety-related areas including the hippocampus, mPFC, PVN, ovBNST, adBNST and dPAG indicated no differences among the groups, potentially indicating that AR in these areas is not necessary for the modulation of anxiety-related behaviors. That cFos quantification did not differ between the groups in these areas does not prove that cell activity did not differ between the groups since other immediate early gene markers could be expressed in response to the anxiogenic stimulus. However, the idea that AR does not affect the response of these brain regions during anxiety is supported by my previous finding that knocking out AR in the hippocampus, mPFC, BNST and dPAG did not affect anxiety-related behaviors in mice (Chen et al., in preparation).

Chapter 5: DISCUSSION

Results of the experiments in this dissertation indicate that testosterone (T) exerts its anxiolytic effects in mice through functional androgen receptor (AR), very likely by modulating cell activity in the basolateral amygdala (blAMY), suprachiasmatic nucleus of the hypothalamus (SCN) and, potentially, other brain regions not related to anxiety. The findings demonstrate that hypothalamic-pituitary-adrenal (HPA) axis function is also modulated by T activating ARs and that this is unlikely due to AR action in either the hippocampus or the medial prefrontal cortex (mPFC).

Testosterone Modulates Anxiety-Related Behavior Through Functional AR

Group differences in all tests of anxiety-related behavior between wild type (WT) and *i*Tfm (*induced* testicular feminization mutation) mice indicated that T requires functional ARs to exert its anxiolytic effects. Specifically, T reduced anxiety-related behavior only in WT mice and failed to affect *i*Tfm males. Since neither T nor AR alone reduced anxiety levels in *i*Tfm+T or WT+B, respectively, close to those found in WT+T animals, these findings make it unlikely that other modes of action are involved. As mentioned in an earlier chapter, T can be aromatized to estrogen (E) to activate estrogen receptor β (ERβ), and the reduced form of dihydrotestosterone (DHT), 3β-diol, can activate ERβ or act as a neurosteroid agonist at the GABAA receptor (Frye et al., 1996). What's more, both ERβ and GABA receptor activation have been shown to reduce anxiety (Choleris et al., 2008; Griebel and Holmes, 2013). However, the findings here show that, although ERβ or GABA could be involved, T's anxiolytic effects require functional AR. Our *i*Tfm

mice recapitulate the human complete androgen insensitivity syndrome (CAIS), where genetic males present a female phenotype due to dysfunctional AR. There is no evidence that CAIS individuals show higher anxiety levels. To my knowledge, the question has not been addressed. However, there are studies that suggest that AR in humans plays a role in modulating anxiety. Specifically, individuals with AR polymorphisms with differing number of CAG repeats show that those with a greater number of repeats, which has been associated with less effective AR action, display greater symptoms of anxiety (Su et al., 2007; Harkonen et al., 2003).

That functional AR is required for T to exert its anxiolytic effects in mice was demonstrated in several behavioral tests during the animal's resting and active phases. In the case of the L/D box, however, that effect was only seen during the animals' active phase. It is very likely that this effect of time of testing is due to the animals' reaction to light. During the resting phase, animals had already been exposed to a high intensity light before being subjected to the L/D box, which uses a lower intensity light as the anxiogenic stimulus, so the stimulus may have been less salient. Contrariwise, during the active phase, mice were in the dark before being exposed to the lit testing chamber, where light would be considered anxiogenic. The manner in which T modulates this photic input in WT mice might be through dampening cell response to light by activating ARs in the SCN. As described in chapter 4, T-treated WT males showed fewer cells in the SCN that responded, as indicated by cFos expression, to exposure to the L/D box compared to T-treated *i*Tfm animals. The SCN is a gateway of photic input to the brain, therefore, if the SCN is interpreting the anxiogenic light stimulus as less intense in WTs, this might be one of the causes of WT males showing less anxiety-related behavior than

*i*Tfm males. Given that about 65% of cells in the SCN are AR positive and are especially concentrated in the SCN core, where photic input first reaches the brain (Karatsoreos and Silver, 2007), activation of AR might be directly modulating SCN neuronal activity.

Androgen receptor activity in the amygdala might also mediate T's anxiolytic effects. In fact, NesARKO mice that did not have AR fully knocked out in the amygdala but had full AR KO in other anxiety-related areas did not differ from their WT counterparts in anxiety-related behavior. Follow up studies confirmed this idea and suggested that AR acts on this behavior in the blAMY. Specifically, while no differences in cell response between WT and *i*Tfm mice were found in the clAMY and cmAMY, WT+T males showed more cell activity in the blAMY in response to a mild anxiogenic stimulus than either *i*Tfm+T or WT+B mice. Analysis of cells in the blAMY determined that about 65% of cells are AR positive, indicating that cells in this area that contain AR might be directly modulating cell response to an anxiogenic stimulus. Since the blAMY sends glutamatergic projections to the clAMY, which in turn sends GABAergic projections to the cmAMY (Tye et al., 2011), it is surprising that cell response did not differ between WT and *i*Tfm males in the clAMY or cmAMY. The blAMY also projects to the BNST (Davis and Shi, 2006; Weller and Smith, 1982) and the cortex (McDonald, 1991). These areas, likewise, did not show differences in cell response between WT and *i*Tfm males, which suggests that glutamatergic cells in the blAMY, which make up about 90% of cells in this area (Tye et al., 2011), might be activating cells in other brain regions not traditionally thought to be related to anxiety.

The fact that no differences were found in cell activity in response to a mild anxiogenic stimulus in the mPFC, oval nucleus and anterodorsal bed nucleus of the stria terminalis,

hippocampus, cl and cmAMY, or dorsal periaqueductal grey, all areas traditionally thought to be related to anxiety, suggests that the anxiolytic effects of T might be achieved through AR activity in other brain regions or circuitry. Possible targets are those brain areas that have been implicated in sex behavior and reproduction. Ecologically speaking, it may be advantageous to be less anxious during the mating season, when T levels are usually elevated, to allow males to venture out and increase their chances of mating.

Testosterone Modulates HPA Activity Through Functional AR

Testosterone requires functional AR to maintain low basal HPA activity in mice, as measured by CORT levels. In response to a stimulus that triggers the activation of an HPA response, T also works through AR to result in a lower response and to hasten the return of CORT to baseline levels. Specifically, WT+T males show lower baseline CORT, an overall lower CORT response and a faster recovery to baseline compared to *i*Tfm+T or WT+B mice.

That T has the capacity to modulate HPA activity is not a new finding. In fact, it is known that hypothalamic-pituitary-gonadal (HPG) activity has opposing interactions with HPA function (Viau, 2002). For instance, androgens lower glucocorticoids and other stress hormones (Viau and Meaney, 1996), and increase mRNA expression of CRH receptor 2 in the hippocampus (Weiser et al., 2008). Conversely, glucocorticoids inhibit gonadotropin releasing hormone production in the hypothalamus (Smith and Vale, 2006), and testicular T-producing Leydig cells posses glucocorticoid receptors and undergo apoptosis when chronically activated, decreasing T production (Wingfield and

Sapolsky, 2003; Chen et al., 2012). Therefore, T's effect on HPA function has been previously established. However, the findings in this dissertation demonstrate for the first time that T's effect on the mouse HPA is achieved through activation of functional ARs.

Studies in this dissertation also demonstrate that AR in the hippocampus and mPFC are not necessary for T's modulation of HPA function. While full AR KO in *i*Tfms impeded the actions of T, T-treated NesARKO males showed no difference in HPA response compared to T-treated WT mice. As mentioned, NesARKO animals show full KO of AR in the hippocampus and mPFC, allowing the conclusion that AR in these areas isn't normally responsible for modulating HPA function. However, since significant AR remained in amygdalar and hypothalamic areas, known to regulate HPA function (Smith and Vale, 2006; Herman et al., 2003; Jankord and Herman, 2008), it is not possible to conclude whether AR in areas such as the blAMY, medial amygdala, and dorsomedial and preoptic area of the hypothalamus are involved in the modulation of HPA activity. Cells in the PVN, on the other hand, possess little gonadal steroid receptors (Simerly et al., 1990; Schuchard et al., 1993; Zhou et al., 1994; Shughrue et al., 1997; Laflamme et al., 1998; Viau, 2002), which may suggest that modulation of HPA function by AR activation is likely to occur elsewhere.

Effects of AR activation by T on Anxiety and HPA Are Activational

Organizational effects of hormones occur during critical periods of development, perinatally or during puberty, when exposure to gonadal hormones can cause permanent changes. Activational effects of gonadal hormones, on the other hand, can

occur throughout life and are acute and present only when the hormone is in the system. WT animals used in studies in this dissertation had seen normal perinatal and pubertal T, and had been castrated and implanted with either T or blank capsules during adulthood. Given that WT+T mice showed less anxiety-related behavior and a milder HPA response than WT+B animals, it can be concluded that the anxiolytic effects of T through AR are activational. Although T's effect on anxiety-related behavior is activational, time is required for the anxiolytic effects to take effect. Only chronic, but not single, exposure to androgens lowers anxiety-related behavior (Fernandez-Guasti and Martinez-Mota, 2005; Penatti et al., 2009) and is blocked by flutamide (Fernandez-Guasti and Martinez-Mota, 2005), suggesting that activation of ARs triggers a cascade of events that in time results in anxiolysis. Potential targets include the GABA system and ERβ, both involved in anxiety (Lydiard, 2003; Rago et al., 1988; Reynolds, 2008; Imwalle et al., 2005; Krezel et al., 2001; Hughes et al., 2008; Rocha et al., 2005; Walf et al., 2008; Walf et al., 2009). In fact, female mice treated with androgens prenatally show greater GABAergic postsynaptic current frequency and larger hypothalamic cells than control females, an effect blocked by the AR antagonist flutamide (Sullivan and Moenter, 2004), suggesting a capacity of AR to modulate GABA function. Similarly, chronic exposure to anabolic androgenic steroids increases selective GABA(A) receptor subunit mRNAs and GABAergic synaptic current decay in the medial preoptic area in WT male mice, an effect that *s*Tfm mice do not show (Penatti et al., 2009). A potential for AR activity to affect ERβ expression or function, however, has yet to be investigated.

Cre-LoxP Technology: The Importance of Validation and Controls

Studies in Chapter 3 demonstrate the importance of validating a mouse model obtained using the Cre-LoxP technology and the significance of including controls in studies using these models.

In trying to understand the role of CNS AR in the anxiolytic effects of T, the Cre-LoxP system was used to breed animals for male offspring with CNS AR KO. The first step of validation, determining where Cre is expressed by crossing Nes-Cre animals with reporter mice, demonstrated that Cre was ubiquitously expressed in the brain, as expected. However, Cre was also expressed in other tissues relevant to our studies such as the adrenals and testes. Later, crossing ARlox with Nes-Cre animals provided male offspring that inherited both transgenes as verified by PCR genotyping, which we identified as our experimental animals, NesARKO. These experimental males should have had no functional AR in the brain. However, after analyzing NesARKO brains for AR immunoreactivity, it was determined that some AR protein remained in the brain even though Cre was expressed throughout. Relevant to this dissertation, AR remained in the amygdala and hypothalamic areas. Other studies have shown similar findings of incomplete excision using this technology (Bao et al., 2013), which could be attributed to loss of expression of Cre recombinase with increasing number of generations (Schulz et al., 2007). This generational loss, however, is not likely the case in our animal model since, through successive generations, I have found the same pattern of AR immunoreactivity in NestnARKO mice. Validation of the model, therefore, is crucial before making conclusions using this system.

Comparing anxiety-related behavior between NesARKO and WT animals indicated that only a single parameter, head dips in the elevated plus maze, might have been affected

by the partial KO. Careful examination of the additional Nes-Cre control group, however, indicated that the group difference was probably due to the Nes-Cre transgene alone, since the same difference was found between Nes-Cre and WT but not between Nes-Cre and NesARKO animals. The apparent influence of the *Cre* transgene on anxiety-related behavior was reinforced when animals carrying the Nes-Cre alone performed fewer rearings than WT males in the OF. Although our animals appeared healthy, studies have reported that Cre can alter the phenotype of cells (Xiao et al., 2012) and can cause defects in brain development (Forni et al., 2006). It is not clear whether behavioral differences seen in this study are due to the *Cre* gene or the recombinase enzyme. In any case, these findings reinforce the need to examine all three control groups accompanying Cre-LoxP studies, namely mice carrying the floxed allele alone, and those carrying the Cre transgene alone, as well as WT animals.

Future Directions

Here, I demonstrate that T requires functional AR to achieve its anxiolytic effects. However, this has been demonstrated by comparing behavior in our WT mice with *i*Tfm animals, which lack AR throughout development. This poses a possible confound because expression of ERα or ERβ, which is known to affect anxiety levels when activated, could have been affected by AR activity, or the lack thereof, in development. To address this question, the expression of brain ERα and ERβ could be assessed using immunohistochemistry in adult WT and *i*Tfm mice. It would be even more revealing to asses ERα and ERβ expression throughout development (perinatally, during and after puberty), given that we know that providing T to adult mice and rats is

anxiolytic only when given to WTs but not *i*Tfms. Interestingly, testicular secretions in the neonatal period appear to be anxiogenic in both WT and *s*Tfm rats as adults, indicating that ERs in development could play a role in the modulation of anxiety.

An additional question that our findings do not address is the role of AR in anxiety-related behavior and HPA function throughout development in mice. AR activity might have different effects depending on time of action. Using conditional AR KO mice to manipulate AR capacity during the prenatal, early post natal, pubertal and post pubertal periods could shed light on the nature of developmental AR influence on anxiety and HPA function.

Findings in chapter 4 suggest that, compared to *i*Tfms, higher cell activity in the blAMY and lower in the SCN in WT mice might be responsible for the down regulation of anxiety-related behavior. This cell activity may very well be modulated directly by AR function since about 65% of cells in both brain regions are AR positive in WTs. However this influence is mediated, it remains to be determined whether AR cells directly influence cell activity in response to a mild anxiogenic stimulus. To answer this question, WT mice could be subjected to the L/D box and their brains processed later for double label AR-cFos fluorescent immunohistochemistry.

It would be interesting to know how all the factors that influence anxiety might work together. For instance, like T and AR, estrogen, GABA, glucocorticoid, mineralocorticoid and their receptors play a role in modulating anxiety. Relevant to findings in this dissertation, it would be interesting to see, for example, how the lack of functional AR might affect the expression of the above mentioned factors as reflected in immunohistochemistry.

Conclusion

Findings presented in this dissertation provide additional understanding of the etiology of anxiety. Specifically, I replicated previous findings and determined that T requires functional AR to decrease anxiety-related behavior and reduce overall HPA function in adult mice. I found through KO and cell activity studies that AR activation may affect anxiety-related behavior by modulating cell activity in the blAMY and SCN. I also demonstrated that AR in the hippocampus, mPFC, ov- and adBNST, PVN, and dPAG are not necessary for T to modulate anxiety-related behaviors and HPA function.

APPENDIX

Table 1.

No differences in cFos expression were found between treatment groups in the cmAMY (centromedial amygdala), PVN (periventricular nucleus of the hypothalamus), ovBNST (oval nucleus of the bed nucleus of the stria terminalis), adBNST (anterodorsal BNST), and dPAG (dorsal periaqueductal grey) in response to the L/D box anxiogenic stimulus.

	WT+T	***i*Tfm+T**	**WT+B**
	Means + Std. Error	*Means + Std. Error*	*Means + Std. Error*
cmAMY	47.68+5.46	57.72+5.76	56.17+7.05
PVN	395.6+18.19	420.2+17.34	405+20.33
ovBNST	97.65+6.51	80.46+6.21	73.14+7.78
adBNST	191.95+14.53	170.99+13.86	168.86+17.37
dPAG	325.9+30.2	307.6+26	306.1+22.7

Figure 1.

The external phenotype of *i*Tfm males is feminine. AGD: anogenital distance. Compared to WT males, *i*Tfm males have a visibly shorter anogenital distance (A vs. B) and have much smaller and undescended testis (arrow in C vs. D). *i*Tfm males also have external nipples (arrows in A) typical of WT females but not WT males (B). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

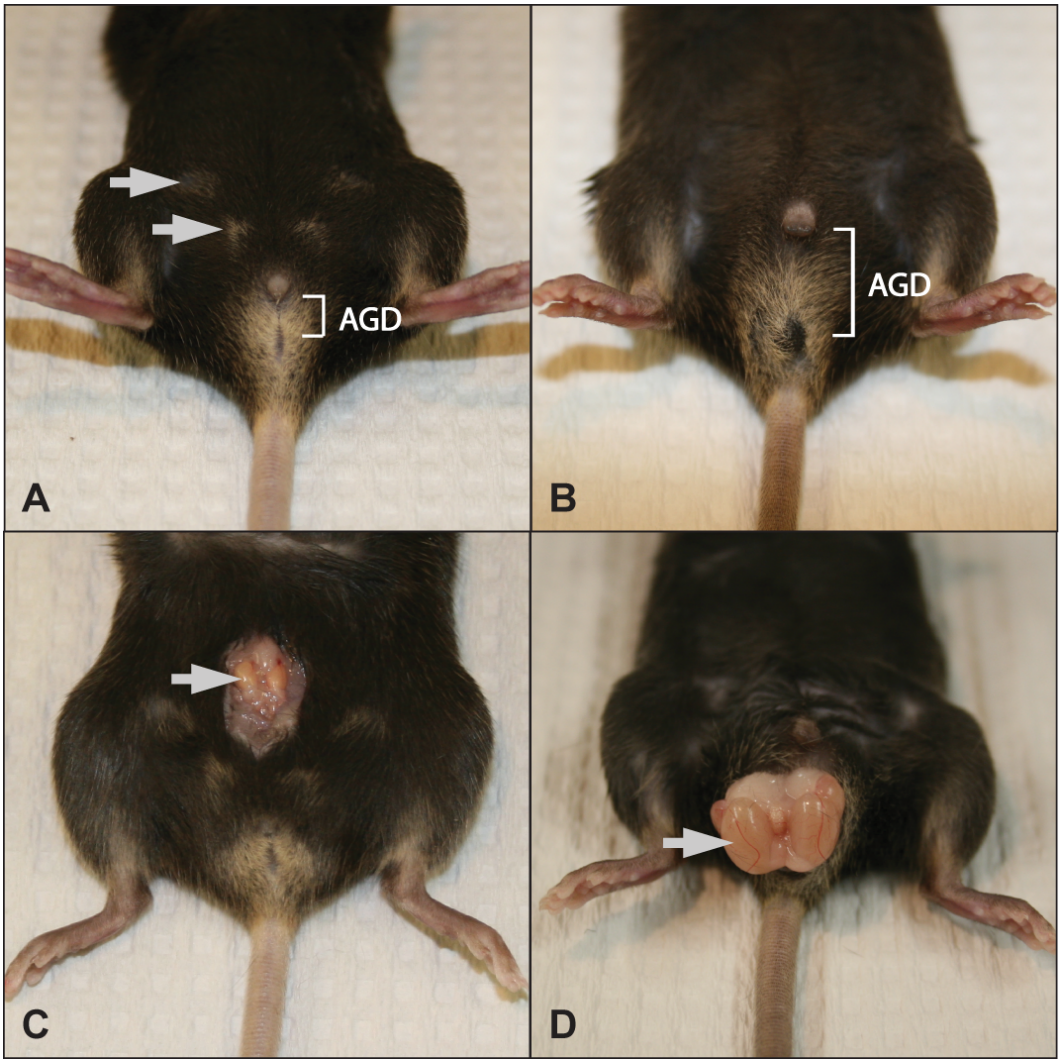


Figure 2.

Brain AR immunoreactivity confirms full AR KO in *i*Tfm animals. Robust nuclear AR immunoreactivity is present in the amygdala (A) and hippocampus (C) of WT males, while absent in the amygdala and hippocampus (B and D, respectively) of *i*Tfm males. Black scale bar: 200μm; white scale bar: 40μm.

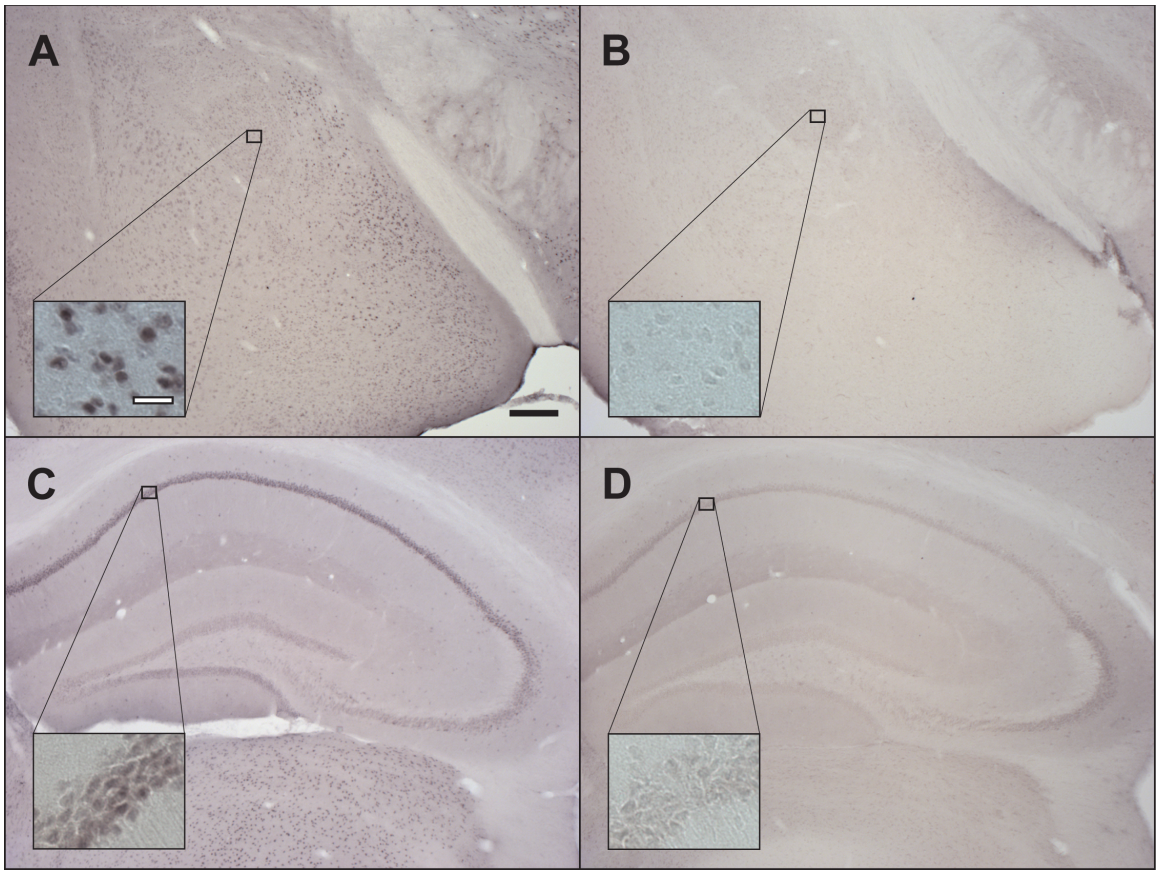


Figure 3.

ARs are necessary to alleviate anxiety-related behavior in mice tested during the resting phase (lights on). Number of rears in the open field (OF) test (A) and the novel object (NO) test (B) both show a main effect of genotype only, with testosterone (T)-treated *i*Tfms showing fewer rears than T-treated WTs. Latency to visit the object in the NO test (C) and total time spent in the open arms of the elevated plus maze (EPM; D) were affected by T treatment only in WT males. WT males given T made more open arm entries than *i*Tfms given T (E). For OF, NO and EPM tests, T treatment has anxiolytic effects only in WT males, not *i*Tfm males, indicating these effects of T are normally mediated through AR. There was no effect of T treatment or genotype in the light dark box (LD) when tested during the resting phase (F, G). These results replicate our previous findings of anxiolytic effects of T in sTfm mice, validating the *i*Tfm model. *p<.05.

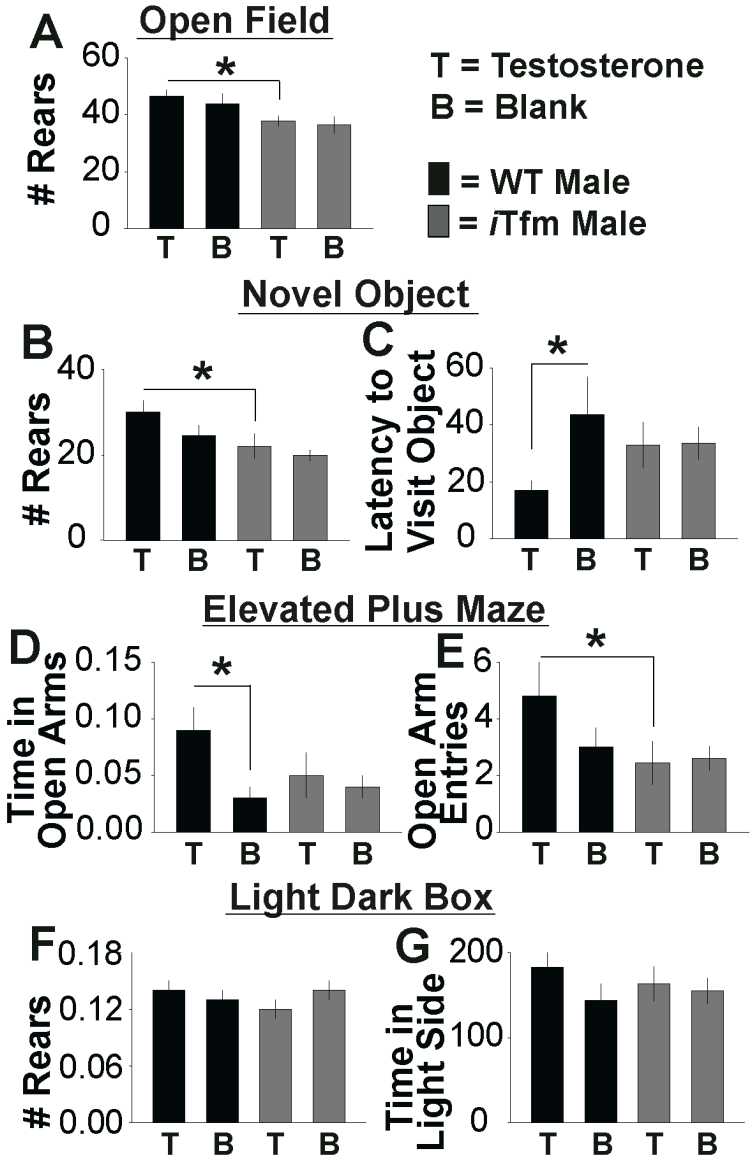


Figure 4.

Anxiety-related behavior is also heightened in intact *i*Tfm male mice compared to intact WT males tested during their active phase (lights off). With one exception (latency to visit object in the novel object test, C), *i*Tfm males show significantly increased levels of anxiety-like behavior compared to WT males based on performance in the open field (A), novel object (B), elevated plus maze (D), and light/dark box (E, F) tests. These results further support the idea that AR plays a role in the modulation of anxiety. Testing during the active phase reveals group differences in the light dark box that were not observed during the resting phase (see Figure 3). *p<.05.

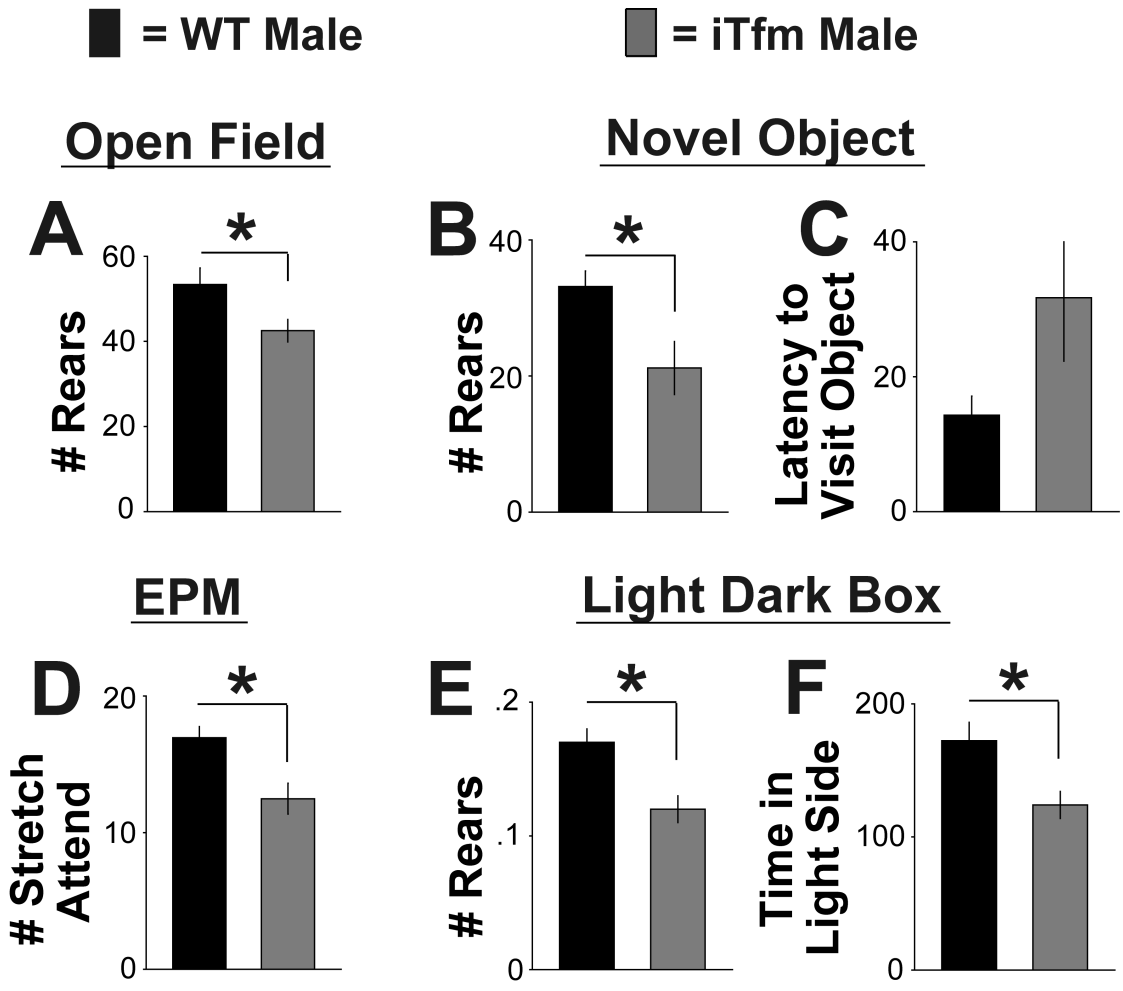


Figure 5.

Functional AR is required for T to regulate basal levels of CORT as well as CORT response to an anxiety-provoking stimulus (light dark box). Basal levels of CORT (time point 0) are highest in *i*Tfm males given T and lowest in WT castrates given T, with control-treated WT males intermediate, indicating that T exposure in adulthood normally reduces basal CORT levels by activating ARs. Additionally, despite equivalent T treatment, *i*Tfm males show an even more elevated response and a hastened CORT return to baseline compared to WT males after exposure to an anxiety-provoking situation. *indicates significantly different from *i*Tfm+T males, p<.05.

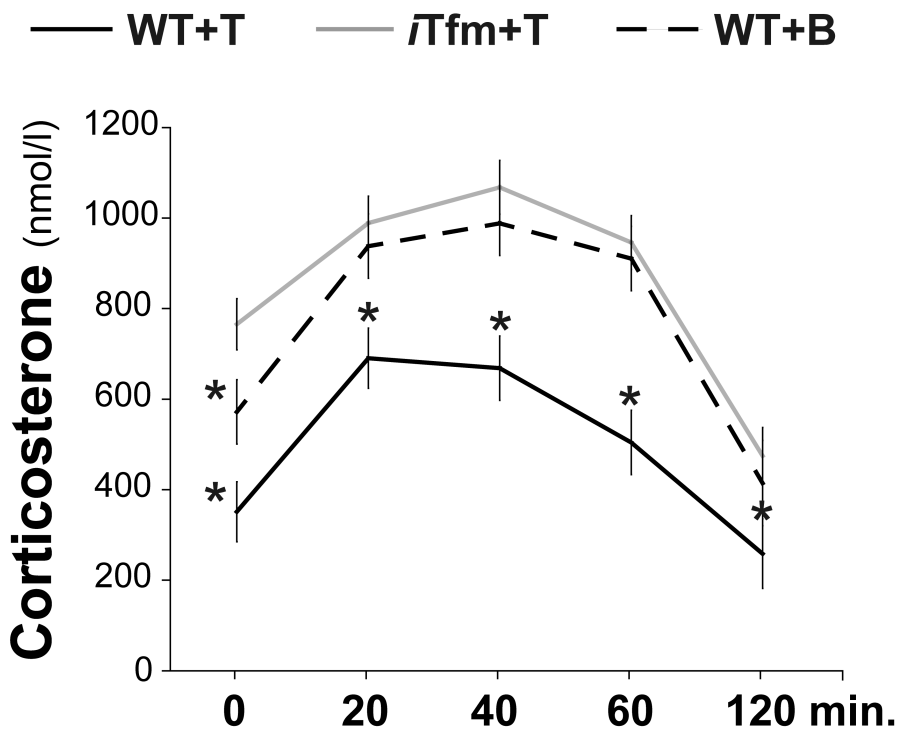


Figure 6.

Brain Cre expression and AR immunoreactivity in WT, NesARKO and *i*Tfm mice. Mice with NestinCre and LacZ transgenes showed robust Cre expression throughout the brain, including anxiety-related areas such as the hippocampus (B), mPFC (C), and amygdala (A). Though Cre was ubiquitously expressed in the brain, AR immunohistochemistry showed that AR was only partially knocked out in the brain of NesARKO mice. Anxiety-related areas such as the hippocampus (H) and mPFC (I) show complete AR KO, while others like the amygdala (G) still show some AR-ir and only partial AR KO. Positive control WT males showed normal AR-ir in all typical brain areas including those mentioned above (D-F), and the negative control *i*Tfm mice showed no AR-ir (J-L).

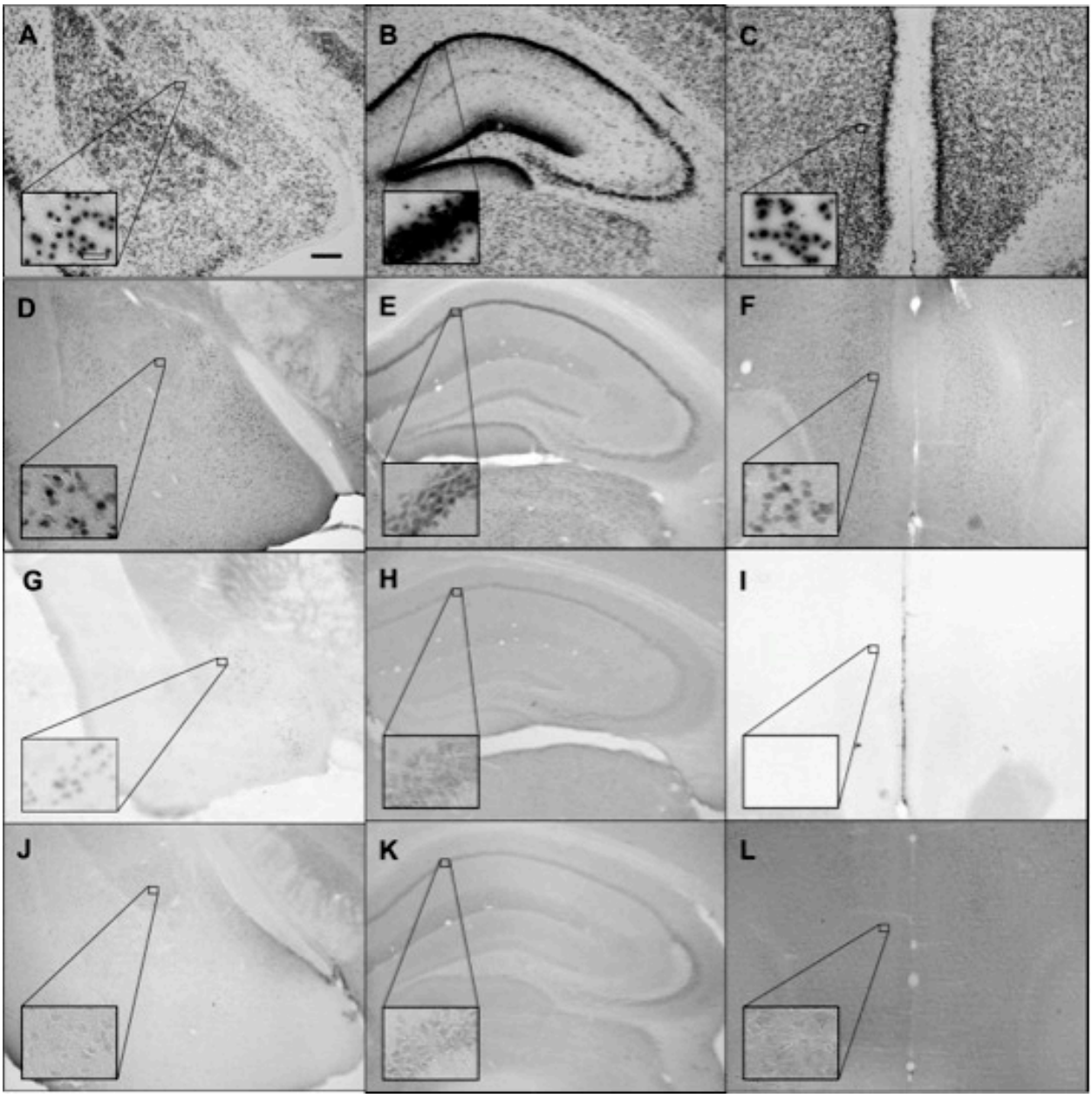


Figure 7.

Anxiety-related behavior in WT, NesARKO, ARlox and Nes-Cre: (A) number of rears; (B) time spent in the center area; (C) time spent with the novel object; (D) number of visits to the novel object; (E) number of head dips; (F) time spent in the open arm; (G) number of light side entries and time spent in the light side when the L/D box test was run after and before the EPM test. For most tests, NesARKO males did not show differences in anxiety-related behavior compared to WT mice. However, WT males performed more head dips compared to NesARKO and Nes-Cre mice in the EPM (E) and more rears in the OF compared to NesARKO (A), suggesting a potential effect of Cre transgene alone on certain anxiety-related behaviors. * = p<.05.

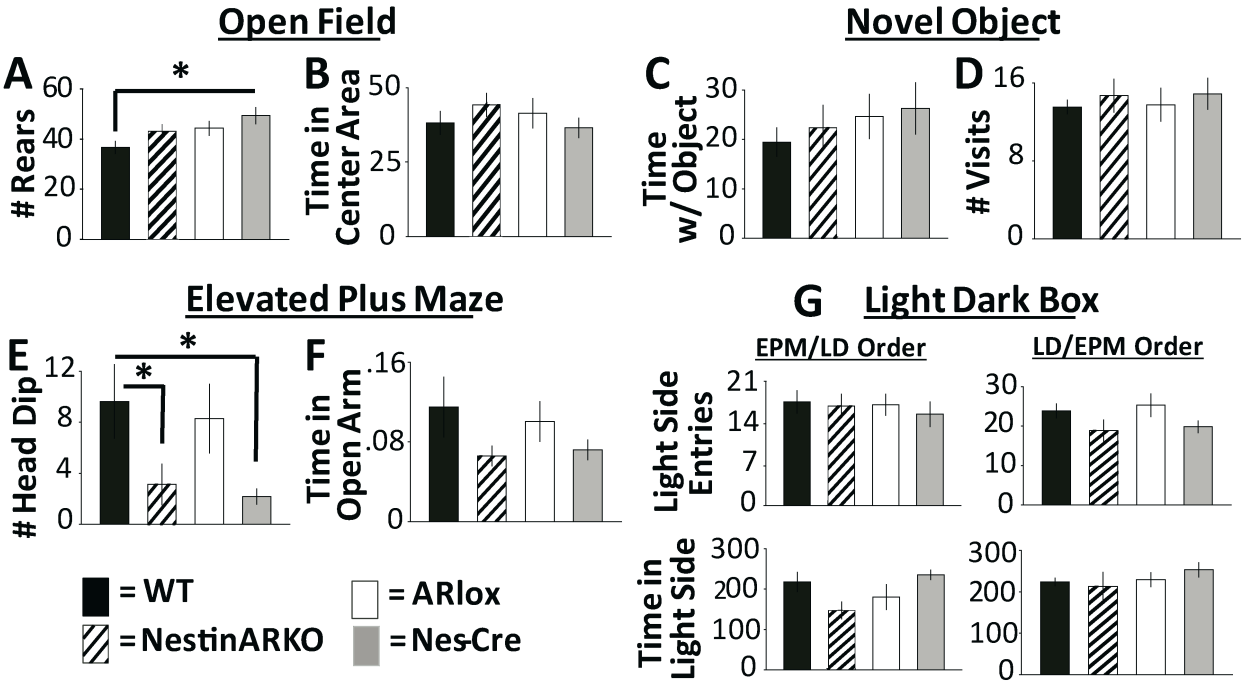


Figure 8.

HPA response to L/D box exposure in WT, NesARKO, ARlox and Nes-Cre mice. No group differences were found on basal CORT levels, nor on the response and recovery of HPA activity after exposure to the anxiety-provoking L/D box.

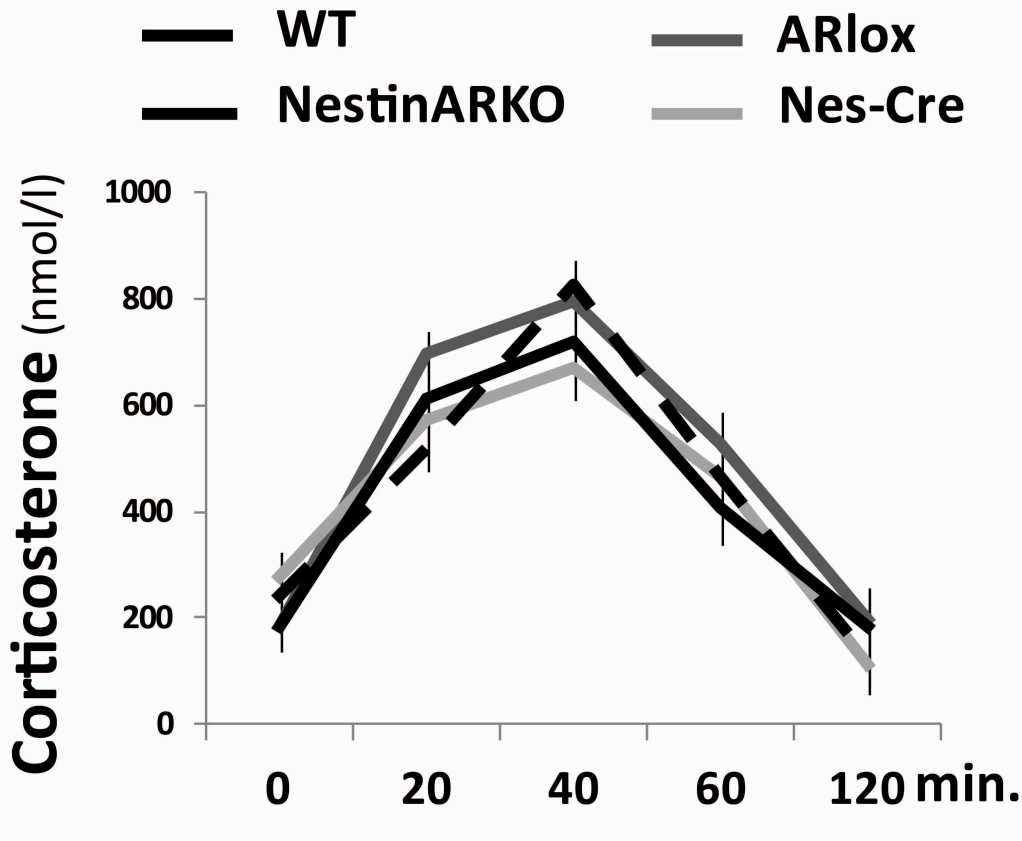


Figure 9.
Brain areas related to anxiety-related behavior and their circuitry.

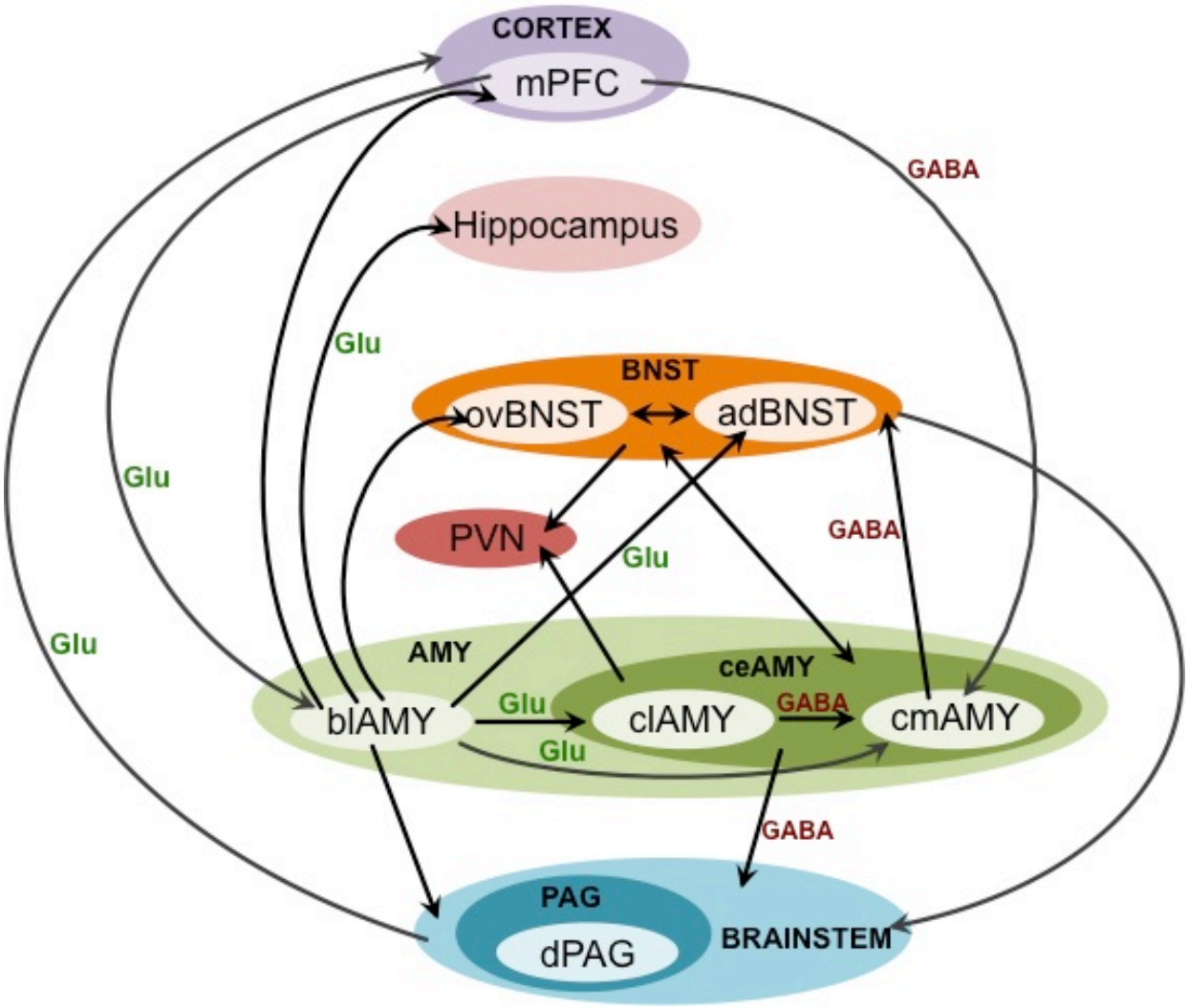


Figure 10.

Testosterone acts through functional AR to modulate cell response to a mild anxiolytic stimulus: (A) T-treated castrated WT males show more cell activity in the blAMY compared to *i*Tfm+T and WT+B males. (B) WT+T males showed less cell response in the SCN than did *i*Tfm+T and WT+B males. (C) No group differences were found in cell activity in the clAMY; however, a laterality difference was found in *i*Tfm+T males, where the left hemisphere showed less cell activity compared to the right hemisphere. No group differences were found in cell response in the (D) hippocampus and (E) mPFC.

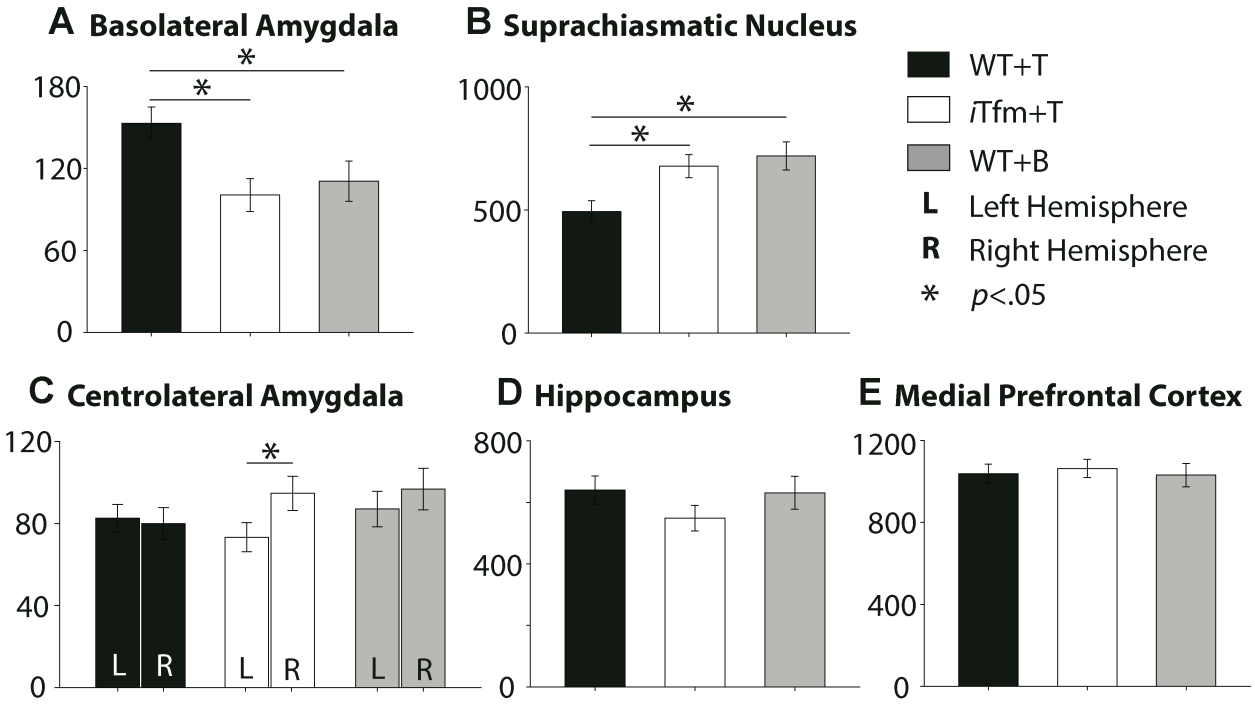


Figure 11.

Differences in cell response to a mild anxiogenic stimulus in the blAMY are not due to cell size differences. No group differences in cell size were found in cFos+ or cFos- cells in the blAMY.

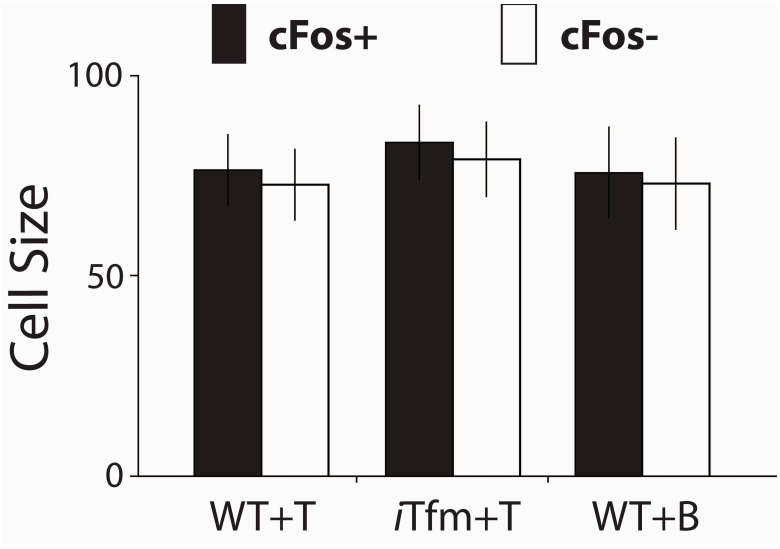
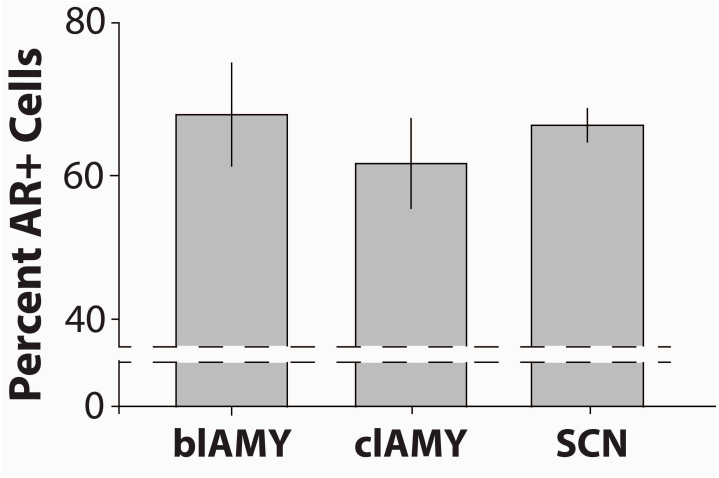


Figure 12.

Cell activity in the blAMY, clAMY and SCN in response to a mild anxiogenic stimulus might be directly modulated by AR activity. Around 65% of cells in the blAMY, clAMY and SCN are AR positive, suggesting the possibility that AR directly modulates cell activity in response to a mild anxiogenic stimulus in these areas.



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